PHOSPHOFRUCTOKINASES FROM EXTREMELY THERMOPHILIC MICROORGANISMS

A thesis submitted

in partial fulfillment of the requirements

for the Degree of Doctor of Philosophy

in Biological Sciences

at the University of Waikato

by

Yan-Huai R Ding

The University of Waikato
Te Whare Wānanga o Waikato

The University of Waikato
Hamilton, New Zealand
2000

ABSTRACT

The distribution of phosphofructokinase (PFK) phosphoryl donor subtypes [ATP-, ADP- and pyrophosphate (PP_i)] in the deeply rooted phylogenetic lineages of thermophiles is of interest with respect to the evolution of PFK activity and of the Embden-Meyerhof (EM) pathway. To gain additional insight into the understanding of this key enzyme in the central metabolism within the three domains of life, PFKs with different phosphoryl donor specificities were studied from some extremely thermophilic bacteria, archaea and non-thermophilic bacteria.

Results from a survey of species from the order Spirochaetales showed that all of the tested species of *Spirochaeta*, both thermophilic and mesophilic species/strains, possessed a PP_i-dependent PFK (PP_i-PFK) activity. However, ATP dependent-PFK activities were found to be predominant in some strains of *Leptospira* and *Treponema*. Overall, the results suggest that the presence of a PP_i-PFK might be a reliable phenotypic marker for the genus *Spirochaeta* and that there are some potentially interesting differences in how the catabolism of saccharides is controlled among members of genera within the Spirochaetales.

The PP_i-PFK from an extremely thermophilic bacterium *Dictyoglomus thermophilum* Rt46 B.1 has been purified and characterised. Biochemical studies with the *Dictyoglomus* native enzyme showed that this enzyme possesses some properties that are similar to other bacterial PP_i-PFKs. The enzyme is homodimeric, non-allosteric and possesses an acidic pH optimum for the forward reaction and a neutral to slightly alkaline optimum for the reverse reaction. The enzyme requires Mg^{2+} for optimal activity and has significant activity with tripolyphosphate (PPP_i) and polyphosphate (polyP, n=15±3). The *Dictyoglomus* enzyme is extremely sensitive to Cu^{2+}.

The *Dictyoglomus* PP_i-PFK-encoding gene (*pfp*) was also sequenced, cloned and the enzyme expressed in *Escherichia coli*. The full-length sequence of the *pfp* gene was obtained using degenerate PCR and inverse-PCR. Sequence analysis and a phylogenetic comparison suggest that the *Dictyoglomus* PP_i-PFK represents an ancient lineage and is closely related to those PP_i-PFKs from the archaeal *Thermoproteus tenax*, bacterial *Mycobacterium tuberculosis* and *Amycolatopsis methanolica*, and the ATP-PFK from *Streptomyces coelicolor*, all of which belong to group III PFKs.

A biochemical comparison between the *Dictyoglomus* native and recombinant enzymes demonstrated that they possessed a high degree of similarity in most of properties examined, e.g. the optimal pH values and Mg^{2+} concentration for activity, lack of allosteric response to metabolites, thermostability, most kinetic parameters and extreme sensitivity to Cu^{2+}.

An ATP-PFK from the hyperthermophilic crenarchaeon *Desulfurococcus amylolyticus* was also purified to homogeneity and characterised. The enzyme was confirmed as an ATP-dependent enzyme possessing no activity with either ADP or PP_i. The *Desulfurococcus* enzyme was not significantly affected by traditional allosteric modulators, e.g. phosphoenolpyruvate (PEP), citrate, succinate or fructose-2,6-bisphosphate (F-2,6-P_2). This enzyme similar to what was found for the *Dictyoglomus* PFK is also extremely sensitive to Cu^{2+}.

Two genes from the hyperthermophilic bacterium *Thermotoga maritima*, one encoding a PP_i-PFK and the other an ATP-PFK, were cloned, expressed and characterised. Both enzymes were shown to be extremely thermostable, activated by KCl and strongly inhibited by Cu^{2+} and Zn^{2+}. The PP_i-PFK enzyme is indicated to be

a non-allosteric homodimer which catalyses a near-reversible reaction. Significantly, the apparent K_m values for the phosphoryl donors PP_i, PPP_i and polyP (n=15±3) for the forward reaction were 67 μM, 10 μM and 3.8 μM respectively, and thus the enzyme might operate *in vivo* as a polyP-dependent PFK. The ATP-PFK exhibited significant activity with other nucleotide triphosphates, GTP (42% of control activity with ATP), UTP (14%), CTP (13%) and TTP (10%), but its activity was not significantly affected by common allosteric effectors, i.e. PEP, although it was partially inhibited by citrate. Surprisingly, the ATP-PFK was strongly inhibited by PP_i, PPP_i and polyP. The inhibition by PP_i could be partially relieved by nucleotide diphosphates including ADP, GDP, TDP and UDP. The *T. maritima* ATP-PFK is thus likely to be modulated by PP_i and/or polyP which would represent a novel mechanism for controlling glycolytic flux.

Taken together, the data suggests that the distribution, subtypes and regulatory mechanisms of PFK in the control of the EM pathway in extremely thermophilic organisms are much more complex than we expected. As originally intended, the results from this work has provided additional insights into the understanding of the evolution of this key enzyme in glycolysis.

ACKNOWLEGEMENTS

I would like to thank my supervisors Professor Hugh Morgan and Dr. Ron Ronimus for their encouragement, help and supervision through the whole range of laboratory work, academic activity and thesis writing.

Special thanks to my wife Chun-Ling (Lucy) Zhu, our children Yi Ding and Nick Boning Ding and our family in China. It would have been impossible to complete this course of study in time without their full support. I would like to present this thesis as a special gift to them.

I am grateful to Lynne Parker and Colin Monk for their technical assistance throughout the whole project and Dr. Stanley Moore for protein crystallisation. I am also grateful for the technical assistance by Judy Bragger, Raewyn Towers, Pat Gread and Cameron Evans.

Many thanks for the friendship, help and support of my lab mates and friends. I am also grateful to Jenny Lanning for her excellent proofreading of the thesis.

I am also thankful to the Royal Society of Marsden Foundation and the University of Waikato for research financial support.

Finally, I am grateful for the favour, mercy and love of the Triune God in whom I have faith during this study.

TABLE OF CONTENTS

ABSTRACT ii
ACKNOWLEDGEMENTS v
LIST OF CONTENTS vi
LIST OF TABLES xiii
LIST OF FIGURES xv
LIST OF ABBREVIATIONS xix

CHAPTER ONE
REVIEW OF LITERATURE 1

1.1 Glycolysis 1
1.1.1 Glycolysis in eukaryotes 4
1.1.2 Glycolysis in bacteria and archaea 5
1.1.3 Evolution of central metabolic pathways 6
1.2 Phosphofructokinase 7
1.2.1 ATP-dependent phosphofructokinases 8
1.2.2 Pyrophosphate-dependent phosphofructokinases 12
1.2.2.1 The history of PP_i-PFKs 12
1.2.2.2 PP_i-PFK activity in primitive eukaryotes 14
1.2.2.3 PP_i-PFK activity in plants 15
1.2.2.4 PP_i-PFK activity in bacteria and archaea 17
1.2.3 ADP-dependent phosphofructokinase 19
1.3 Phylogeny and evolution of PFK 19
1.4 The organisms studied in this work 22
1.4.1 *Dictyoglomus thermophilum* Rt46 B.1 23
1.4.2 *Thermotoga maritima* 24
1.4.3 The order Spirochaetales 25
1.5 Hypothesis and the aim of this thesis 26

CHAPTER TWO
GENERAL MATERIALS AND METHODS 28

2.1 Microorganisms studied in this thesis 28
2.2 Chemicals and reagents 29
2.3 Phosphofructokinase assays 30
2.4 Cell growth, harvesting and screening of PFK activities 33
2.5 Assessment of the purity of PFK preparations 33

2.6 Determination of native molecular masses by gel filtration chromatography 33
2.7 Determination of molecular masses of PFKs by mass spectrometry 34
2.8 Protein assays 35
2.9 Electrophoresis 35
2.9.1 SDS-PAGE 35
2.9.2 SDS-PAGE sample preparation and eletrophoresis 35
2.9.3 Isoelectric focusing (IEF) standards and isoelectric point (*p*I) determination 36
2.9.4 Protein silver staining for SDS-PAGE and IEF 37
2.10 Characterisation of purified PFKs 38
2.10.1 Determination of optimum pH for activity 38
2.10.2 Determination of optimum concentration of Mg^{2+} for activity 39
2.10.3 Determination of enzyme thermostability 39
2.10.4 Determination of activity with alternate phosphoryl donors 39
2.10.5 Determination of kinetic parameters 39
2.10.6 Determination of activators/inhibitors 40
2.10.7 Determination of N-terminal sequences 40
2.11 DNA manipulations 41
2.11.1 Bacterial strains and plasmids used in this study 41
2.11.2 Preparation of reagents used in DNA isolation and purification 42
2.11.3 Genomic DNA isolation and quantification 43
2.11.4 Isolation of plasmid DNA 46
2.11.4.1 Minipreps for plasmid isolation 46
2.11.4.2 Large-scale plasmid isolation 47
2.12 Gene cloning methods 48
2.12.1 Preparation of competent cells 49
2.12.2 Restriction enzyme digestions 49
2.12.3 Agarose gel electrophoresis 50
2.12.4 Southern blotting 52
2.12.5 DNA fragment isolation and purification 53
2.12.6 Preparation of de-phosphorylated vector 53
2.12.7 DNA ligation 54
2.12.8 Probe preparation 55
2.12.9 Hybridisation of radioactive probes 55
2.12.10 Autoradiography and film development 56

2.12.11 Electroporation 56
2.12.12 Identification of bacterial colonies containing recombinant plasmids 57
2.13 PCR and inverse-PCR amplifications 58
2.14 DNA sequencing, computer analyses and database searches 58
2.15 Gene expression 59
2.15.1 Construction of expression clones 59
2.11.3 Small-scale isolation of cloned vectors and induction of expressed enzymes 60
2.15.3 Large scale induction of recombinant enzymes 60
2.15.4 Purification of recombinant enzymes 61
2.16 Crystallographic studies 61

CHAPTER THREE
SCREENING OF PFK ACTIVITY FROM BACTERIA AND ARCHAEA 62

3.1 INTRODUCTION 62
3.2 MATERIALS AND METHODS 64
3.3 RESULTS 65
3.3.1 Strain cultivation 65
3.3.2 PFK activities within the Spirochaetales 66
3.3.3 PFK activities within thermophilic bacteria 67
3.3.4 PFK activities within thermophilic archaea 67
3.4 DISCUSSION 68
3.4.1 Cultivation of microorganisms 68
3.4.2 Distribution of PFK subtypes within the Spirochaetales 69
3.4.2 PFK activity in extremely thermophilic bacteria and archaea 70

CHAPTER FOUR
PURIFICATION AND CHARACTERISATION OF A PP_i-PFK FROM AN EXTREMELY THERMOPHILIC BACTERIUM *DICTYOGLOMUS THERMOPHILUM* 74

4.1 INTRODUCTION 74
4.2 MATERIALS AND METHODS 75
4.2.1 Bacteria and culture conditions 75

4.2.2 Determination of PFK reaction rates and protein concentrations 75
4.2.3 Purification of the *Dictyoglomus* PP_i-PFK 75
4.2.3.1 Preparation of cell-free extract 76
4.2.3.2 Phenyl-Sepharose hydrophobic interaction chromatography 77
4.2.3.3 Q-Sepharose anion exchange chromatography 77
4.2.3.4 Chromatofocusing 78
4.2.3.5 Gel filtration chromatography 78
4.2.3.6 Reactive red 120 dye-ligand chromatography 78
4.2.4 SDS-PAGE gel electrophoresis 79
4.2.5 Determination of the native molecular mass and thermostability 79
4.2.6 Determination of N-terminal sequence and BLAST search 79
3.4.3 Determination of kinetic parameters and activator/inhibitor effects 80
4.3 RESULTS 80
4.3.1 Purification of the *Dictyoglomus* PP_i-PFK 80
4.3.2 Subunit structure of the enzyme and thermostability 86
4.3.3 Substrate specificity, cation effects and kinetic properties 88
4.3.4 Determination of N-terminal sequence 91
4.4 DISCUSSION 91

CHAPTER FIVE
SEQUENCING, CLONING AND HIGH-LEVEL EXPRESSION OF THE *pfp* GENE ENCODING A PP_i-PFK FROM THE EXTREMELY THERMOPHILIC BACTERIUM *DICTYOLOMUS THERMOPHILUM* 96

5.1 INTRODUCTION 96
5.2 MATERIALS AND METHODS 97
5.2.1 Bacterial strains and plasmids 97
5.2.2 Protocols used for gene cloning, sequencing and expression 97
5.2.3 Gene cloning by colony lifting method 98
5.2.3.1 PCR amplification of a 350 bp region of the gene encoding the PP_i-PFK of *D. thermophilum* 99
5.2.3.2 Analysis of the sequence data in the 350 bp fragment 99
5.2.3.3 Genomic DNA restriction digests

5.3.7 Crystallisation of the recombinant *Dictyoglomus* PP_i-PFK 124
5.4 DISCUSSION 125

CHAPTER SIX
PURIFICATION AND CHARACTERISATION
OF A HYPERTHERMOPHILIC ARCHAEAL
ATP-PFK FROM *DESULFUROCOCCUS AMYLOLYTICUS* 130

6.1 INTRODUCTION 130
6.2 MATERIALS AND METHODS 131
6.2.1 Source of strains and large-scale culture conditions 131
6.2.2 Determination of PFK reaction rates 131
6.2.3 Purification of the *D. amylolyticus* ATP-PFK 132
6.2.3.1 Preparation of cell-free extract 133
6.2.3.2 Ammonium sulfate precipitation 133
6.2.3.3 Phenyl-Sepharose hydrophobic interaction 133
6.2.3.4 Q-Sepharose anion exchange chromatography 133
6.2.3.5 Orange dye-ligand chromatography 134
6.2.3.6 Hydroxyapatite 134
6.2.4 Gel electrophoresis, gel filtration chromatography and protein determinations 134
6.2.5 Determination of the thermostability of the ATP-PFK 135
6.2.6 pH optimum for activity 135
6.2.7 Substrate specificity and kinetic parameters 135
6.2.8 N-terminal determination 136
6.3 RESULTS 136
6.3.1 Purification and properties of the *Desulfurococcus* ATP-PFK 136
6.3.2 Characterisation of the *Desulfurococcus* ATP-PFK 141
6.4 DISCUSSION 145

CHAPTER SEVEN
CLONING, EXPRESSION AND CHARACTERISATION OF A PP_i-PFK
AND AN ATP-PFK FROM HYPERTHERMOPHILIC BACTERIUM
THERMOTOGA MARITIMA 148

7.1 INTRODUCTION 148
7.2 MATERIALS AND METHODS 148
7.2.1 Bacterial strains and plasmids 148
7.2.2 DNA techniques 149

7.2.3 Sequence and restriction map analysis for both genes 149
7.2.4 Construction of the *Thermotoga* PP_i-
and ATP-PFK expression clone 149
7.2.5 Gene expression and enzyme purification 151
7.2.6 Determination of molecular sizes and enzyme thermostabilities 152
7.2.7 pH dependence, cation specificities and molecular
properties of recombinant enzymes 152
7.2.8 Determination of kinetic parameters, activators and inhibitors 153
7.3 RESULTS 153
7.3.1 Sequence analysis of the *Thermotoga* PP_i- and ATP-PFKs 153
7.3.2 Amplification of the two genes by PCR 154
7.3.3 Molecular sizes and thermostabilities of both enzymes 156
7.3.4 pH optima, cation specificities and molecular properties 157
7.3.5 Phosphoryl donors, kinetics parameters,
activators and inhibitors 161
7.3.6 Sequence alignment and phylogenetic study for
both the *Thermotoga* enzymes 165
7.4 DISCUSSION 166

CHAPTER EIGHT
GENERAL DISCUSSION,CONCLUSION
AND FUTURE RESEARCH 172

APPENDICES
Appendix A Media for microorganisms 179
Appendix B
B.1-2 Programme for SDA-PAGE and IEF 190
B.3 Protocol for plasmid purification 190
B.4 Sequences of *Dictyoglomus pfp* gene 192
B.5 Mass spectrometry data 198
Appendix C Sequences of *Thermotoga pfp* and *pfk* genes 200
Appendix D Ni-NTA resin regeneration 202
Appendix E Double reciprocal ($1/v$ versus $1/[S]$) Lineweaver-Burk plots
for determination of the apparent K_m values
of the *Thermotoga* enzymes 203
REFERENCES 223

PUBLICATIONS, CONFERENCE ABSTRACT
AND ACADEMIC AWARD 248

LIST OF TABLES

Chapter 1

1.1	Summary of properties of some ATP-PFKs from primitive eukaryotes, bacteria and archaea	9
1.2	Properties of some PP_i-PFKs from primitive eukaryotes	14
1.3	Properties of some PP_i-PFKs from bacteria and archaea	18

Chapter 2

2.1	Source of strains for screening of PFK activity	28
2.2	Source of chemicals and materials	29
2.3	Low molecular weight standard marker SDS-PAGE	36
2.4	Modified method of silver staining for SDS-PAGE with PhastGel gradient media	37
2.5	Silver staining method optimised for IEF gel with PhastGel gradient media	38
2.6	Bacterial strains and plasmids used in this study	41
2.7	Restriction enzymes used in this study	49
2.8	Reagents for hybridisation	56

Chapter 3

3.1	PFK activities in crude extracts of representative thermophilic and nonthermophilic bacteria and archaea	65

Chapter 4

4.1	Purification of the PP_i-PFK from the *D. thermophilum* Rt46 B.1	86
4.2	Phosphoryl donors for the *D. thermophilum* PP_i-PFK	88
4.3	Kinetic parameters of the *D. thermophilum* PP_i-PFK	89
4.4	Effect of some cations on the *Dictyoglomus* PP_i-PFK activity	90
4.5	N-Terminal sequencing of the *D. thermophilum* PP_i-PFK from BLAST search results	91

Chapter 5

5.1	The main strategies used for sequencing, cloning and expression of the *pfp* gene from *D. thermophilum* Rt46 B.1	98
5.2	Homologues of the 350 bp fragment identified with BLASTX	106
5.3	Amino acid residues in the active sites of selected PFKs	116
5.4	Amino acid composition of the *D. thermophilum* PP_i-PFK	117
5.5	Codon usage for the *D. thermophilum* PP_i-PFK	118
5.6	Purification of the recombinant PP_i-PFK from *D. thermophilum* Rt46 B.1	121

5.7	Comparative data between the native and the expressed *D. thermophilum* PP_i-PFK	124

Chapter 6

6.1	Purification table for the ATP-PFK from *D. amylolyticus*	141
6.2	Summary of properties for the *Desulfurococcus* ATP-PFK	143
6.3	Phosphoryl donors and activators/inhibitors tests for the ATP-PFK from *D. amylolyticus*	144

Chapter 7

7.1	Effects of KCl on the apparent K_m values of PP_i and F-6-P from the *Thermotoga* PP_i-PFKs	160
7.2	Cation effects on the *T. maritima* PP_i- and ATP-PFK	160
7.3	Kinetic parameters from the *Thermotoga* PP_i-PFK	161
7.4	Kinetic parameters from the *Thermotoga* ATP-PFK	162
7.4	Effects of some compounds for the PP_i inhibition on the *Thermotoga* ATP-PFK	165

LIST OF FIGURES

Chapter 1

1.1	The Embden-Meyerhof or glycolytic pathway	2
1.2	Schematic views of two subunits in the *B. stearothermophilus*-PFK tetramer	11
1.3	A phylogenetic tree based on 16S rRNA sequence comparisons	21

Chapter 2

2.1	Modified coupled assays for PFK activity based on (a) F-1,6-P_2 production and (b) ADP formation	32

Chapter 4

4.1	Purification procedure used with the *Dictyoglomus* PP_i-PFK	76
4.2	The elution profile of the *Dictyoglomus* PP_i-PFK from a phenyl-Sepharose column	81
4.3	The elution profile of the *Dictyoglomus* PP_i-PFK from a Q-Sepharose column	82
4.4	The elution profile of the *Dictyoglomus* PP_i-PFK from a Mono-P column	83
4.5	The elution profile of the *Dictyoglomus* PP_i-PFK from gel filtration	84
4.6	The elution profile of the *Dictyoglomus* PP_i-PFK from a reactive red 120 dye-column	85
4.7	SDS-PAGE for purified *Dictyoglomus* PP_i-PFK	87
4.8	Determination of native molecular mass of the *Dictyoglomus* PP_i-PFK	87

Chapter 5

5.1	PCR amplification of the 350 bp fragment to be used as probe	105
5.2	Restriction map of the 350 bp PCR fragment	106
5.3	Restriction digestions of genomic DNA with 5 different enzymes and 10 double enzyme combinations	107
5.4	Autoradiography after Southern hybridisation	108
5.5	Digestions of the *Dictyoglomus* DNA by 3 restriction enzymes with different combinations	109
5.6	Examination of restriction digestions using different times	110
5.7	Colony lifting experiment for the *Dictyoglomus pfp* gene cloning	111

5.8	Restriction digests for inverse-PCR amplification	112
5.9	Southern hybridisation after 11 restrition digests for inverse-PCR amplification	112
5.10	Inverse-PCR amplification products with *Sau*3A I, *Rsa* I and *Sau*96 I digested *Dictyoglomus* DNA	113
5.11	Inverse-PCR amplification map of the *D. thermophilum pfp* gene	114
5.12	Nucleotide and deduced amino acid sequence of the cloned *pfp* gene from *D. thermophilum* Rt46 B.1	115
5.13	Multiple alignment of amino acids with the *Dictyoglomus* PP_I-PFK amino acid sequence	119
5.14	Phylogenetic relationships of PP_i- and ATP-PFKs from some organisms	122
5.15	SDS-PAGE of the recombinant *Dictyoglomus* PP_i-PFK	123
5.16	The crystallisation of the recombinant *Dictyoglomus* PP_i-PFK	125

Chapter 6

6.1	Purification procedure of the *Desulfurococcus* ATP-PFK	132
6.2	The elution profile of the *Desulfurococcus* ATP-PFK from a phenyl-Sepharose column	137
6.3	The elution profile of the *Desulfurococcus* ATP-PFK from a Q-Sepharose column	138
6.4	The elution profile of the *Desulfurococcus* ATP-PFK from an orange dye-ligand column	139
6.5	The elution profile of the *Desulfurococcus* ATP-PFK from a HPHT column	140
6.6	SDS-PAGE for the *Desulfurococcus* ATP-PFK	142
6.7	Determination of pH optimum from the *Desulfurococcus* ATP-PFK	143

Chapter 7

7.1a	Restriction map of the *Thermotoga* PP_i-PFK	154
7.1b	Restriction map of the *Thermotoga* ATP-PFK	154
7.2	PCR amplification of both *pfp* and *pfk* genes from *T. maritima*	155
7.3	Miniprep plasmid analysis of both the *Thermotoga pfp* and *pfk* genes from transformed *E. coli* clones	156
7.4	SDS-PAGE for both purified PP_i- and ATP-PFK from *T. maritima*	157
7.5	Determination of pH optima for the *Thermotoga* PP_i-	

	and ATP-PFK	158
7.6	Effect of KCl on enzyme activity from the *Thermotoga* PP_i- and ATP-PFK	159
7.7	Effects of PP_i, PPP_i, polyP and PEP on the *Thermotoga* ATP-PFK activity	164

Appendix E

E.1	Double reciprocal ($1/v$ versus 1/[S]) Lineweaver-burk plots for apparent K_m value of PP_i from the *Thermotoga* PP_i-PFK	203
E.2	Double reciprocal ($1/v$ versus 1/[S]) Lineweaver-Burk plots for apparent K_m value of F-6-P from the *Thermotoga* PP_i-PFK	204
E.3	Double reciprocal ($1/v$ versus 1/[S]) Lineweaver-Burk plots for apparent K_m value of F-1,6-P_2 from the *Thermotoga* PP_i-PFK	205
E.4	Double reciprocal ($1/v$ versus 1/[S]) Lineweaver-Burk plots for apparent K_m value of P_i from the *Thermotoga* PP_i-PFK	206
E.5	Double reciprocal ($1/v$ versus 1/[S]) Lineweaver-Burk plots for apparent K_m value of PPP_i from the *Thermotoga* PP_i-PFK	207
E.6	Double reciprocal ($1/v$ versus 1/[S]) Lineweaver-Burk plots for apparent K_m value of F-6-P using PPP_i as phosphoryl donor from the *Thermotoga* PP_i-PFK	208
E.7	Double reciprocal ($1/v$ versus 1/[S]) Lineweaver-Burk plots for apparent K_m value of polyP (n=15±3) from the *Thermotoga* PP_i-PFK	209
E.8	Double reciprocal ($1/v$ versus 1/[S]) Lineweaver-Burk plots for apparent K_m value of F-6-P usng polyP (n=15±3) as phosphoryl donor from the *Thermotoga* PP_i-PFK	210
E.9	Double reciprocal ($1/v$ versus 1/[S]) Lineweaver-Burk plots for apparent K_m value of ATP from the *Thermotoga* ATP-PFK	211
E.10	Double reciprocal ($1/v$ versus 1/[S]) Lineweaver-Burk plots for apparent K_m value of F-6-P from the *Thermotoga* ATP-PFK	212
E.11	Double reciprocal ($1/v$ versus 1/[S]) Lineweaver-Burk plots for apparent K_m value of GTP from the *Thermotoga* ATP-PFK	213
E.12	Double reciprocal ($1/v$ versus 1/[S]) Lineweaver-Burk plots for apparen K_m value of F-6-P with 0.1 mM PEP from the *Thermotoga* ATP-PFK	214
E.13	Double reciprocal ($1/v$ versus 1/[S]) Lineweaver-Burk plots for apparent K_m value of F-6-P with 0.5 mM PEP	

from the *Thermotoga* ATP-PFK 215
E.14 Double reciprocal (1/*v* versus 1/[S]) Lineweaver-Burk plots for apparent K_m value of F-6-P with 1.0 mM PEP from the *Thermotoga* ATP-PFK 216
E.15 Double reciprocal (1/*v* versus 1/[S]) Lineweaver-Burk plots for apparent K_m value of F-6-P with 5.0 mM PEP from the *Thermotoga* ATP-PFK 217
E.16 Double reciprocal (1/*v* versus 1/[S]) Lineweaver-Burk plots for apparent K_m value of F-6-P with 0.01 mM PP_i from the *Thermotoga* ATP-PFK 218
E.17 Double reciprocal (1/*v* versus 1/[S]) Lineweaver-Burk plots for apparent K_m value of F-6-P with 0.025 mM PP_i from the *Thermotoga* ATP-PFK 219
E.18 Double reciprocal (1/*v* versus 1/[S]) Lineweaver-Burk plots for apparent K_m value of F-6-P with 0.05 mM PPP_i from the *Thermotoga* ATP-PFK 220
E.19 Double reciprocal (1/*v* versus 1/[S]) Lineweaver-Burk plots for apparent K_m value of F-6-P with 0.05 mM polyP from the *Thermotoga* ATP-PFK 221
E.20 Double reciprocal (1/*v* versus 1/[S]) Lineweaver-Burk plots for apparent K_m value of F-6-P with 1.0 mM citrate from the *Thermotoga* ATP-PFK 222

ABBREVIATIONS

ADP	adenosine 5′-diphosphate
AMP	adenosine 5′-monophosphate
ATCC	American Type Culture Collection
ATP	adenosine 5′-triphosphate
Bis-Tris	Bis-(2-hydroxyethyl)imino-tris-[hydroxymethyl]methane
bp	base pair
BSA	bovine serum albumen
CIAP	calf intestinal alkaline phosphatase
CTP	cytidine 5′-triphosphate
cAMP	cyclic adenosine 5′-monophosphate
dATP	2′-deoxyadenosine 5′-triphosphate
dCTP	2′-deoxcytidine 5′-triphosphate
DNA	deoxyribonucleic acid
dGTP	2′-deoxyguanosine 5′-triphosphate
DMSO	dimethyl sulphoxide
dsDNA	double stranded DNA
DSM	Deutsche Sammlung von Mikroorganismen
DTT	dithiothreitol
dTTP	2′-deoxythymidine 5′-triphosphate
EB	ethidium bromide
ED	Entner-Doudoroff
EDTA	ethylenediaminetetra acetic acid
EM	Embden-Meyerhof
FPLC	fast protein liquid chromatography

g	gravitational constant
GDP	guanosine 5′-diphosphate
GTP	guanosine 5′-triphosphate
h	hour
HEPES	N-[2-hydroxylethyl]piperazine-N'-[2-ethanesulfonic acid]
IEF	isoelectric focusing
IPA	isopropyl alcohol
IPTG	isopropylthiogalactoside
kb	kilobase pairs
kDa	kilodalton (protein molecular mass)
Klenow	large fragment *of E. coli* DNA polymerase I
K_m	Michaelis-Menton constant
*lac*Z	β-galactosidase gene
LB	Luria-Broth
LBA	Luria-Broth agar with ampicillin
LMP	low melting point
M	moles per litre
MES	2-[N-morpholino]ethanesulfonic acid
mM	milli Molar
MOPS	3-[N-morpholino]propane sulphonic acid
M_r	relative molecular mass (molecular weight)
MW	molecular weight
NADH	nicotinamide-adenine dinucleotide (reduced)
$NADP^+$	NAD phosphate (oxidised)
OD	optical density
PCR	polymerase chain reaction

PEG	polyethylene glycol
PEP	phosphoenolpyruvate
PFK	phosphofructokinase
P_i	inorganic orthophosphate
*p*I	isoelectric point
PMSF	phenylmethylsulfonyl fluoride
PolyP	polyphosphate
PP_i	inorganic pyrophosphate
rpm	revolution per minute
RT	room temperature
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSC	standard sodium citrate buffer
SSPE	standard salt phosphate EDTA buffer
STD	standard molecular marker
TAE	Tris-acetate EDTA electrophoresis buffer
TBE	Tris-borate EDTA electrophoresis buffer
TE	Tris-EDTA buffer
Tris	tri-hydroxymethyl amino methane
TRUCC	thermophile research unit culture collection
TTP	thymidine 5′-triphosphate
UDP	uridine 5′-diphosphate
UTP	uridine 5′-triphosphate
UV	ultraviolet light
V_{max}	maximum velocity
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indoly-β-D-galactoside

CHAPTER ONE

REVIEW OF LITERATURE

1.1 Glycolysis

The birth of modern biochemistry was at the beginning of the 20th century with the demonstration that alcoholic fermentation could be observed in mammalian muscle and yeast cells (Uyeda, 1979; Garrett and Grisham, 1995). Two German biochemists, Otto Meyerhof and Gustav Embden independently reported that organic phosphates are intermediates in alcoholic fermentation and showed that these organic phosphates are also intermediates in the conversion of glucose to lactate by muscle cells (Garrett and Grisham, 1995). Embden isolated fructose-1,6-bisphosphate (F-1,6-P_2) from muscle extracts and demonstrated that the phosphorylated sugar is an intermediate in lactate formation. He also proposed that F-1,6-P_2 is cleaved to form two triose phosphate molecules, glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, by both muscle cells and yeasts (Garrett and Grisham, 1995). Meyerhof using a cell-free muscle extract showed that it was able to convert glycogen into lactate, these establishing a relationship between glucose metabolism and lactate production. The work of Embden and Meyerhof was largely responsible for elucidating the glycolytic pathway, which is called either the glycolytic or the Embden-Meyerhof (EM) pathway (Uyeda, 1979; Garrett and Grisham, 1995).

In glycolysis, glucose is hydrolysed into two, three-carbon molecules and eventually to pyruvate (Fig. 1.1). Two ATPs are consumed and four ATPs are synthesised, which results in a net yield of two ATPs. Four electrons are also transferred to two molecules of NAD^+ (an electron carrier) resulting in the formation of two molecules of NADH. Glycolysis serves two general functions. One is that glucose is hydrolysed to generate ATP, reductant and pyruvate, and it produces building blocks for anabolism; the other function is as an amphibolic

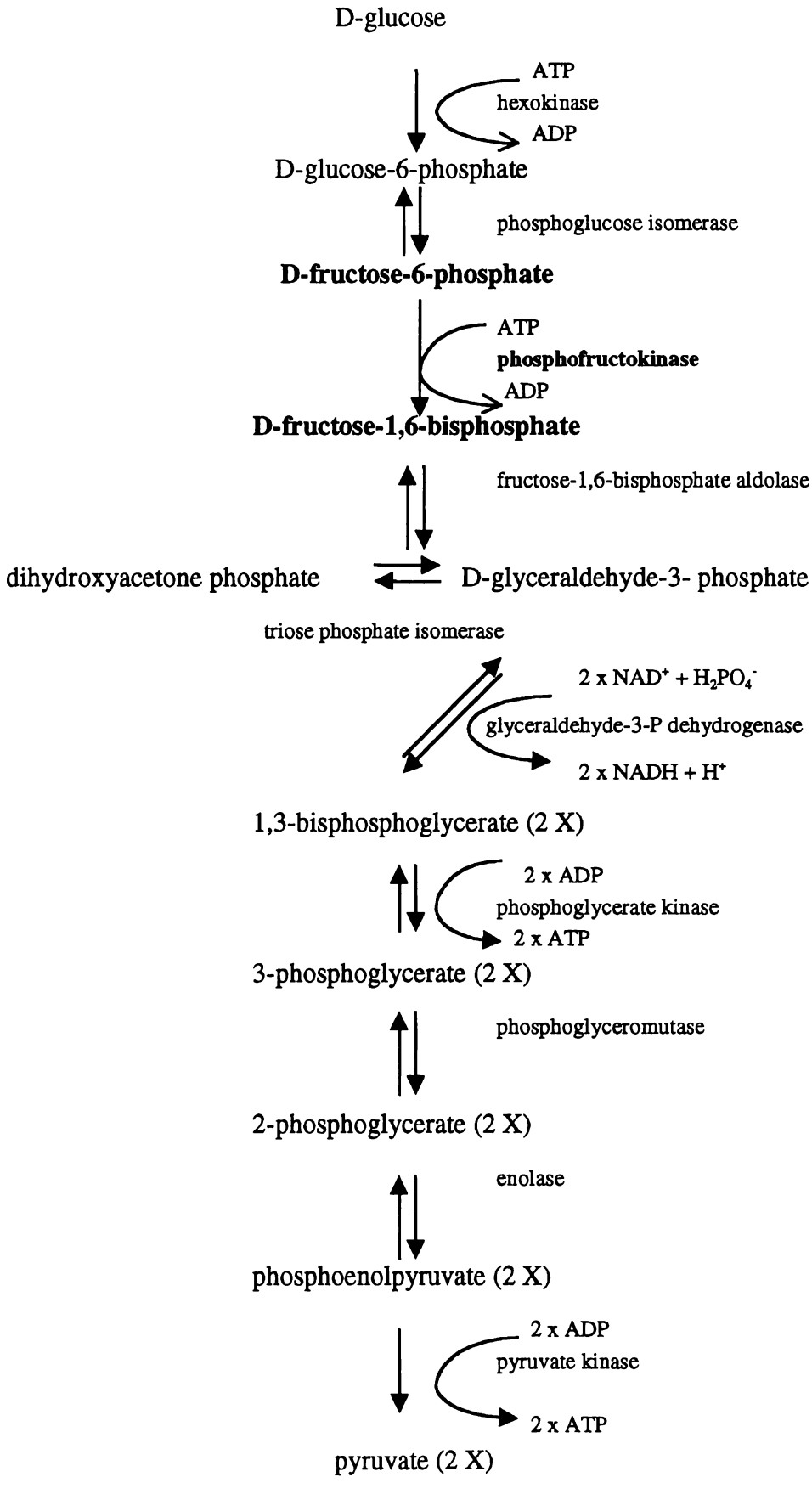


Figure 1.1 The Embden-Meyerhof or glycolytic pathway

pathway because it can function in reverse to generate hexoses from various low-molecular-weight compounds in energy-dependent gluconeogenesis in some organisms (Garrett and Grisham, 1995). In microorganisms, plants and animals (including humans), this pathway is carried out by 10 enzyme reactions in more or less similar fashion, although the rates of the individual reactions and the means by which they are regulated differ from species to species (Brock et al., 1994; Garrett and Grisham, 1995; Plaxton, 1996). The most significant difference among species, however, is the way in which the product pyruvate is utilised (Danson et al., 1998).

Glycolysis is regarded as an ancient metabolic process, as life is generally thought to have first appeared in an environment nearly devoid of oxygen (Fothergill-Gilmore and Michels, 1993). Glycolysis served as an important pathway for extracting energy from organic molecules and has played a central role in anaerobic metabolic processes in the early years of biological evolution on earth. Modern organisms still employ glycolysis to provide precursor molecules for aerobic catabolic pathways, i.e. the tricarboxylic acid cycle and as a short-term energy source when oxygen is limiting (Fothergill-Gilmore and Michels, 1993; Garrett and Grisham, 1995).

In addition of the EM pathway, there are another two pathways of conversion of sugars to pyruvate. They are the pentose phosphate pathway (also called the hexose monophosphate shunt) and the Entner-Doudoroff (ED) pathway (Stanier et al., 1986). The pentose phosphate pathway does not lead directly to pyruvate but it provides for the oxidation of one of the carbon atoms of glucose (Stanier et al., 1986). After three cycles, one glucose is degraded into one molecule of glyceraldehyde-3-phosphate and 3 molecules of CO_2.

The ED pathway only occurs in prokaryotes. In this pathway, one glucose is converted into 2 pyruvate by 4 enzymes. A unique intermediate of this pathway is 2-keto-3-deoxy-6-phosphogluconic acid (KDPG). The net yield of ATP from this pathway is one molecule (Stanier et al., 1986). Interestingly, in many archaea, the ED pathway is utilised but a semi-phosphorylated version of this pathway is found in halophiles, and some nonphosphorylated versions are found in the thermophilic genera of *Thermoplasma*, *Sulfolobus*, *Thermoproteus* and *Pyrococcus* (Danson et al., 1998). No net yield of ATP is generated by the nonphosphorylated ED route.

1.1.1 Glycolysis in eukaryotes

In plants, glycolysis is carried out in the cytoplasm of cells and begins with the cleavage of sucrose and proceeds via alternative parallel steps in a network-like fashion to pyruvate (Sung et al., 1988). In primitive eukaryotes, a similar catalytic process performs the EM pathway as in higher eukaryotes, except that a pyruvate:ferredoxin oxidoreductase complex, rather than the usual pyruvate dehydrogenase has been identified in some amitochondriate eukaryotes (Martin and Müller, 1998). Anaerobic glycolysis in muscle is utilised for ATP production during exercise when the oxygen supply is low and there is a large ATP requirement for muscle contraction. However, the liver controls the rate of glucose flux through the glycolytic pathway as a function of its need to replenish blood or to provide intermediates from glucose for the synthesis of other cellular constituents, e.g. fatty acids (Lemaigre and Rousseau, 1994). Glycolysis and gluconeogenesis in livers of adults are controlled at three substrate steps, i.e. the glucose/glucose-6-phosphate, F-6-P/F-1,6-P_2 and phosphoenolpyruvate (PEP)/pyruvate cycles. The individual reactions of each cycle are catalysed by different enzymes, which represent the main targets for the regulatory mechanism. Lemaigre and Rousseau (1994) have described two types of regulation. One is a short-term regulation related to both the supply of glycolytic or gluconeogenic

substrates and the control of the catalytic properties of the enzymes through allosteric changes and phosphorylation. The other is a long-term regulation and involves changes in gene expression and protein synthesis.

1.1.2 Glycolysis in bacteria and archaea

The EM pathway has been investigated in various species of bacteria, both aerobic and anaerobic and from thermophilic to mesophilic bacteria (O'Brien et al., 1975; Alves et al., 1996; Selig et al., 1997). The major characteristics of this pathway in the bacterial domain are essentially the same as those in eukaryotes. Interestingly, some properties of this pathway from thermophilic bacteria, i.e. the hyperthermophilic bacteria *Thermotoga maritima,* have also been well-studied (Schröder et al., 1994). Enzyme studies with cell-free extracts indicated that *T. maritima* ferments sugars via the classical EM pathway (85%) with an ATP-dependent glucokinase, ATP-dependent phosphofructokinase (ATP-PFK) and a classic glyceraldehyde-3-phosphate dehydrogenase. In addition, the ED pathway (15%) has been found to be operative with 2-keto-3-deoxy-6-phosphogluconate being identified, a product of the key enzyme of the conventional phosphorylated ED pathway (Selig et al., 1997). A glyceraldehyde-3-phosphate:ferredoxin oxidoreductase is not precent in *T. maritima* as is common in other hyperthermophilic archaea (Selig et al., 1997) but a pyruvate:ferredoxin oxidoreductase was found in this organism (Smith et al., 1994).

Archaea are phylogenetically deeply rooted and are considered to possess primitive physiological and metabolic traits, especially life at high temperatures and life without oxygen (Kengen et al., 1994). Their metabolic capabilities have allowed them to survive under conditions, which may be not too different from the early physical and chemical environment thought to have been present on earth some four billion years ago (Achenbach-Richter et al., 1987). Recently, an

increasing number of hyperthermophiles with the ability to utilise sugar as source for carbon and energy have been isolated (Kengen et al., 1994, 1996; Selig et al., 1997). For example, the proteolytic *Thermococcus litoralis* and *Thermococcus celer* showed good growth on starch (Canganella et al., 1994). The species belonging to the Desulfurococcales (*D. mucosus* and *D. mobilis*) which were thought to use only peptides were recently found to also grow on starch (Canganella et al., 1994). In a review by Kengen et al. (1996), the authors provided data showing that 27 hyperthermophiles could use different carbohydrates. In addition, enzymological studies from *Pyrococcus furiosus* have shown that this hyperthermophilic archaeon possesses a modified EM pathway, which involves an unprecedented ADP-dependent hexokinase, ADP-dependent phosphofructokinase and a unique glyceraldehyde 3-phophate:ferredoxin oxidoreductase.

1.1.3 Evolution of central metabolic pathways

The presence of glycolysis in all current domains of life is interpreted to indicate an origin of the pathway that was prior to the evolution of the last universal common ancestor (Fothergill-Gilmore and Michels, 1993). It is generally believed that the separation of life forms into three domains of life (Bacteria, Archaea and Eukarya) took place at a time when the average temperature on earth was much higher than today (Achenbach-Richter et al., 1987; Woese, 1990; Morgan and Ronimus, 1998). The EM pathway is particularly suitable for testing theories of enzyme evolution (Fothergill-Gilmore and Michels, 1993). Since this pathway is virtually ubiquitous, at least in part in all three domains of life, it is possible, therefore, to compare enzymes isolated from phylogenetically distant organisms (Fothergill-Gilmore and Michels, 1993). In addition, many amino acid sequences exist for each individual enzyme in this pathway and crystal structures are also available. Fothergill-Gilmore and Michels (1993) suggested that

glycolytic enzymes are probably among the most highly conserved enzymes known and are changing at a relatively low rate compared to the other cellular enzymes. In addition, some glycolytic enzymes catalyse similar types of reactions, which makes it possible to examine the proposal that an ancestral kinase may have given rise to the four glycolytic kinases (Fothergill-Gilmore and Michels, 1993). For example, for several of the enzymes bind the same ligand, e.g. ATP or NAD^+, it may be possible to resolve whether they have converged to provide similar functions or have diverged from common ancestors.

Before the discovery of PFK activity in the archaeal domain, it was generally believed that the central metabolic pathway in archaea was carried out through either the ED pathway or a modified ED pathway, as most of these archaea used non-carbohydrate substrates as their energy and carbon sources (Danson, 1993). Some early studies (Danson, 1989, 1993; Romano and Conway, 1996) also suggested that the ED pathway was of a more ancient origin than the EM pathway. In contrast, Kengen et al. (1996) proposed that the hypothesis of the ED pathway being regarded as more primitive is not true, as an increasing number of enzymes in the EM pathway have being identified in various archaea. This study and others strongly indicate that the EM pathway, or variations of the pathway, is also present in other extremely thermophilic archaea (De Rosa et al., 1984; Siebers and Hensel, 1993; Kengen et al., 1994; Selig and Schönheit, 1994) and bacteria (Janssen and Morgan, 1992).

1.2 Phosphofructokinase (PFK)

The primary site of regulation of the glycolytic flux in higher organisms is believed at the step from F-6-P to F-1,6-P_2 which is catalysed by a physiologically irreversible ATP-dependent PFK (Uyeda, 1979). This reaction is regarded as the first unique step of the pathway and the enzyme is normally subject to strong metabolic regulation (Kemera et al., 1975; Uyeda, 1979). So far, three subtypes

of PFK, with respect to their phosphoryl donor, have been investigated within three domains of life: an ATP-, a PP_i- and an ADP-dependent form.

1.2.1 ATP-dependent phosphofructokinases (ATP-PFKs)

ATP-PFKs are widely distributed in the Eukarya and Bacteria as mainly a tetrameric, allosteric enzyme and possess significant similarities in their amino acid sequences from bacteria to mammals (Deville-Bonne et al., 1991a, 1991b; Li et al., 1993; Kopperschläger, 1994). The reaction catalysed by PFK is strongly regulated by a variety of activators and inhibitors (Uyeda, 1979). The ATP:D-fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.11) catalyses the following reaction (Morgan and Ronimus, 1998).

$$ATP + F\text{-}6\text{-}P \longrightarrow F\text{-}1,6\text{-}P_2 + ADP \ (\Delta G^{\circ'} = -18.45 \text{ kJ/mol})$$

ATP-PFKs are found in most highly evolved organisms, primitive eukaryotes, bacteria and one member of archaea (Table 1.1). For example, the ATP-PFKs have been identified from rabbit muscle (Walker et al., 1976) and human erythrocytes (Layzer, 1975), higher plants (Häusler et al., 1989), yeast (Stellwagen and Harvey, 1975), *Dictyostelium discoideum* (Martínez-Costa et al., 1994), primitive eukarya, *Schistosoma mansoni* (Su et al., 1996), *Trypanosoma brucei* (Cronin and Tipton, 1985), *Bacillus stearothermophilus* (Byrnes et al., 1994), *Escherichia coli* (Deville-Bonne et al., 1991a, 1991b; Kemerer et al., 1975), *Clostridium pasteurianum* (Uyeda and Kurooka, 1975), *Lactobacillus bulgaricus* (Le Bras et al., 1991), *Thermotoga maritima* (Selig et al., 1997), *Thermus* strain X-1 (Yoshida, 1972) and the crenarchaeon *Desulfurococcus amylolyticus* (Selig et al., 1997). Most ATP-PFKs are tetramers except one example of an octomeric enzyme ($\alpha_4\beta_4$) in yeast (Uyeda, 1979). PFKs from mammals are similar to those from bacteria in their responses to the F-6-P concentration in that they display cooperatively (Kemp 1975; Su and Storey, 1993; Byrnes et al., 1994).

Table 1.1 Summary of properties of some ATP-PFKs from primitive eukaryotes, bacteria and archaea

Organisms & PFK subtypes	M_r (kDa)	Native	K_m (μM) F-6-P	K_m (μM) P-donor	pH (F) [a]	K_m (μM) F-1,6-P_2	K_m (μM) P_i	pH (R) [b]	Reference
ATP-PFKs in primitive eukarya									
D. discoideum	96	tetramer	22	16	7.6	-	-	-	Martínez-Costa et al., 1994
S. mansoni	86	tetramer	3,500	150	8.2	-	-	-	Su et al., 1996
T. brucei	53	tetramer	999	47	6.7	-	-	-	Cronin and Tipton, 1985
ATP-PFKs in bacteria									
B. stearothermophilus	36	tetramer	30	70	8.2	-	-	-	Byrnes et al., 1994
E. coli	34	tetramer	60	49		-	-	-	Johnson and Reinhart, 1992
L. bulgaricus	35	tetramer	300	70	8.2	-	-	-	Le Bras et al., 1991
T. maritima	-	-	2800	90	-	-	-	-	Schröder et al., 1994;
Thermus sp. X-1	-	-	600	20	9.0	-	-	-	Yoshida, 1972
ATP-PFK in archaea									
D. amylolyticus	-	-	5,000	60	-	-	-	-	Selig et al., 1997

[a] forward reaction; [b] reverse reaction; -: not determined

However, mammalian PFKs possess differences to bacterial enzymes. For instance, three types of isoenzyme subunits (M for muscle, L for liver and C for the third type) have been found based on structure and enzymology (Brennan et al., 1974; Kemp, 1975; Uyeda, 1979; Gekakis et al., 1994). The liver PFK is similar to the muscle enzyme in pH optimum. In addition, the liver enzyme is more inhibited by ATP, less sensitive to the de-inhibiting action of AMP, ADP, and cAMP, less inhibited by citrate, phosphoenolpyruvate (PEP), phosphocreatine, and 3-phosphoglycerate, and more inhibited by 2,3-diphosphoglyceric acid than muscle PFK (Kemp, 1975).

The subunit molecular weights of ATP-PFKs from *B. stearothermophilus*, *D. discoideum* and rabbit muscle are 36,000, 96,000 and 82,000, respectively (Walker et al., 1976; Byrnes et al., 1994; Martínez-Costa et al., 1994). Most eukaryotic ATP-PFKs have relatively high apparent K_m values for F-6-P and neutral or slightly alkaline (7.2-8.5) optimal pH values for activity (Uyeda, 1979). An interesting control mechanism is found with the catalytically active muscle enzyme, which is a tetramer of identical subunits but dissociates into inactive dimers in the presence of citrate (Uyeda, 1979). The muscle and liver enzymes are encoded by different genes and have distinct kinetic and structural properties. This may reflect, at least in part, the different roles of glucose metabolism in the two tissues (Uyeda, 1979; Lemaigre and Rousseau, 1994).

The ATP-PFK subunits from *B. stearothermophilus* and *E. coli* (Evans and Hudson, 1979; Shirakihara and Evans, 1988) each consists of two domains and each domain has a control β-sheet sandwiched between α-helices, a type of fold which has been seen in a number of proteins (Fig. 1.2). Each subunit forms close contacts with only two of the other subunits in the tetramer. Each subunit has an interface with two other subunits, one of these is bridged by F-6-P, the other by the effector, and thus, catalysis and effector control requires the whole tetramer (Evans and Hudson, 1979). The active site is close to the effector site, and the part of the enzyme that lies between the site, particularly residues 151 to 159, has side chains pointing in one direction to the F-6-P site and in the opposite direction to the effector site. All three binding sites in ATP-PFK bind phosphate groups, but there is interesting difference between them. Sites A and C both bind an inorganic phosphate ion as well as their specific ligands, however, site B does not bind inorganic phosphate and does not have such a clearly positively charged nature (Evans and Hudson, 1979).

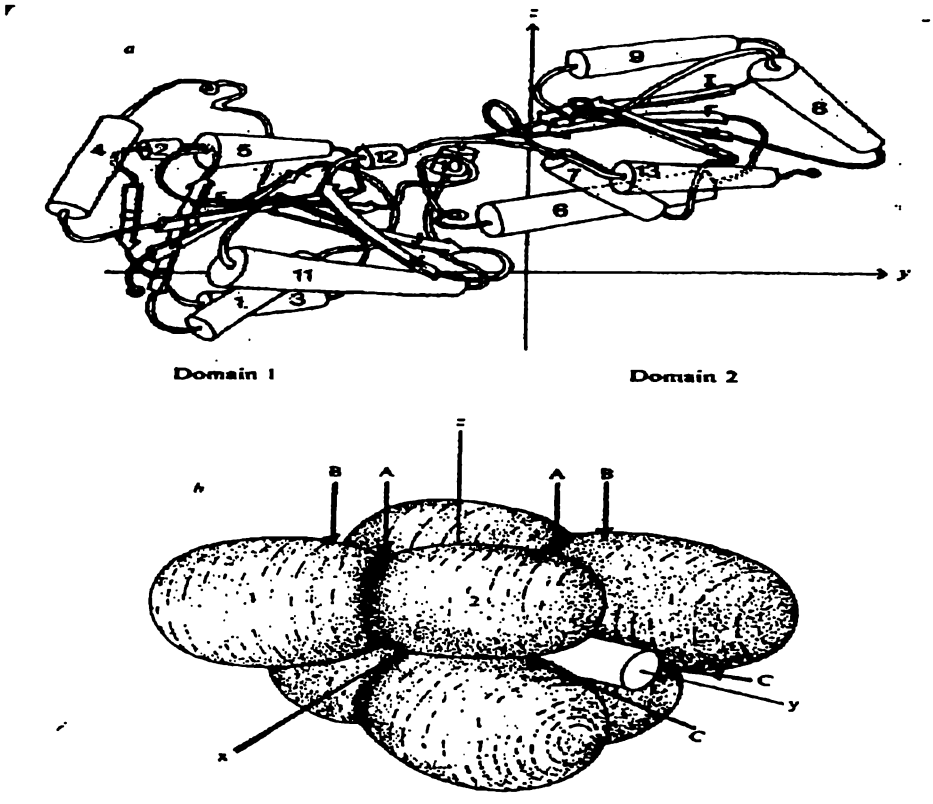


Figure 1.2 Schematic views of two subunits in the *B. stearothermophilus*-PFK tetramer from Evans and Hudson (1979). *a*, schematic drawing of the polypeptide chain of one subunit, viewed along the x-axis. Arrows represent β-sheet strands (A-K) and cylinders represent α-helices (1-13). *b*, schematic drawing of the tetramer of PFK. Each subunit is shown divided into two domains, numbered 1 and 2. Four subunits are related by three orthogonal dyad axes (222 symmetry), which coincide with the crystallographic symmetry axes. There is a solvent filled hole of ~7 Å diameter through the centre of the tetramer along the y-axis, indicated by a cylinder. The positions of some of the sites A, B and C are marked, showing that sites A and C lie between subunits.

MgATP is the substrate, whereas uncomplexed ATP binds at an allosteric, inhibitory site, thereby increasing the apparent K_m value for F-6-P. Most ATP-PFKs are inhibited by a relatively high ATP concentration at pH below 7.1 but show no inhibition at all at pH 9 (Uyeda, 1979). The effect of pH on the inhibition by ATP of the yeast and *E. coli* enzymes is the opposite to that of mammalian PFKs. The tested ATP-PFKs from yeast, plants and mammals are also inhibited by citrate, 3-phosphoglycerate, 2-phosphoglycerate, 2,3-

phosphoglycerate and PEP when the pH is at or below neutral and in the presence of an inhibitory concentration of ATP (Uyeda, 1979). Van Schaftingen et al. (1987) discovered that F-2,6-P_2 can act as a potent regulator of liver PFK. In contrast to these inhibitors, some modulators can activate ATP-PFKs, e.g. AMP, cAMP, ADP, P_i, F-6-P and F-1,6-P_2.

Although most ATP-PFKs are allosterically controlled and homotetrameric, some examples of non-allosteric ATP-PFKs have been identified from the slime mold *D. discoideum* (Martínez-Costa et al., 1994), *L. bulgaricus* (Branny et al., 1996) and a dimeric PFK-2 from *E. coli* (Uyeda, 1979; Kotlarz and Buc, 1981). PFK-2 has been biochemically characterised from *E. coli* (Kotlarz and Buc, 1981). The *E. coli* PFK-2 activity is inhibited by high concentrations of ATP, which also provokes the tetramerisation of the dimeric native enzyme (Kotlarz and Buc, 1981).

1.2.2 Pyrophosphate-dependent phosphofructokinases (PP_i-PFKs)

1.2.2.1 The history of PP_i-PFKs

In 1974, Reeves et al. reported the first PP_i-PFK (inorganic pyrophosphate:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.90) activity in the amoebic parasite *Entamoeba histolytica.* This discovery was based on their previous work on PP_i-dependent enzymes from *E. histolytica* (Reeves and Malin, 1969; Reeves, 1970) and in a propionic acid bacterium (Cagen and Friedmann, 1968). Subsequently, PP_i-PFKs have been reported to be present in many anaerobic bacteria, primitive eukaryotes and in some plants (O'Brien et al., 1975; Yan and Tao, 1984; Mertens et al., 1990; Janssen and Morgan, 1992; Morgan and Ronimus, 1998). PP_i-PFK catalyses the transfer of phosphate from PP_i to F-6-P forming F-1,6-P_2 and P_i (Morgan and Ronimus, 1998).

$$PP_i + \text{F-6-P} \longleftrightarrow \text{F-1,6-}P_2 + P_i \quad (\Delta G^{o'} = -8.70 \text{ kJ/mol})$$

The free energy of hydrolysis of inorganic diphosphate ($\Delta G^{o'}$=-21.9 kJ/mol) is less than that of ATP ($\Delta G^{o'}$=-31.8 kJ/mol) under physiological conditions but it is high enough to provide an energy source for most synthetic reactions (Wood et al., 1977). The PP_i-PFK-mediated reaction is also more reversible and may function as a primitive fructose-1,6-bisphosphatase (FBPase) under physiological conditions (Kemp and Tripathi, 1993).

Kinetic studies have shown that the PP_i-PFKs from the bacterium *Propionibacterium freudenreichii* and plants are allosterically regulated by F-2,6-P_2 (O'Brien et al., 1975; Miyatake et al., 1984; Kruger et al., 1988). Non-allosteric PP_i-PFKs, however, have been identified mainly in bacteria and some primitive eukaryotes (Reeves et al., 1974; Mertens, 1993; Alves et al., 1996). This implies that PP_i-PFKs are unlikely to play a physiological role in metabolic control, which is a primary feature of ATP-PFKs. Interestingly, the PP_i-PFK from protist *Naegleria fowleri* is activated by AMP (Mertens et al., 1993) but the sequencing, cloning and expression results suggested that no AMP binding site was present on the protein (Wessberg et al., 1995). Although most of the characterised PP_i-PFKs are dimeric, some have been found to be either tetrameric or monomeric (Mertens, 1993). In addition, most PP_i-PFKs require a slightly acidic pH (5.6-6.0) for F-1,6-P_2 formation and neutral or slightly alkaline (7.2-7.6) for F-6-P formation, but the enzymes from *E. histolytica*, *Giardia lamblia*, *P. freudenreichii* and *Amycolatopsis methanolica* have a near-neutral pH for both the forward and reverse reactions (Table 1.2 and Table 1.3).

1.2.2.2 PP_i-PFK activity in primitive eukaryotes

Since the first PP_i-PFK was found in the primitive eukaryote *E. histolytica*, a number of this type of PFK has been widely identified from other primitive eukaryotes, i.e. *Eimeria tenella* (Denton et al., 1994, 1996), *Euglena gracilis*

(Enomoto et al., 1988;), *Isotricha prostoma* (Mertens et al., 1989b), *Toxoplasma gondii* (Peng and Mansour, 1992), *Trichomonas vaginalis* (Mertens et al., 1989b) and *Tritrichomonas foetus* (Mertens et al., 1989b). Indeed, the PFKs of almost all amitochondriate organisms studied, including the most deeply rooted organism *G. lamblia*, uses PP_i as the phosphoryl donor instead of ATP (Müller, 1992).

Table 1.2 The properties of some PP_i-PFKs from primitive eukaryotes

Organisms & PFK subtypes	M_r (kDa)	Native	K_m (μM) F-6-P	K_m (μM) P-donor	pH (F) [a]	K_m (μM) F-1,6-P_2	K_m (μM) P_i	pH (R) [b]	Reference
E. tenella	-	-	77	-	6.8	-	-	7.5	Denton et al., 1994
E. histolytica	83	dimer	38	14	7.0	18	800	7.0	Reeves et al., 1974
E. gracilis	110	monomer	300	100	6.5	-	-	-	Enomoto et al., 1988;
G. lamblia	42	dimer	80	11	7.2	8	510	7.0	Li and Phillips, 1995
I. prostoma	48	dimer	80	12	<5.0	10	1250	7.0	Mertens et al., 1989b
N. fowleri	51	tetramer	10	15	<6.0	35	590	7.5	Mertens et al., 1993;
T. gondii	45	dimer	270	33	6-7	-	-	7-8.0	Peng and Mansour, 1992
T. vaginalis	50	tetramer	110	25	<5.0	10	1250	7.5	Mertens et al., 1989b
T. foetus	48	-	120	14	<5.0	-	-	-	Mertens et al., 1989b

[a] forward reaction; [b] reverse reaction; -: not determined

Phylogenetic reconstructions based on the amino-terminal half of the molecule reveal significant divergence of the protist PP_i-PFKs. Müller (1992) suggested that no clear evolutionary trends of PFKs could be discerned in these primitive eukaryotes and it needed to be ascertained whether the transition from one to the other phosphoryl donor (from ATP to PP_i or PP_i to ATP) occurred only once or several times in the various lineages.

1.2.2.3 PP_i-PFK activity in plants

In addition to the primitive eukaryotic examples mentioned above, a number of PP_i-PFKs from higher plants have also been investigated (Carnal and Black, 1979; Yan and Tao, 1984; Kruger et al., 1986; Wong et al., 1988; Hatzfeld et al., 1989; Mahajan and Singh, 1989; Botha and Botha, 1993; Enomoto et al., 1994; Tripodi and Podesta, 1997). It has been suggested that these PP_i-PFKs may play at least as important a role as ATP-PFKs in plant cytoplasmic glycolysis. Sung et al. (1988) described PP_i as an integral component of plant glycolysis and gluconeogenesis and suggested it to have some key functions, i.e. either substituting for ATP through the reversible PP_i-PFK or supporting the breakdown of sucrose via glycolysis (van Schaftingen, 1987).

Two forms of PP_i-PFKs have been purified and characterised from wheat seedlings (Yan and Tao, 1984). The enzymes had very similar kinetic properties with low apparent K_m values for F-6-P but not for PP_i, and the enzymes were activated by F-2,6-P_2. The PP_i-PFK-1 of wheat seedlings was indicated to be a tetramer with two subunits (M_r=67,000 and 60,000 for α and β subunits, respectively). The PP_i-PFK-2 was probably a homodimer with a subunit of M_r=60,000 (Yan and Tao, 1984). The amount of PP_i-PFK activity in watermelon (*Citrullus lanatus*) was shown to be related to gluconeogenic flux (Botha and Botha, 1993). The peak of PP_i-PFK activity coincided with the maximum gluconeogenic flux and high F-2,6-P_2 concentration. The FBPase activity was only one-third of the PP_i-PFK activity. The data suggested that the low cytosolic FBPase activity and high F-2,6-P_2 most probably resulted in inadequate *in vivo* activity to catalyse the observed gluconeogenic flux. The total PP_i-PFK activity was sufficient to catalyse the observed carbon flux (Botha and Botha, 1993). The proposed functions for PP_i-PFK include a role in sucrose hydrolysis and the maintenance of equilibrium between the hexose phosphate and triose phosphate

pools and biosynthetic activity (Black et al., 1986). Mertens et al. (1989) investigated the PP_i-PFK activity in rice seedlings under anoxic conditions and the results showed a preferential involvement of PP_i-PFK rather than of ATP-PFK in glycolysis. The potential advantage of the use of PP_i-PFK during anaerobiosis could allow an increase of up to 50% in the ATP yield of glycolysis. However, this energetic advantage is likely to be minimal during aerobic conditions (Mertens et al., 1990).

The presence and relative activities of PP_i- and ATP-PFK found in higher plants are of interest. Botha and Small (1987) studied both of the enzymes from germinating bean (*Phaseolus vulgaris*) seeds and found that the activity of the PP_i-PFK and ATP-PFK varied depending on the stage of germination. The cytosolic isoenzyme of ATP-PFK exhibits hyperbolic kinetics with respect to F-6-P and ATP. The PP_i-PFK also exhibited hyperbolic kinetics both in the presence and absence of the activator F-2,6-P_2. The activation of F-2,6-P_2 was due to an increase in V_{max} and affinity for both F-6-P and PP_i (Botha and Small, 1987). In addition, both the PP_i- and ATP-PFK activity was investigated during germination of rice seeds by Enomoto et al. (1994). The PP_i-PFK activity in the rice seedlings was always higher than that of the ATP-PFK, which was almost constant during germination. The high PP_i-PFK activity was detected in the growing point of seedlings, where cell division was active associated with biosynthesis, and low activity, equal to ATP-PFK activity, found in the base of these organs (Enomoto et al., 1994). It was reported that glycolysis was regulated by the cellular activity of PP_i-PFK under anaerobic conditions when ATP generation is required at a high rate for biosynthetic reactions in tissues where cell division is active (Enomoto et al., 1994).

In addition, the relative ratio of the maximum activity of PP_i- and ATP-PFK, and the relationship between the activity of PP_i-PFK and the capacity for biosynthesis

of macromolecules were also examined in different segments of seedlings of *Vigna mungo* (Ashihara and Sato, 1993). A high relative ratio of the maximum activity of PP_i-PFK to that of ATP-PFK was found in young tissues and lower ratios were found dependent on the degree of differentiation and age of the tissues. The highest level of F-2,6-P_2 was also observed in the youngest part of hypocotyls of *Vigna mung*. This result supported the hypothesis that PP_i-PFK is particularly important in young, metabolically active tissues and functions as a glycolytic enzyme, which is closely related to biosynthetic pathways (Ashihara and Sato, 1993).

1.2.2.4 PP_i-PFK activity in bacteria and archaea

The first bacterial PP_i-PFK identified was from *P. shermanii* later renamed *P. freudenreichii* (O'Brien et al., 1975). Subsequently, a large number of PP_i-PFKs have been studied from other bacteria (Table 1.3), i.e. *Alcaligenes sp.*, *Pseudomonas marina* (Sawyer et al., 1977), *A. methanolica* (Alves et al., 1996), *Anaeroplasma intermedium*, *Clostridium inoculum* (Petzel et al., 1989), *Rhodospirillum rubrum* (Pfleiderer and Klemme, 1980), *Spirochaeta thermophila* (Janssen and Morgan, 1992) and a crenarchaeon *Thermoproteus tenax* (Selig et al., 1997). A particularly well studied bacterial PP_i-PFK is from *P. freudenreichii* (Bertagnolli and Cook, 1984; Green et al., 1994). Surprisingly, the entire amino acid sequence comparison between the *P. freudenreichii* PP_i-PFK and potato PP_i-PFK showed only 19% identity (Fothergill-Gilmore and Michels, 1993). A phylogenetic tree based on 16S rRNA sequence comparison reveals that the hyperthermophilic crenarchaeon *Thermoproteus tenax* is deeply rooted (Woese, 1990). This organism is able to grow chemolithoautotrophically on H_2, CO_2 and S° as well as chemoorganotrophically in the presence of S° (Zillig et al., 1981). It can also use glucose and several polysaccharides by the EM pathway with an ATP-dependent glucokinase and a PP_i-PFK (Selig et al., 1997). The discovery of

a PP_i-PFK from this organism was very interesting as it suggested that the EM pathway could also play a major function in this archaea. Interestingly, the non-phosphorylated ED pathway was also identified to be present in this organism as well (Siebers and Hensel et al., 1993).

Table 1.3 Properties of some PP_i-PFKs from bacteria and archaea

Organisms & PFK subtypes	M_r (kDa)	Native	K_m (μM) F-6-P	K_m (μM) P-donor	pH (F) [a]	K_m (μM) F-1,6-P_2	K_m (μM) P_i	pH (R) [b]	Reference
PP_i-PFK in bacteria									
Alcaligenes sp.	-	-	-	-	-	-	-	-	Sawyer et al., 1977
A. methanolica	43	tetramer	400	100	7.5	25	840	7.5	Alves et al., 1996
A. intermedium	-	-	-	-	-	-	-	-	Petzel et al., 1989a
C. inoculum	-	-	-	-	-	-	-	-	Petzel et al., 1989a
P. freudenreichii	43	dimer	100	69	7.0	51	600	7.4	O'Brien et al., 1975;
P. marina	-	-	-	-	-	-	-	-	Sawyer et al., 1977
R. rubrum	40	dimer	380	25	7.2	-	-	8.6	Pfleiderer and Klemme, 1980
S. thermophila	-	-	-	-	-	-	-	-	Janssen and Morgan, 1992
PP_i-PFK in archaea									
T. tenax	37	dimer/ trimer	53	23	-	33	1,430	-	Selig et al., 1997

[a] forward reaction; [b] reverse reaction; -: not determined

1.2.3 ADP-dependent phosphofructokinase (ADP-PFK)

A unique ADP-PFK has been identified in the genera *Pyrococcus* (*P. furiosus*) and *Thermococcus* (*T. celer, T. litoralis* and *T. zilligii*) of the archaeal kingdom Euryarchaeota (Kengen et al., 1995, 1996; Selig et al., 1997). This enzyme catalyses the following reaction:

$$ADP + F\text{-}6\text{-}P \longrightarrow F\text{-}1,6\text{-}P_2 + AMP$$

No $\Delta G^{\circ'}$ value has been given for the ADP-driven reaction but presumably it lies near the values of the ATP reactions. In addition, a novel ADP-dependent glucokinase (Kengen et al., 1995) has been identified in *P. furiosus* which suggested that modifications of the classical EM pathway at one or more of these steps have occurred. The discovery of an ADP-PFK in these organisms is of particular interest because it represents a further modification of the normal glycolytic pathway. In addition, it has been found that the conversion of glyceraldehyde 3-phosphate to 3-phosphoglycerate proceeds via a ferredoxin oxidoreductase, without the intermediate 3-diphosphoglycerate that would otherwise lead to the formation of ATP (Danson et al., 1998). Danson et al. (1998) also proposed that the energy yields from these archaeal pathways were low as no net yield of ATP was generated in either *Thermococcus* or *Pyrococcus*.

1.3 Phylogeny and evolution of PFK

Molecular phylogeny started in the 1960s when some protein sequences became available, e.g. ferredoxins and cytochromes (Smith, 1968). In the mid-1970s, Woese and his collaborators began to assemble an extensive database of sequence information on small subunit ribosomal RNAs (SSU rRNA), as this molecule possesses some advantages for determining phylogeny (Woese et al., 1990). For example, it contains both slowly and quickly evolving sequences and it represents an ancient and essential functions in the traditional machinery.

By studying the evolution of PFKs, an enhanced understanding of how glycolysis evolved may be achieved. It might be possible to resolve the evolution of the proteins as far back as the last common ancestor of life by comparing the amino acid sequences of PFKs. Crystallographic structures of related proteins suggest that the tertiary structure of a particular protein from distantly related species changes less rapidly than its amino acid sequences during evolution by natural

selection (Fothergill-Gilmore and Michels, 1993). Some unique properties of PFKs (described in Section 1.13) led to this enzyme being used as a candidate to study the evolution of enzymes and glycolysis itself.

The current hypotheses to account for the distribution of PP_i-PFKs in different organisms make quite different assumptions about the phylogeny of the enzyme (Morgan and Ronimus, 1998). Mertens (1991) proposed that "The distribution of PP_i-PFK in the living world appears to correlate better with metabolic characteristics (anaerobiosis) than with phylogenetic relationships. An interesting possibility is, therefore, that PP_i-PFK derived from ATP-PFK, probably not once but on several independent occasions". Mertens (1991) explanation of the occurrence of the enzyme in quite diverse and unrelated phylogenetic lineages was the evidence of its spontaneous derivation from an ATP-PFK on many separate occasions; a likely reason for this was adaptation to an anaerobic mode of existence (Morgan and Ronimus, 1998). Mertens (1991) argued further that the prevalence of PP_i-PFKs in obligate anaerobes (including the anaerobic protists *Giardia* and *Entamoeba*) and its absence from aerobic bacteria was support for this contention.

The reverse argument, that PP_i-PFKs preceded and gave rise to ATP-PFKs, has been made by Alves et al. (1996, 1997), Michels et al. (1997), Morgan and Ronimus, (1998) and Siebers et al. (1998). The amino acid sequence comparisons of the ATP-PFKs from *S. coelicolor* and *T. brucei* have strongly suggested that

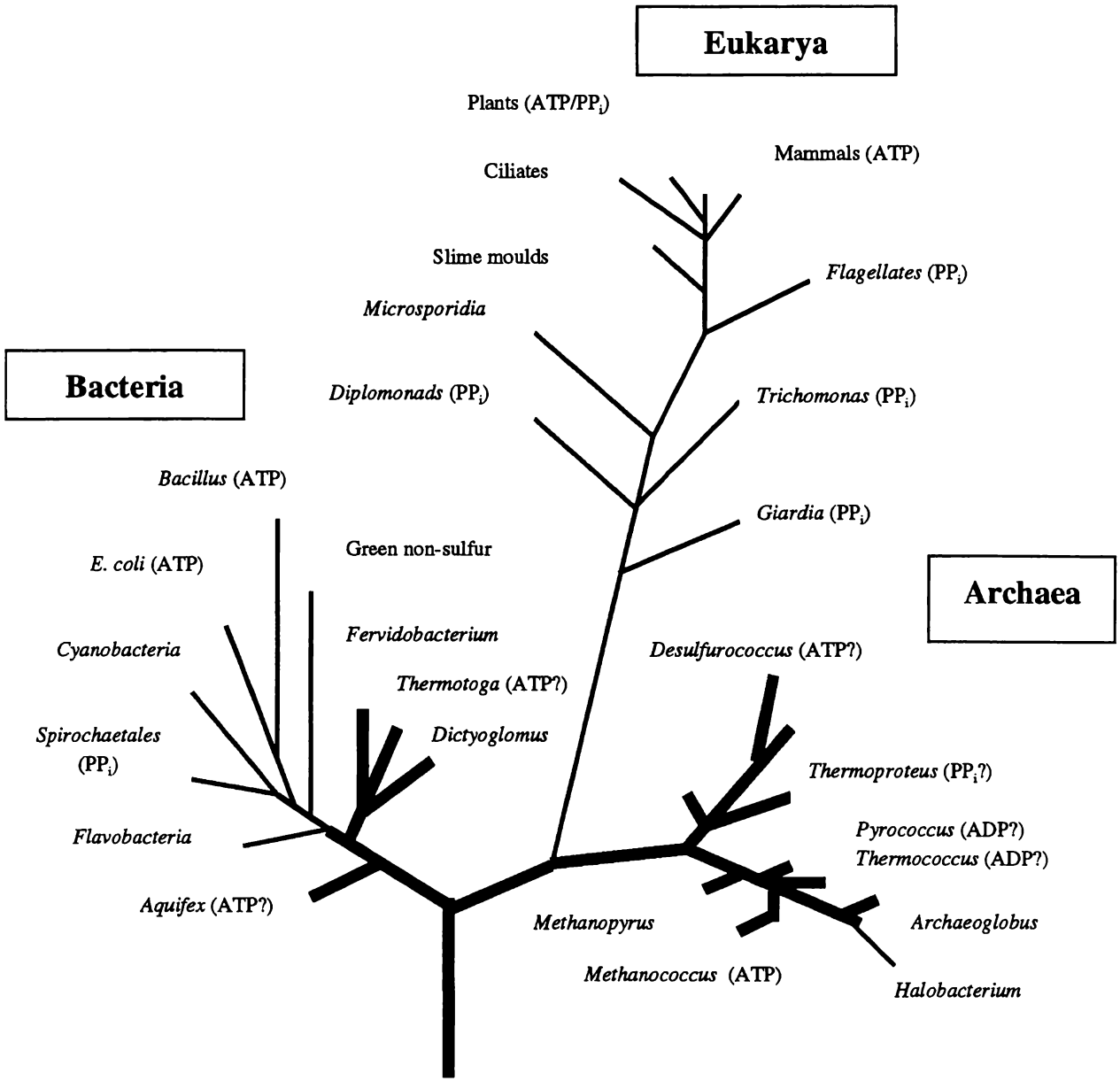


Figure 1.3 A phylogenetic tree based on 16S rRNA sequence comparisons and distribution of PFK subtypes prior to thesis. The division of organisms into three domains of life and subdivisions are shown. The tree also shows the distribution of PFKs within the three domains before 1997. ATP: represents ATP-PFK; PP_i: represents PP_i-PFK; ADP: represents ADP-PFK; ?: represents enzyme activity detected in cell-free extracts or putative PFK genes without biochemical characterisation. Thicker and bold lineages are extremely or hyperthermophilic organisms.

the ATP-PFKs are derived from PP_i-PFK ancestors (Alves et al., 1997; Michels et al., 1997). In addition, Baltscheffsky (1996) also suggested that the ATP world likely evolved from a PP_i world based on the analysis of the three prenucleotide

"worlds" (thioester, iron-sulfur and PP_i). Furthermore, Yamagata et al. (1991) studied volcanic activity and demonstrated the continuous formation of PP_i, tripolyphosphate (PPP_i) and tetrapolyphosphate in condensates from volcanic magma. They obtained direct evidence for the production of polyphosphate (polyP, n=4) by collecting condensates of gas from several fumaroles at temperatures of between 540 and 690°C. The concentration of orthophosphate in the samples after hydrolysis was found to be 6-9 μM, whereas before hydrolysis it was about 1 μM. This result indicated that a high proportion of polyP was present. Yamagata et al. (1991) proposed that the volcanic production of polyP could be related to prebiotic evolution. The use of PP_i as phosphoryl donor has been suggested to reflect its use in energy conversion processes leading to its role in the origin of metabolism and early evolution of life (Baltscheffsky, 1996). The presence of PP_i in prebiotic environments could have provided both the phosphate and energy source for evolution of early metabolic pathways (Yamagata et al., 1991; Baltscheffsky, 1996). As PP_i formation was a feature of hydrothermal and volcanic systems, there was the possibility that thermophilic bacteria and archaea might have retained the use of this energy source to a greater degree than other nonthermophilic organisms, because of its ready availability in their environment. It has been reported that enzymes in photosynthetic bacteria using PP_i are used to drive essential metabolic processes, e.g. *R. rubrum* and related organisms (Keister and Minton, 1971). In these organisms, PP_i is an energy donor for cytochrome reduction, pyridine nucleotide transhydrogenation, NAD^+ reduction and ATP synthesis reactions indicating that PP_i may play an important role in energy conservation.

1.4 The organisms studied in this work

Thermophilic organisms can be classified into different subgroups based on their optimum temperature of growth. Organisms growing optimally from 55-60°C and

over 70°C are defined as thermophiles and extreme thermophiles, respectively (Stetter et al., 1990). Organisms with optimum growth of 80°C or higher are termed hyperthermophiles (Huber et al., 1986; Stetter et al., 1993). These organisms are found and isolated from geothermal areas, e.g. acidic hot springs in solfatara fields, neutral to alkaline fresh-water hot springs and geysers, and deep-sea hydrothermal vent systems (Huber et al., 1986; Patel et al., 1987; Rainey et al., 1991). In addition to optimal growth temperatures, pH is also an important physical factor in thermal biotic environments. Some extremely thermophilic organisms were the object of study in this thesis. Their properties of these organisms are described in following sections.

1.4.1 *Dictyoglomus thermophilum* Rt46 B.1

The first isolate *D. thermophilum* (Greek/Latin derivation: Dictyo=net, glomus=ball; thermus=heat, philum=loving), was reported from a Japanese hot spring and was described as a non-spore forming, non-motile, obligately anaerobic, rod-shaped, Gram negative bacterium (Saiki et al., 1985). This organism may occur singly, in pairs, in filaments or in bundles as its name suggests with several hundred cells which are surrounded by a common outer wall or membrane (Saiki et al., 1985). *D. thermophilum* is extremely thermophilic and has a temperature range for growth from 50-80°C, with an optimum temperature at 73°C. The pH range for growth is between 5.9-8.3. The G+C% nucleotide content of the genome is 29%. The organism can utilise soluble starch, glucose, lactose, galactose, maltose, sucrose and xylan but will not grow on cellulose or insoluble starch (Saiki et al., 1985; Mathrani and Ahring, 1991).

D. thermophilum Rt46 B.1 was isolated by Patel et al. (1987) from a geothermal hot spring at Kuirau Park, Rotorua, New Zealand. This organism is an obligate anaerobe and was originally described as "*Fervidothrix*" but was later determined

to be a strain of *D. thermophilum* based on its morphology, carbohydrate utilisation, G+C% (29.5%) content and growth conditions. The pH for growth for strain Rt46 B.1 was pH 6.0-8.5 with optimal growth at pH 7.5. Growth was inhibited by penicillin, tetracycline and chloramphenicol, indicating that the organism was a bacterium. The organism could use numerous carbohydrates for growth and fermentation products included ethanol, lactate, acetate and H_2 (Patel et al., 1987). The distribution of *Dictyoglomus* strains appears to be restrictive. Saiki et al. (1985) obtained a positive enrichment in only one of the six springs sampled. Patel et al. (1987) also found that only three positive enrichment from 370 springs were obtained, which suggested that strains of *Dictyoglomus* might be inhibited by other fast growing thermophiles (Patel et al., 1987). Love et al. (1993) reported that *D. thermophilum* strains Rt46 B.1 was phylogenetically positioned within the order Thermotogales. This placement was based on a complete SSU rRNA gene sequence, which indicates that *Dictyoglomus* forms a deep branch within the order of the Thermotogales.

1.4.2 *Thermotoga maritima*

T. maritima, a non-spore-forming, rod-shaped, Gram-negative and anaerobic bacterium belonging to the order Thermotogales, was originally isolated from geothermally heated marine sediments at Vulcano, Italy (Huber et al., 1986) and has an optimum growth temperature of 80°C. *Thermotoga* is one of the few bacteria, which can grow at temperatures above 85°C. Cells of *T. maritima* are surrounded by a characteristic sheath-like structure with overballooning at the ends of the bacterium (Huber et al., 1986). Unlike archaeal cell walls, *T. maritima* has a murein cell wall which is sensitive to lysozyme and various antibiotics, e.g. penicillin. *T. maritima* and all other species of the Thermotogales are obligate chemoorganoheterotrophic organisms that metabolise many simple and complex carbohydrates including glucose, sucrose, starch, cellulose and xylan

to produce acetate, H_2 and CO_2 (Huber et al., 1986). Schröder et al. (1994) reported that one mol glucose was fermented via the classical EM pathway to 2 mol acetate, 2 mol CO_2 and 4 mol H_2. All enzymes of the EM pathway from *T. maritima* have been identified (Schröder et al., 1994; Schurig et al., 1995). It has been suggested that *T. maritima* might perform a type of sugar fermentation similar to heterofermentative lactic acid bacteria (Schröder et al., 1994). Sulfur stimulated growth in the presence of glucose, with the S^0 apparently helping to remove inhibitory amounts of H_2. Interestingly, high activities of ATP-dependent hexokinase and ATP-PFK were also found in this organism (Selig et al., 1997).

Phylogenies based on SSU rRNA and the translational elongation factors (Achenbach-Richter et al., 1987) have placed this bacterium as one of the deepest and most slowly evolving lineages within the domain of bacteria. It has been suggested that both *Thermotoga* and *Aquifex* are the earliest and perhaps the most slowly evolving branches in the bacterial domain (Pace, 1997).

1.4.3 The order Spirochaetales

The order Spirochaetales is phylogenetically interesting as it contains a diverse array of phenotypes, which includes strictly anaerobic, facultatively anaerobic, halophilic, alkaliphilic, thermophilic and both free-living and obligately parasitic species (Greenberg and Canale-Parola, 1976; Canale-Parola, 1977; Harwood et al., 1982; Harwood and Canale-Parola, 1984; Aksenova et al., 1992; Zhilina et al., 1996a, 1996b). A phylogeny of the spirochetes based on 16S rRNA gene sequences has suggested that the spirochete phylum falls into five major subclasses: the genera *Spirochaeta*, *Treponema*, *Borrelia*, *Leptospira* with *Treponema hydysenteriae* as an outlier (Paster et al., 1991). Spirochetes are a group of anaerobic bacteria with helical shape and motility mechanisms. These organisms are unique among bacteria and all except for species of *Leptospira* are

resistant to rifampicin (Leschine and Canale-Parola, 1986). Under anaerobic conditions, spirochetes such as *S. litoralis*, *S. halophila* ferment carbohydrates to pyruvate via the EM pathway. Janssen and Morgan (1992) reported the presence of a PP_i-PFK activity in *S. thermophila,* however, this organism possesses an ATP-dependent 3-phosphoglycerate kinase, an ATP-dependent pyruvate kinase and an ATP-dependent acetate kinase. Three mol of ATP per mol of glucose were suggested to be produced, rather than the 2 mol of ATP per mol of glucose that would be expected if a normal ATP-PFK activity were present (Janssen and Morgan, 1992).

1.5 Hypothesis and the aim of this thesis

It is generally believed that the temperature in the prebiotic environment was much higher than that at present and 16S rRNA sequence phylogeny also suggests the last common ancestor was a hyperthermophile (Woese et al., 1990). Significantly, studies indicate that PP_i and shortchain polyP are formed continuously in volcanic condensates and are geochemically stable (Yamagata, 1991). Consequently, these chemicals could have been available as energy sources to drive reactions in the prebiotic environment. In addition, ATP is relatively unstable in hyperthermophilic environments (with a half-life about 115 min at 90°C at pH 7.0) whereas PP_i and polyP are stable (with a half-life about 750 min at 90°C at pH 7.0), suggesting that either PP_i or polyP might be a more ancient energy source of energy for metabolism than ATP was during more early evolution of metabolism (Morgan and Ronimus, 1998). Therefore, it is likely that ATP-PFKs evolved from PP_i-PFKs based on the above considerations than the reverse. Some biochemical properties of PP_i-PFKs, i.e. their activity to catalyse the reversible reaction, provides additional support for that PP_i-PFKs preceded ATP-PFKs and the theory that life/metabolism utilised a gluconeogenic process (Wächterhäuser, 1990). Although the fact is that PP_i-PFKs have been found in

members of the most ancient lineage of primitive eukaryotes, i.e. *G. lamblia*, the general lack of biochemical and sequence information of PFKs from hyperthermophilic organisms at the start of this thesis represented an obstacle to our understanding of the evolution of this key enzyme in glycolysis.

The aims of this thesis were initially to screen for PFK activities from the species with diverse physiologies from the order Spirochaetales and representative extremely thermophilic microoganisms. Subsequently, the goal was to purify, characterise, clone and express PP_i- and ATP-subtypes of PFKs from extremely thermophilic bacteria and/or archaea to further study their biochemical properties and phylogenies in order to examine the evolution. It was hoped that, through this study that gaps in our understanding of the biochemistry and phylogenetic relationships of PFKs from extremely thermophilic microorganisms, which are considered to represent the most ancient lineages of life, would be filled. Furthermore, it was envisaged that the study would help us to understand how PFKs function in life in thermophilic environments and, indeed, within central metabolism.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Microorganisms studied in this thesis

The microorganisms studied in this thesis are listed in Table 2.1. These organisms were obtained from either the American Type Culture Collection (ATCC), Deutsche Sammlung von Microorganismen und Zellkulturen GmbH (DSM) or the Thermophile Research Unit Culture Collection (TRUCC), University of Waikato, New Zealand. Media numbers refer to catalogue numbers from the equivalent institutional source and all the media for bacterial and archaeal growth are listed in Appendix A.

Table 2.1 Source of strains for screening of PFK activity

Strain	Source	Medium Number	T^{a}_{opt}	Carbon Source
Non-thermophilic organisms				
Spirochaeta				
S. alkalica	DSM 8900	700	37°C	sucrose
S. asiatica	DSM 8901	700	37°C	sucrose
S. halophila	ATCC 29478	937	37°C	maltose
S. isovalerica	DSM 2461	273	30°C	glucose
S. litoralis	DSM 2029	173	30°C	glucose
S. zuelzerae	DSM 1903	169	30°C	glucose
Treponema				
T. bryantii	ATCC 33254	602	37°C	glu+mal[b]
T. denticola	ATCC 33520	1357	37°C	glucose
T. pectinovorum	ATCC 33768	1223	34°C	pectin
Leptospira biflexa	ATCC 22582	1470	30°C	FA&AA[c]

Table 2.1 (continued)

Thermophilic bacteria and archaea				
C. saccharolyticum [d]	ATCC 35040	1118	70°C	cellobiose
Clostridium fervidus	ATCC 43204	1190	68°C	glucose
D. amylolyticus	DSM 3822	395	80°C	starch
D. thermophilum	ATCC 35947	1464	73°C	starch
Fervidobacterim nodosum	ATCC 35602	1465	70°C	glucose
S. thermophila	TRUCC	SE	55°C	maltose
S. thermophila Rt118 B.1	TRUCC	SE	55°C	maltose
S. thermophila GAB 76	TRUCC	SE	55°C	maltose
T. maritima	DSM 3109	343	80°C	starch
T. zilligii AN1	TRUCC	AN1 medium	75°C	trypticase
Thermococcus sp Wai 21 S1	TRUCC	ATCC 1464	90°C	starch

[a] optimal temperature; [b] glu + mal: glucose + maltose; [c] FA+ AA: fatty acids and amino acids; [d] *Caldicellulosiruptor*; SE: *Spirochaeta* enrichment medium.

2.2 Chemicals and reagents

Enzymes, chemicals and reagents that were used in this thesis are listed in Table 2.2.

Table 2.2 Source of chemicals and materials

Company	Chemicals and materials
Amersham Pharmacia Biotch, Uppsala, Sweden	Hybond-N^+ membrane, pKK223-3
Bio-Rad, Hercules, CA, USA	Gene Pulser, hydroxyapatite chromatography matrix
Boehringer-Mannheim/Roche, Mannheim, Germany	restriction enzymes, Klenow polymerase, α-^{32}P-dCTP ATP, dNTP, pyruvate kinase, phosphoglucoisomerase, glucose-6-phosphate dehydrogenase, lactate dehydrogenase
Life Technologies, Gaithersburg, MD, USA	DNA primer synthesis, *E. coli* DH5α, pUC18, pPROEX HTb, Ni-NTA resin, UV Cross-Linker

Table 2.2 (continued)

New England Biolabs, Beverly, MA, USA	restriction enzymes
Perkin Elmer, CA, USA	Ampli *Taq* Gold polymerase
Pharmacia Biotch, Uppsala, Sweden	FPLC system, Polybuffers, low molecular marker for SDS-PAGE, IEF standard marker
Promega, Madison, WI, USA	T_4 DNA ligase, Wizard plasmid purification kit, restriction enzymes, *E. coli* strain JM109, pGEM-7Zf(+)
Sigma, St. Louis, MO, USA	general chemicals, reagents and following specific materials: aldolase, α-glycerophosphate dehydrogenase, triosephosphate isomerase, ATP-PFKs (type III from rabbit muscle), PP_i-PFK (*P. freudenreichii*), F-6-P, F-1,6-P_2, F-2,6-P_2, PP_i, PPP_i, polyphosphate (polyP, n=15±3), potential activators and inhibitors, $NADP^+$ and NADH

2.3 Phosphofructokinase assays

The PFK activity from different organisms was assayed spectrophotometrically using an Ultrospect 3000, UV/Visible Spectrophotometer (Pharmacia Biotech) by a variation of the method of Janssen and Morgan (1992). The final concentrations of the standard reaction mixture for PFK screening, in a total volume of 100 µl, were 5 mM F-6-P; 1.0 mM phosphoryl donor (PP_i, ATP or ADP); 5.0 mM $MgCl_2$; 0.2 mM NADH; 0.04 U aldolase; 0.5 U α-glycerophosphate dehydrogenase and 5.0 U triosephosphate isomerase. Assays were conducted by monitoring by the decrease A_{340} at 50°C (the maximum temperature at which the mesophilic coupling enzyme assay can be utilised) for thermophilic enzymes and at their optimal temperature either 30, 34 or 37°C for mesophilic enzymes (Table 2.1). The following enzymes were used as positive controls during the investigation: the ATP-PFK (EC. 2.7.1.11) positive control was derived from rabbit muscle; the PP_i-PFK (EC 2.7.1.90) positive

control was derived from *P. freudenreichii* and the ADP-PFK positive control consisted of the purified enzyme from *T. zilligii* strain AN1 (Ronimus et al., 1999). Other control reactions were performed using the following conditions: either no F-6-P; no coupling enzymes; or no phosphoryl donors in the reaction mixture, particularly for the screening of PFK activities from cell-free extracts. The experimental conditions for kinetic studies for both purified and recombinant enzymes are described individually in their respective chapters. All assays were conducted for at least 90 sec in duplicate except those for determining kinetic parameters, which were done in triplicate. Specific activities are expressed in units/mg of protein. One unit (U) is defined as that amount of enzyme required to convert 1 μmole of F-6-P into F-1,6-P_2 per min at optimal temperature, and for the reverse reaction, the conversion of F-1,6-P_2 into F-6-P per min. The reverse reaction was assayed for PP_i-PFK by monitoring the increase of A_{340} in a total volume of 100 μl using phosphoglucoisomerase (0.4 U) and glucose-6-phosphate dehydrogenase (0.1 U) as described by Mertens et al. (1993), except that the phosphoglucoisomerase concentration was doubled. The concentrations of other components are described in each respective chapter. Control experiments were conducted for both forward (1.5 mM F-1,6-P_2) and reverse (1.5 mM F-6-P) reactions to ensure that the coupling enzymes were not rate limiting (shown in Fig. 2.1).

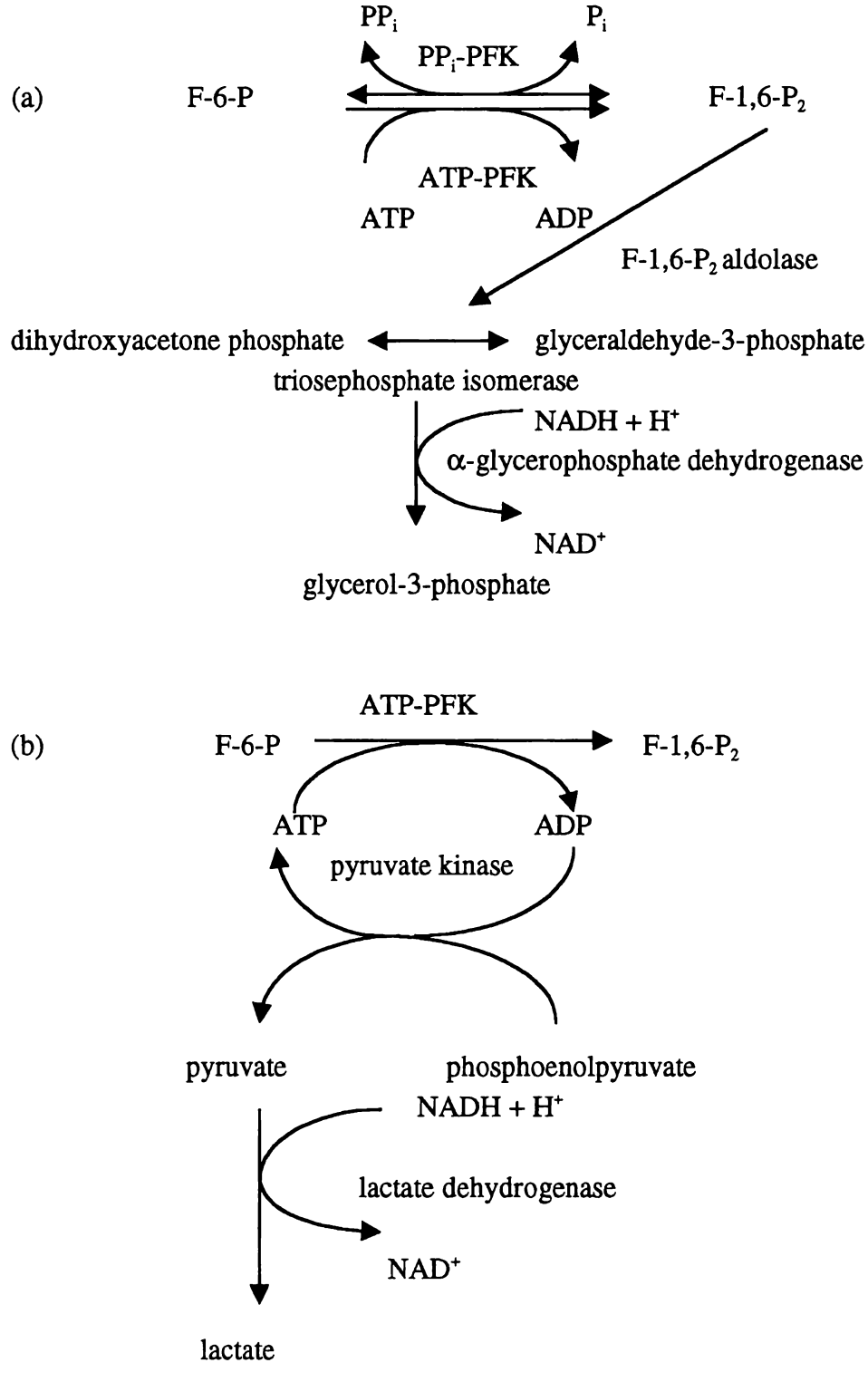


Figure 2.1 Modified coupled assays for PFK activity based on (a) F-1,6-P_2 production and (b) ADP formation. ATP-PFKs were assayed using method either (a) or (b). PP_i-PFKs and ADP-PFKs were assayed using method (a).

2.4 Cell growth, harvesting and screening of PFK activities

All the organisms were grown in their appropriate media and at their optimal temperatures (Table 2.1). For the screening of PFK activity from various organisms, cells were grown in small-scale volumes of media (100-500 ml). When the cell density reached approximately 0.6 or over (A_{600}), cultures were harvested by centrifugation at 4,000 x *g* for 20 min at room temperature (RT). All the centrifugations were performed with either a Sorvall RC 26 Plus (USA) or an Eppendorf Centrifuge 5415 (Germany). The standard PFK assay protocol utilised for PFK screening is described in Section 2.3. The large-scale protein purifications of PFKs from *D. thermophilum* and *D. amylolyticus* are described individually in Chapters 4 and 6, respectively.

2.5 Assessment of the purity of PFK preparations

The purity of purified PFKs from different organisms was determined by one or a combination of several methods. Electrophoresis was carried out by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 10-15% gradient Phast gels or using an isoelectric focusing (IEF) gel on pH 4-9 gradient gels (Pharmacia Phast System). After electrophoresis, gels were silver stained (Section 2.9.4) as described in the manufacturer's instructions. The N-terminal sequence, mass spectrometry and gel-filtration data could also be used to assess the purity of the protein.

2.6 Determination of native PFK molecular masses by gel filtration chromatography

The molecular masses of the purified PFKs were determined by gel filtration chromatography using a BIOSEP-SEC3000 column (Phenomenex, USA) for both the

PP_i-PFK and ATP-PFK from *D. thermophilum* and *D. amylolyticus*, respectively. Bio-Gel 200 (Bio-Rad) was used for determination of molecular masses for both the PP_i- and ATP-PFKs from *T. maritima*. The buffer used for gel filtration contained 25 mM Bis-Tris, 200 mM NaCl, 1 mM $MgCl_2$, pH 6.7, for all purified native and recombinant enzymes in this thesis. The standard proteins used for the calibration of the column were thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), lactate dehydrogenase (140 kDa), carbonic anhydrase (29 kDa), and α-lactate albumin (14 kDa). The elution volumes of the standards were compared to the elution volume of the purified PFK. All injections were of a constant size (100 µl, 50-200 µg of protein) and the proteins were eluted at a flow rate of 0.75 ml/min in the buffer used for equilibration. Blue-dextran 2,000 (2,000 kDa) was used to determine the column void volume.

2.7 Determination of molecular mass by mass spectrometry

The molecular mass of the *Dictyoglomus* native and recombinant enzymes was also measured using mass spectrometry (VG Platform II, Fisons Instrument, UK) described by Yates (1996) and Banks and Whitehouse (1996). The enzyme preparations were initially dialysed against MilliQ water (Millipore: equivalent to double-distilled water with a monitored resistance level of >18 µOhms) for 16 h at 4°C. An aliquot (5 µl) containing approximately 20 µg of native or recombinant enzyme of dialysed enzyme solution was mixed with 5 µl of 50% formic acid (which was required for ionisation of the proteins) and the same injected. The mobile phase was 50:50 methyl cyanide water ($MeCN:H_2O$) at a continous flow rate of 0.02 ml/min.

2.8 Protein assays

The concentration of protein was determined by a modification of the method of Bradford (Bradford, 1976) using bovine serum albumin (1.0 mg/ml, 0.2 μm filter sterilised) as the standard. The stock solution for the Bradford assay was made by dissolving 100 mg Coomassie blue G250 in 50 ml ethanol. After the dye had dissolved, 100 ml of 85% phosphoric acid was added. For assay, 20 ml of the dye stock reagent was diluted to 100 ml with MilliQ water. This diluted reagent was stable for up to 3-4 weeks at RT.

2.9 Electrophoresis

2.9.1 SDS-PAGE

Two major advantages of SDS-PAGE gel electrophoresis are evident when compared with native electrophoresis. One is that aggregates and insoluble particles often cause poor loading with native gels by blocking the pores; when fully denatured, these aggregates are solubilised and converted to single polypeptides (Scopes, 1994). The other advantage is that the mobility is relative to the polypeptide size, so an immediate indication of molecular weight for each component is provided (Scopes, 1994).

2.9.2 SDS-PAGE sample preparation and electrophoresis

The Pharmacia PhastGel System was used to determine both the purity of isolated enzymes and their molecular weights. The standard used was Pharmacia low molecular weight marker kit prepared for SDS-PAGE 10-15% gradient gels (Table 2.3). The purified native and recombinant enzymes from either *D. thermophilum*, *T. maritima* or *D. amylolyticus* were diluted to a protein concentration between 0.25-8.0 μg for each track on the gel. The gel loading buffer (1 μl of 5x gel loading buffer

containing 12.5% SDS, 25.0% β-mercaptoethanol and 0.05% bromophenol blue) was added to each protein sample (4 µl). The samples were boiled for 5 min, then centrifuged at 4,000 x *g* for 1 min. Aliquots (1 µl of boiled sample mixture for each track) were loaded onto the gel, then electrophoresed with programme #2 (Appendix B.1).

Table 2.3 Low molecular weight standard marker for SDS-PAGE

Standards	Molecular Weight
Phosphorylase b	94,000
Bovine serum albumin	67,000
Ovalbumin	43,000
Carbonic anhydrase	30,000
Soybean trypsin inhibitor	20,100
α-Lactate albumin	14,400

MilliQ water (100 µl) was added to the vial and the protein standard markers were diluted to the appropriate concentration (6 µg/µl) for silver staining. The silver staining protocols are described in Section 2.9.4.

2.9.3 Isoelectric focusing (IEF) standards and isoelectric point (*p*I) determinations

Isoelectric focusing involves setting up a pH gradient and allowing the proteins to migrate in an electric field to the point in the gel where the pH equals their isoelectric point (Scopes, 1994; Righetti et al., 1996). The IEF Phast Gel pH 3-9 gradient, Pharmacia IEF standards for pH 3-10 and Pharmacia Phast System were used for isoelectric point determinations of the native and recombinant enzymes. The separation method included the following three steps: first, was the prefocusing step which formed the gel pH gradient; second, the samples were loaded onto the centre of the IEF 3-9 gel; and third, the proteins were focused to their isoelectric points. The

procedure was performed at RT with programme #4 (Appendix B.2) according to the manufacturer's instructions (Pharmacia).

The *p*I standard contained the following markers (isoelectric point): amyloglucosidase (3.50), methyl red (3.75), soybean trypsin inhibitor (4.55), β-lactoglobulin A (5.20), bovine carbonic anhydrase B (5.85), human carbonic anhydrase B (6.55), horse myoglobin-acidic (6.85), horse myoglobin-basic band (7.35), lentil lectin-acidic band (8.15), lentil lectin-middle band (8.45), lentil lectin-basic band (8.65) and trypsinogen (9.30).

2.9.4 Protein silver staining for SDS-PAGE and IEF

Silver staining is a sensitive method for the detection of proteins after electrophoresis. The first step is to fix proteins by denaturation in acid, i.e. a mixture of trichloroacetic acid/ethanol/acetic acid. After fixing the proteins, the gel is treated with silver nitrate. Silver staining is about 10-20 times more sensitive than staining with Coomassie Blue (which tends to bind to positively charged groups, i.e. lysines, arginines and histidines on the proteins). Very small amounts of impurities can be detected by silver staining.

Table 2.4 Modified method of silver staining for SDS-PAGE with PhastGel gradient media

Step	Solution	Time (min)	Temperature
1	50% ethanol, 10% acetic acid	5	50°C
2	8.3% glutaraldehyde	10	50°C
3	MilliQ water	1	50°C
4	MilliQ water	2	50°C
5	0.5% silver nitrate	15	50°C
6	MilliQ water	1	50°C
7	MilliQ water	2	50°C

Table 2.4 (continued)

8	developer*	5-15	25°C
9	10% acetic acid, 5% glycerol	5	25°C

*Developer was made of 20 ml of 12.5% Na_2CO_3 and 40 µl of formaldehyde to 79.6 ml of MilliQ water. (The temperature from step 1 to 7 was changed from 40°C to 50°C in this study as it gave much better background). The original protocol was obtained from the manufacturer's instructions.

Table 2. 5 Silver staining method optimised for IEF gel with PhastGel gradient media

Step	Solution	Time (min)	Temperature
1	20% trichloroacetic acid	2	20°C
2	50% ethanol, 10% acetic acid	2	50°C
3	10% ethanol, 5% acetic acid	2	50°C
4	10% ethanol, 5% acetic acid	4	50°C
5	8.3% glutaraldehyde	6	50°C
6	10% ethanol, 5% acetic acid	3	50°C
7	10% ethanol, 5% acetic acid	5	50°C
8	MilliQ water	2	50°C
9	MilliQ water	2	50°C
10	0.5% silver nitrate	10	50°C
11	MilliQ water	0.5	30°C
12	MilliQ water	0.5	30°C
13	developer*	5-15	25°C
14	developer	5	25°C
15	5% acetic acid	5	50°C
16	10% acetic acid, 5% glycerol	5	25°C

Developer was the same as described in Table 2.4. This protocol was obtained from the manufacturer's instructions.

2.10 Characterisation of purified PFKs

2.10.1 Determination of optimum pH for activity

The pH optima of enzymes were measured between pH of 5.2 and 8.8 at 50°C using either citric acid (5.2-5.6), Bis-Tris (5.6-6.8), triethanolamine (6.8-7.4), Tris-HCl (7.4-8.0) or diethanolamine (8.0-8.4) buffer (each 100 mM for final concentration).

2.10.2 Determination of the optimal concentration of Mg^{2+} for activity

The optimal Mg^{2+} concentrations were determinated using the conditions of standard assays with the Mg^{2+} concentrations varied between 0 to 10 mM Mg^{2+}.

2.10.3 Determination of enzyme thermostabilities

The thermostabilities of different purified native and recombinant PFKs were determined by incubating the enzymes (100-150 μg/ml each enzyme) at a constant set temperature between 80-100°C in solution containing different buffers (described in each separate enzyme section), 3.0 mM $MgCl_2$ and 0.02% Triton X-100. The pH values were maintained at about pH 7.0 at the appropriate temperatures and the mixtures were incubated under a mineral oil overlay for different incubation times (0, 10, 20, 30, 40, 60, 90, 120, 160, 220, 280 min, until the enzyme activity decreased to under 10-20% remaining activity). Remaining activity was then assayed at the optimal pH for the individual enzymes at 50°C and compared to the unheated control.

2.10.4 Determination of activities with alternate phosphoryl donors

ATP, ADP, GTP, GDP, TTP, TDP, CTP, CDP, UTP, UDP, PP_i, PPP_i and polyP (n=15±3) were checked as possible phosphoryl donors for either purified or recombinant enzymes from either *D. thermophilum*, *D. amylolyticus* or *T. maritima*. The final concentrations of each were varied from 0.1-1.0 mM in the standard assay and are described individually in their respective chapters.

2.10.5 Determination of kinetic parameters

The apparent K_m values of the purified and recombinant enzymes from *D. thermophilum*, *D. amylolyticus* and *T. maritima* were measured by fixing one substrate at saturating concentration while varying the other substrate concentration.

Forward reaction kinetic parameters for PP_i- and ATP-PFK activities were determined at their optimal pH values (at 50°C) by varying either the concentration of F-6-P or PP_i (or other phosphoryl donors) in the presence of saturating quantities of PP_i or ATP or of F-6-P, respectively. Reverse reaction parameters for PP_i-PFK were determined at their optimal pH values (at 50°C) by either varying the concentration of F-1,6-P_2 or phosphate (P_i) in the presence of saturating quantities of P_i or of F-1,6-P_2, respectively.

2.10.6 Determination of activator/inhibitor effects

Potential allosteric effectors for the purified native and the recombinant PFKs were investigated by adding between 0.1 to 5.0 mM (final concentration in assay) of the potential modulator, e.g. ADP, AMP, cAMP, F-2,6-P_2, succinate, citrate, PEP, ATP, GTP, GDP, TTP, TDP, CTP, CDP, UTP, UDP, PP_i or polyP to the assays (using substrate conditions close to K_m values). Some particular experimental conditions are also described in related chapters.

2.10.7 Determination of N-terminal sequences

N-terminal sequencing of the purified native *Dictyoglomus* PP_i-PFK was carried out using Edman degradation chemistry on an Applied Biosystem Procise 492 protein sequencer at the School of Biological Sciences, Auckland University, New Zealand. The N-terminal sequencing of the native *Desulfurococcus* ATP-PFK was determined at the Australian Proteome Facility, MacQuarie University, New South Wales, Australia. The residues were used for database searching and incorporated the BLASTP search program at the National Centre for Biotechnology Information, National Library of Medicine, National Institute of Health, Bethesda, Maryland, USA (Altschul et al., 1997).

2.11 Deoxyribonucleic acid (DNA) manipulations

All the work relating to gene cloning and expression reported in thesis was approved (GM099-UOW 006) by the Environmental Risk Management Authority (ERMA), New Zealand.

2.11.1 Bacterial strains and plasmids used in this study

The properties of *E. coli* stains and plasmids for gene cloning and expression in this thesis are described in Table 2.6.

Table 2.6 Bacterial strains and plasmids used for gene cloning and expression

***E. coli* strains**	**Genotype and properties**
JM109	*endA*1, *recA*1, *gyrA*96, *thi*, *hsdR*17 (r_k^-, m_k^+), *relA*1, *supE*44, Δ(*lac-proAB*), [F', *traD*36, *proAB*, *lacI*^{q}ZΔ M15]. This strain is *rec*A$^-$ and lacks the *E.coli* K restriction system. For blue and white color screening of vectors, it is deficient in β-galactosidase activity due to deletions in both genomic and episomal copies of the *lacZ* gene.
DH5α	F' ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA*1 *endA*1, *hsd*R17 (r_k^-,m_k^+) *phoA supE*44, λ$^-$, *thi*-1 *gyrA*96 *relA*1] It has a high transformants/mg efficiency (>1x10^8) and helps ensure insert stability due to the *recA*1 mutation.
Cloning and expression vectors	**Properties**
pGEM-7Zf(+)	3.0 kb, RNA synthesised by T7 RNA polymerase and complementary to RNA synthesised by SP6 RNA polymerase, with a *lacZ* start codon, *lac* operator, β-lactamase gene (Ap^r), a multiple cloning site of one of the M13 mp sequencing vectors.

Table 2.6 (continued)

pKK223-3	4.584 kb, with a strong *tac* promoter and a strong *rrn*B ribosomal terminator for control of protein expression. *tac* promoter is inducible with 1-5 mM isopropyl-β-D-thiogalactoside (IPTG). The promoter is so strong that may show a low level of expression even in uninduced cells. The *rrn*B transcription terminators stabilise the plasmid by inhibiting read-through transcription initiated from the *tac* promoter in the parent plasmid. A host with *lacI*^{q}, e.g. JM109 is recommended.
pPROEX HTb	4.775 kbp, with a powerful *trc* promoter for high level expression in *E. coli*, a *lac*I^q repressor for regulated expression with IPTG and 6 x histidine affinity tag for one-step affinity purification of the protein on nickel nitrilo-tri-acetic acid (Ni-NTA) resin. DH5α is recommended.

2.11.2 Preparation of reagents used in DNA isolation and purification

Phenol (2.0 litre) was re-distilled in a chemical fume hood. A round-bottom distillation flask containing some boiling chips was filled to no more than three-quarters full with liquified phenol. Purified phenol emerged from the condenser (180°C) and was collected in brown bottles and stored at –20°C. 8-hydroxyquinoline was added to the distilled phenol (1 μg/ml). 8-hydroxyquinoline can retard the rate of phenol oxidation and partially inhibits the activity of ribonuclease. It has a bright yellow colour for easy identification of the phenol-containing phase. Aliquots (250 ml) of distilled phenol were buffered with the same volume of 1.0 M Tris-HCl (pH 8.0), 0.5 M Tris-HCl (pH 8.0), and 0.1 M Tris-HCl (pH 8.0), respectively. The pH of buffered phenol should be above pH 7.6 or DNA can be retained in the phenol phase and interphase. β–mercaptoethanol was added to a final concentration of 7.0 mM and the solution stored in a brown bottle at 4°C.

Proteinase K and sodium ampicillin (Sigma) were made to a final concentration of 20 and 10 mg/ml, respectively, in MilliQ water and 0.2 μm filtered and then stored aliquots at –20°C.

Denatured and fragmented salmon sperm DNA was prepared according to Sambrook et al. (1989). Salmon sperm DNA (Sigma type III, sodium salt) was dissolved in MilliQ water at a concentration of 10 mg/ml. Sodium chloride was added to 0.1 M and the DNA solution then extracted with phenol, phenol:chloroform and chloroform. The DNA was sheared by passing rapidly it 12 times through a 17-gauge hypodermic needle. The DNA was precipitated by 100% ethanol, quantified by absorption at 260 nm and diluted to a concentration of 100 μg/ml and stored in aliquots at -20°C.

2.11.3 Genomic DNA isolation and quantification

The method for genomic DNA isolation was a modified version of methods from Wallace (1987) and Ronimus (1993). Bacterial cells (usually 1-5 g, wet weight) were resuspended in 5-25 ml of 50 mM Tris-HCl, pH 8.0, 100 mM NaCl and 100 mM ethylenediaminetetraacetic acid (EDTA). SDS and Proteinase K were added to 1% and 100 μg/ml, respectively, then the same volume of 10 mM Tris-HCl, 1.0 mM EDTA, pH 8.0 (TE) was added. SDS is an anionic detergent and employed to promote the dissociation of proteins from nucleic acids when extracting from biological materials. An equal volume of buffered phenol was added to remove protein and other contaminants. The solution was mixed gently to avoid genomic DNA shearing by inverting 4 times. After centrifugation (4,000 x *g* for 5-10 min at RT), the top aqueous phase was removed to a new tube. The remaining phenol phase was re-extracted with 10% of the original volume of DNA solution. The top aqueous phases were pooled, then re-extracted with phenol:chloroform:isoamyl alcohol

(24:25:1), then extracted with chloroform:isoamyl alcohol (25:1) each time with gentle inversion followed by centrifugation. In conjunction with phenol, chloroform improves the efficiency of nucleic acid extractions due to its ability to denature protein and assist the dissociation of protein from nucleic acid. The high density of chloroform also enhances phase separation, facilitating the removal of the aqueous phase with little cross-contamination with organic material and lipid. Isoamyl alcohol is added to the chloroform to prevent foaming. The DNA solution was transferred to a clean sterilised centrifuge tube, NaCl added to a final concentration of 200 mM, and 2.5 volumes of 100% ethanol gently added and the DNA left to precipitate at 4°C overnight. Ethanol precipitation is probably the most versatile method of concentrating nucleic acid and is commonly used to recover DNA or RNA after extraction from biological sources or to retrieve phenol-extracted DNA products of *in vitro* enzymatic manipulations.

The DNA was collected by centrifugation at 15,000 x *g* for 30 min and the aqueous phase discarded. The DNA pellet was washed carefully with 80% ethanol, then air dried for 30-60 min and resuspended in an appropriate volume of TE, pH 8.0. Afterwards, ribonuclease (RNase) was added (50 µg/ml), and incubated at 37°C for 10-20 min. To get rid of the RNase, Proteinase K (50 µg/ml), SDS (0.5%) and EDTA (10 mM) were added and incubated at 65°C for 60-90 min, then the DNA was phenol, phenol:chloroform and chloroform extracted, ethanol precipitated, air dried and resuspended in an appropriate volume of TE, pH 8.0. The determination of the DNA integrity, purity and concentration was performed by gel electrophoresis with a 1.0% agarose gel, measuring the A_{260}/A_{280} ratio and the absolute value of the A_{260}, respectively. DNA samples were stored at either –20°C or 4°C. DNA is best stored in TE (pH 8.0) buffer. The EDTA chelates heavy metal ions which are commonly

required for DNase activity, while the use of pH 8.0, minimises deamination. EDTA also chelates the Mg^{2+} which can mediate aggregation of nucleic acids to each other and to proteins.

To determine the DNA concentration, two techniques were used. One was a "spot test" in which a standard DNA (10 μg/ml) was used from the archaeal *T. zilligii* AN1 (supplied by Dr. Ronimus) for a small quantity of DNA (Sambrook et al., 1989). The method was carried out as following steps: the standard DNA was diluted in a final volume of 20 μl containing a series of quantities of DNA (10, 20, 30, 40, 50, 75, 100, 150, 200 ng). An aliquot (2 μl) of EB (10 μg/ml) was added to each standard. The experimental samples were also diluted to 20 μl with an equal amount of EB and pipetted onto a sheet of Parafilm and photographed with the Eagle Eye II Strategene System (USA). The concentration of the DNA samples were then visually estimated by comparison of appropriate dilution factors. This method is very useful for samples containing small quantities of DNA samples.

The other method is that described by Sambrook et al. (1989) which measured the optical absorbance at 260 nm. The concentration of double-stranded DNA (dsDNA) solutions was calculated by multiplying the measured A_{260} by 50 μg/ml per 1.0 A_{260} and an appropriate dilution factor. For single stranded DNA, the calculation was modified with 40 μg/ml per 1.0 A_{260}. The relative purity of DNA samples was obtained from the A_{260}/A_{280} ratio. If the A_{260}/A_{280} of a sample was lower than 1.8, it indicated the DNA sample was contaminated with proteins and/or phenol, and needed further phenol:chloroform and chloroform extraction. If the A_{260}/A_{280} ratio was between 1.8-2.0, it suggested that the DNA was relative pure. If the A_{260}/A_{280} ratio was higher than 2.0, the sample might contain some RNA and this would have been

confirmed by agarose electrophoresis. In general, most DNA preparations were checked by agarose gel electrophoresis to determine of the preparations still contained significant quantities of RNA or whether they had been degraded by nucleases during purification.

2.11.4 Isolation of plasmid DNA

2.11.4.1 Minipreps for plasmid isolation

During this work, two methods were used for isolation of plasmid DNA on a small scale. One is described as a mini-Birnboim (Birnboim, 1983; Ronimus, 1993) and the other is used a Wizard *plus* SV Minipreps DNA Purification Kit (Promega, USA).

The procedure for small-scale isolations was as follows: a single, well-isolated colony grown on a fresh Luria-Bertani agar plate containing 100 µg/ml ampicillin (LBA) was inoculated to 10 ml LB broth containing 100 µg/ml ampicillin. The culture was incubated with vigorous shaking (200 rpm) at 37°C overnight. Cells were then pelleted from 1.5 ml of culture by centrifugation at 5,000 x *g* for 1 min in a microfuge (eppendorf, Centrifuge 5415D). The supernatant was decanted into a container (for autoclaving), and the cell pellet re-suspended in 1.0 ml solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA). The cell suspension was re-centrifuged as before. The washed cell pellet was re-suspended by pipetting in 200 µl of solution I and 400 µl of solution II (1% SDS, 0.2 N NaOH). After gentle mixing, 300 µl of solution III (3 M potassium, 5 M acetate, pH 4.8) was added. The preparation was mixed and held for 5 min at RT, then centrifuged at 11,000 x *g* for 10 min, and the supernatant (750 µl) transferred to a clean Eppendorf tube containing 750 µl isopropyl alcohol (IPA) followed immediately by a further 7 min centrifugation at 11,000 x *g* to pellet the plasmid DNA. After decanting the supernatant, the DNA

pellet was washed in 1.5 ml of 70-80% ethanol and centrifuged at 11,000 x *g* for 2 min. This ethanol washing step was repeated and after decanting the supernatant, the pellet was air dried in an inverted position for 20-30 min in a laminar flow hood. Finally, the pellet was resuspended in 100 µl TE, pH 8.0.

DNA of suitable quality for sequencing (dsDNA) was also isolated using Wizard *plus* SV Minipreps DNA Purification Kit (Promega, USA). The method combines an alkaline lysis step in combination with a silica based column, which binds DNA under low salt conditions allowing the purification of supercoiled DNA from contaminating genomic DNA, RNA and protein. The procedure for this purification followed the manufacturer's instruction (Appendix B.3).

2.11.4.2 Large-scale plasmid isolation

Four plasmids [pUC18, pGEM-7Zf(+), pKK223-3 and pPROEX HTb] were isolated in large-scale quantities in this study. Four overnight cultures (500 ml) of *E. coli* strain JM109 (each containing one of the above plasmids) were grown in LB broths containing 100 µg/ml ampicillin and harvested by centrifugation at 11,000 x *g* for 20 min at 4°C. The supernatants were removed and the cell pellets resuspended in 18 ml of ice-cold solution I as described above (Section 2.11.4.1). Two ml of ice-cold lysozyme (10 µg/ml in solution I) were added. The resuspended cells were transferred to an appropriate sized tube and solution II (40 ml) was added and mixed by gentle inversion and the samples allowed to stand for 5 min at RT. Solution III (20 ml) was added and the samples mixed, then tubes stored on ice for 10 min followed by centrifugation at 11,000 x *g* at 4°C for 15 min. The supernatants were filtered through two layers of Miracloth and 0.6 volumes of isopropanol (IPA) added. The solutions were centrifuged at 11,000 x *g* for 15 min and the pellets washed twice

with 80% ethanol and allowed to air dry until all traces of ethanol had disappeared. The pellets were then resuspended in 2.5 ml of TE, pH 8.0. Two and a half grams of caesium chloride (CsCl) were added and the sample volumes determined. Ethidium bromide (EB, 10 mg/ml) was added (0.8 ml/10 ml DNA:CsCl solution) to the sample. After the samples were mixed, centrifugation was carried out at 4,000 x *g* for 5 min to remove EB:protein complexes. This was one of the crucial steps for successful large scale plasmid isolation during this study. The supernatants were transferred to thick-walled polycarbonate tubes. Tubes were balanced to within 1 mg and centrifuged for at least 16 h at 165,700 x *g* in a Beckman benchtop ultracentrifuge (Model TL-100) at 15°C. The supercoiled plasmid band was carefully removed and placed in a new tube to which was added CsCl/EB solution in the same proportions (0.8 ml/10 ml DNA:CsCl solution). The tubes were recentrifuged at 165,700 x *g* for 16 h at 15°C. The appropriate bands were transferred to Eppendorf tubes and extracted in relative darkness with 4 M CsCl-saturated IPA to remove the EB. The extraction was repeated until no colour derived from the presence of EB could be discerned, and then extracted one final time. The remaining aqueous phases were apportioned into new Eppendorf tubes, diluted 1:3 in TE and ethanol precipitated using IPA. The resulting pellets were washed twice in 80% ethanol, dried, resuspended in a total of 500 µl TE, pH 8.0, and stored at –20°C for long-term storage or at 4°C if the DNA was to be used relatively often during the subsequent weeks/months.

2.12 Gene cloning methods

Two methods of gene sequencing and cloning for PP_i- and ATP-PFKs from *D. thermophilum* and *T. maritima* were used in this thesis. The general procedure is described in this chapter and the detailed protocols are described in Chapters 5 and 7.

2.12.1 Preparation of competent cells

From an overnight fresh culture of the appropriate *E. coli* strain, 10 ml of culture was added to one litre of LB broth and incubated with vigorous shaking (200 rpm) at 37°C until the A_{600} was approximately 0.6. Cells were harvested by centrifugation in a cold rotor (4°C) at 4,000 x *g* for 15 min. After decanting the supernatant, the pellets were gently re-suspended in a total of 1.0 litre of ice-cold sterile 10% glycerol in MilliQ water. The cell pellets were centrifuged again and the process repeated twice once with 0.5 and once with 0.1 litre of ice-cold sterile 10% glycerol. The final cell pellets were re-suspended in 2-3 ml of ice-cold 10% glycerol. The cell concentration was about 1-3 x 10^{10} cells/ml which was split into 400 µl aliquots and snap-frozen in liquid N_2 and stored at -70°C.

2.12.2 Restriction enzyme digestions

The nineteen restriction enzymes used in this study are listed in Table 2.7. The quantities of enzymes used for 1.0 µg of DNA were varied between 3-5 units and are described in Chapters 5 and 7. These enzymes were used according to the manufacturer's instructions.

Table 2.7 Restriction enzymes used in this study

Restriction enzymes	Recognition sequences	Buffer	Heat[a] inactivation	Source
Ava I	C(T/C)CG(A/G)G	B	+/-	BM[b]
Ban II	G(A/G)GCT/C) ↓ C	A	+	BM
Bgl II	A ↓ GATCT	D	+	BM
Cla I	AT ↓ CGAT	Buffer 4	+	NEB[c]
Dde I	C ↓ TNAG	H	+/-	BM
*Eco*R I	G ↓ AATTC	H	+	BM
*Eco*R V	GAT ↓ ATC	B	+	BM
*Hin*d III	A ↓ AGCTT	REACT 2	+	LT[d]

Table 2.7 (continued)

Kpn I	GGTAC ↓ C	L	+/-	BM
Msp I	C ↓ CGG	L	+	BM
Pst I	CTGCA ↓ G	REACT 2	+	LT
Rsa I	GT ↓ AC	L	+	BM
*Sau*3A I	↓ GATC	REACT 4	+	LT
*Sau*96 I	G ↓ GNCC	A	-	BM
Sal I	G ↓ TCGAC	H	+	BM
Sca I	AGT ↓ ACT	H	+	BM
Sfo I	GGC ↓ GCC	Buffer 2	+	NEB
Sty I	C ↓ C(A/T)(A/T)GG	H	+	BM
*Tru*9 I	T ↓ TAA	M	-	BM

[a] Thermostability: + 95% inactivation by incubation at 65°C for 15 min; -: no inactivation; +/-: partial inactivation.

[b] Boehringer Mannheim; [c] New England Biolabs; [d] Life Technologies;

N=A (deoxyadenine), C (deoxycytosine), G (deoxyguanine), or T (deoxythymine)

Double digests were generally carried out using the following principles. Two enzymes were usually added simultaneously. The choice of buffer to be used for double enzyme digests was determined from the % activities of the various enzymes and by choosing a buffer in which both enzymes would have at least 50% of activity. If one enzyme activity was lower than 50%, a two-fold excess of enzymes was used to compensate for the reduced activity.

2.12.3 Agarose gel electrophoresis

Electrophoresis of DNA in this study was performed using agarose gels (0.8-1.2%) with Seakem LE agarose (USA), in 1 x Tris-borate EDTA electrophoresis buffer termed TBE (89 mM Tris-HCl, 89 mM boric acid, 1.0 mM EDTA, pH 8.3) as described by Sambrook et al. (1989). The agarose-buffer mixture was boiled in microwave oven for 2-3 min until fully solublised, then cooled to around 55°C before pouring onto the gel platforms and allowed to set. A volume of 1 x TBE buffer was added into the gel box to a height sufficient (0.5-1.0 cm) to allow for adequate buffer

circulation between the cathode and anode reservoirs. Preparative gels with 1 x Tris-acetate-EDTA electrophoresis buffer termed TAE buffer (40 mM Tris-HCl, 40 mM acetate, 1.0 mM EDTA, pH 8.3) were used for isolation or purification of particular DNA fragments for cloning or other specialised purposes.

DNA samples were mixed with approximately 0.2 volumes of gel loading buffer (0.04% bromophenol blue, 30% glycerol) and loaded into the wells. Separation was initiated by electrophoresing the DNA slowly into the gel at 20-30 volts for 10-15 min. The voltage used for DNA purification from preparative gels was under 40 volts for overnight runs but for analytical gels was 60-120 volts. After electrophoresis, agarose gels were stained in a solution of EB (0.25 mg/l) for 20-30 min. The solution after staining was poured into a large flask with activated charcoal for decontamination. The gels were then destained using tap water or in 1.0 litre distilled water for 20-30 min. Nucleic acids are not fluorescent themselves, but when EB binds to nucleic acids in solution, the dye, upon excitation in the ultraviolet range, fluoresces intensely in the visible range. The destained gels were photographed under UV (260 nm) excitation with the Eagle Eye gel documentation system (Stratagene, USA).

After agarose electrophoresis, DNA purification was performed by the "freeze and squeeze" method from the preparative gels. After electrophoresis, staining and destaining, the DNA bands of interest from the preparative digest(s) were excised and placed between sheets of Parafilm and frozen at -20°C for at least 2 h. Firm, constant pressure was applied to the gel slice(s) and the exudate collected into 1.5 ml Eppendorf tubes. Iso-butanol was used to reduce the volume of the aqueous phase to approximately 500 µl. DNA was purified by extraction with phenol:chloroform and chloroform followed by ethanol precipitation. The integrity and yield of the DNA

recovered was checked by gel electrophoresis and the DNA spot-test, respectively (Ronimus, 1993).

2.12.4 Southern blotting

Southern blotting, named after its inventor (Southern, 1979), is a method for transferring size-fractionated DNA from a gel matrix to a solid support followed by hybridization with a labelled probe (Wahl et al., 1987; Sambrook et al., 1989). This technique allowed fractionated DNA from an agarose gel to be transferred to Hybond-N^+ membranes by passive diffusion and the transfers were accurate replicas of the high-resolution gel patterns. The Southern blot technique consists of the following steps: DNA agarose electrophoresis, denaturation, neutralisation, transfer, fixation onto a suitable membrane and detection of specific immobilised sequences by hybridization (Sambrook et al., 1989). The procedure was conducted in the following manner.

DNA samples were digested with appropriate restriction enzymes and after electrophoresis, the gels were stained, destained and photographed. The gels were then immersed twice in 500 ml of 0.25 M HCl for 5 min. The gels were transferred twice to 500 ml of 0.4 M NaOH for 15 min. The gels were placed upside down onto a plastic sheet on a flat surface. A piece of Hybond-N^+ membrane cut to the size of the gel and marked with pencil was wetted in MilliQ water and placed squarely on the gel. A glass rod was gently rolled back and forth on the membrane to remove any air bubbles that would prevent transfer of DNA. Following this, 3 sheets of 3 MM chromatography paper wetted with MilliQ water and 3 sheets of dry 3MM paper and a stack (6-10 cm) of Hygenix paper towels were placed on top of the membrane in sequence. Finally, a glass plate and an appropriate weight (about 300-500 g) were

placed on top. The transfer of DNA was allowed to proceed overnight and the thickness of the gels was reduced from 7-8 mm to 2-3 mm. After the DNA transfer, the membrane was rinsed in 2 x standard sodium citrate buffer (SSC, 1.5 M NaCl, 50 mM sodium citrate, pH 7.0) for 2 min, then the blots air-dried and stored at 4°C.

2.12.5 DNA fragment isolation and purification

Two methods were employed for isolating the DNA fragments separated by gel electrophoresis. One was a "freeze and squeeze" techniques described earlier in Section 2.12.3 and the other used a low melting point (LMP) agarose procedure.

For LMP agarose gel extraction, after electrophoresis, staining and de-staining, the bands of interest were excised and transferred to 10 ml centrifuge tubes. The volumes of the recovered gel slices were determined and three volumes of 20 mM Tris-HCl, 1 mM EDTA, pH 8.0, were added. The samples were incubated in a waterbath at 70°C until the agarose was dissolved and cooled to 37°C. The DNAs were purified by extraction with phenol, phenol:chloroform and chloroform, followed by ethanol precipitation. Iso-butanol was also used to reduce the volume of the aqueous phase to approximately 500 µl, then the DNAs were ethanol precipitated. This method had some advantages compared to the freeze-and-squeeze technique when maximum yield of DNA was desired.

2.12.6 Preparation of de-phosphorylated vector

All cloning vectors, except those used for expression, were de-phosphorylated by calf-intestinal alkaline phosphatase (CIAP). CIAP is a dimeric enzyme which is used for removal of 5'-phosphate groups from linear DNA thus helping to prevent self-ligation of the digested vector molecules (Sambrook et al., 1989). The DNA (10-20

μg) was digested with the appropriate restriction enzyme and the extent of digestion analysed by electrophoresis. When the digestion was completed, the sample was extracted with phenol:chloroform and chloroform, followed by ethanol precipitation. The DNA was re-suspended in 100 μl of 1 x CIAP buffer containing 1.0 mM $ZnCl_2$, 1.0 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.3. The appropriate amount of CIAP (approximately 0.027 U/μg DNA; the amount of enzyme varies dependent on type of end) was added depending on the type of ends (Sambrook et al., 1989). After incubation at 37°C for 30 min, SDS, EDTA, and Proteinase K were added to 0.5% (w/v), 5 mM and 100 μg/ml, respectively, and then incubated at 56°C for 30 min. The DNA was phenol, phenol:chloroform and chloroform extracted then ethanol precipitated followed by re-suspension in 80-100 μl TE, pH 7.6.

2.12.7 DNA ligation

After the vector and insert DNA had been prepared for ligation, the concentration of each was estimated by the DNA spot-test. The mass ratio of vector:inserts (1:3, 1:1, 3:1) was prepared in order to obtain the optimal experimental ratio for the particular samples of vector and insert. The ligation buffer (1 x) contained 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 1 mM ATP and 10 mg/ml gelatin. The calculation of the optimum ratios was based on the manufacturer's instructions (Promega, USA). Ligations were usually performed in a small volume (either 10 or 20 μl). Both blunt-end and sticky-end ligations of DNA (200-400 μg DNA/1.0 unit ligase) were carried out at RT overnight. The conditions for different ligations are described in individual sections in Chapters 5 and 7. Following the overnight ligations, the mixtures were heat-treated at 65°C for 15 min to denature the ligase. Before transformations, portions of these ligation solutions (2 μl) were loaded

onto agarose gels to check that the ligations had occurred. This, on occasion, was found to be an important step for successful cloning in this study.

2.12.8 Probe preparation

The DNA probe used for cloning the *Dictyoglomus* PP_i-PFK was amplified by PCR with degenerate primers derived from the N-terminal sequence of the purified native PFK enzyme and a highly conserved sequence for the F-6-P binding site, which is described in Chapter 5. The standard method for radioactive labelling in this thesis was the random hexamer labelling protocol (Boehringer Mannheim, Germany). The DNA sample (30 ng) and 1.0 kb standard marker (5.0 ng, Life Technologies) in a PCR tube was denatured at 98°C for 10 min in a PCR thermocycler (eppendorf, Germany). After DNA denaturation, the tube was placed in ice-water immediately and the following reagents quickly added: 2.0 µl 10 x hexanucleotide mixture (0.5 M Tris-HCl, 0.1 M $MgCl_2$, 1.0 mM DTT, 2.0 mg/ml BSA, hexanucleotides 62.5 A_{260} units/ml, pH 7.2, Boehringer Mannheim); 3.0 µl dNTP (0.5 mM dGTP, 0.5 mM dTTP, 0.5 mM dATP in 10 mM Tris-HCl, pH 8.0); 2.5 µl α-^{32}P-dCTP (50 µCi [α-^{32}P]) and 1.0 µl Klenow enzyme (2.0 U/µl). The sample was mixed briefly by pipetting and then incubated at 37°C overnight.

2.12.9 Hybridisation of radioactive probes

A high stringency protocol was used for perfectly homologous probes, i.e. for the *D. thermophilum pfp* (PP_i-PFK) gene cloning experiments. The Hybond-N^+ membranes, after Southern blotting or colony lifting (described in Chapter 5), were pre-hybridised at 60°C for 30-60 min in 0.5% SDS, 10% de-ionised formamide, 10% polyethylene glycol (PEG) 8000, 5 x SSPE (Table 2.8) and 10 µg/ml denatured salmon-sperm DNA. The radioactively labelled probe was denatured by either boiling or heating to

98°C in a PCR thermocycler for 10 min and immediately put the tube in icewater, then transferred to the hybridisation solution. Hybridisation was carried out at 60°C overnight, then the hybridisation solution was transferred to a plastic tube with cap and stored at -20°C for possible re-use. The membrane was washed with 1 x SSC , 0.5 x SSC and 0.1 x SSC, each for 20 min at 60°C. During each washing, the remaining amount of radioactivity on the blots was checked with a Geiger counter (Panax Monitor type TM64B).

Table 2.8 Reagents for hybridisation

Reagent	Contents
50 x Denhardt's	1% (w/v) BSA, 1% (w/v) polyvinylpyrrolidone, 1% (w/v) Ficoll, type 400DL (M_r 400,000)
20 x SSC	3 M NaCl, 0.3 M Na_3citrate, pH 7.0
20 x SSPE	3 M NaCl, 0.3 M NaH_2PO_4, 20 mM EDTA, pH 7.4

2.12.10 Autoradiography and film development

After post-hybridisation washing, the membranes were wrapped in clingfilm, placed in an autoradiograph cassette and exposed to X-ray film (Kodak, USA). The cassette was then left at -70°C for a few hours, the total time being dependent on the intensity of radioactivity on the membrane. After autoradiography, the film was developed for 5 min and fixed for 5-10 min followed by extensive rinsing with tap water.

2.12.11 Electroporation

The method of electroporation was developed originally to introduce DNA into eukaryotic cells and later it was used to transform *E. coli* (Dower, 1988). During this work, a Gene Pulser apparatus was used (Bio-Rad). This method results in higher transformation efficiencies than chemical-mediated transformation methods, is time-saving and is easy to use. Competent *E. coli* cells prepared by the method in Section

2.12.1 were thawed gently at 0°C and then immediately placed on ice. The sterile cuvettes (Gene Pulser, 0.1 cm cuvettes) were placed on ice for 15 min. It is very important to keep all of the samples close to 0°C to maintain the competency of the cells. Two µl of the plasmid solution with an appropriate amount of DNA were mixed with 80 µl of either DH5α or JM109 *E. coli* cells (mass ratios of insert:vector using 1:3, 1:1, 3:1) and allowed to sit on ice until the suspension was transferred to the chilled electroporation cuvette. The Gene Pulser apparatus was set to a voltage of 1.80 kV and a capacitance of 25 µF. The Pulse Controller was set to 200 Ω and the cuvette was placed in a chilled safety chamber slide. Within 1-2 seconds of the pulse, the cuvette was removed from the chamber and the cells were transferred to a 1.5 ml Eppendorf tube containing 1.0 ml of LB broth followed by incubation with gentle shaking (100 rpm) at 37°C for 45-60 min. The cell pellet was gently re-suspended in 100 µl of LB broth and transferred to LBA plates containing IPTG (0.8 mg/plate) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal, 0.8 mg/plate). The LBA plates were sealed with Parafilm and incubated in an inverted position at 37°C overnight.

2.12.12 Identification of bacterial colonies containing recombinant plasmids

Four methods are commonly used to identify bacterial colonies that contain recombinant plasmids. These are: complementation, insertional inactivation, screening by hybridisation and restriction analysis of small-scale preparations of plasmid DNA (Hanahan and Meselson, 1983). The latter two techniques were used in this study. White colonies from LBA plates containing IPTG and X-gal were transferred with sterilised toothpicks to the surface of another LBA plate in a laminar flow hood in a grid pattern. Each plate contained about 100 colonies. The plates were sealed with Parafilm and incubated in an inverted position at 37°C overnight.

Hybond-N^+ membranes were cut to the same size of the petri dish and marked asymmetrically, then placed on the surface of the LBA plates with the bacterial colonies. The membranes were gently removed with blunt-ended forceps. The bacteria adhering to the membrane were lysed (colony side-up) with denaturing agent (0.5 N NaOH, 1.5 M NaCl), then the blots neutralised with 0.5 M NaCl, 0.5 M Tris-HCl, pH 7.4. Finally, the membrane was washed with 2 x SSC, and then fixed at 80°C for 2 h or by UV crosslinking (0.127 joule/cm^2 for 30 sec). The colonies associated with positive hybridisation signals were selected from the duplicate plates, inoculated into LB broth containing 100 µg/ml ampicillin and incubated at 37°C overnight with vigorous shaking (200 rpm). The overnight culture was used for miniprep plasmid isolation (Section 2.11.4.1). The DNA samples were loaded onto an agarose gel for electrophoresis. Those that appeared to have inserts were analysed further by restriction digests and those with inserts of the correct sizes were purified and quantified for sequencing.

2.13 PCR and inverse-PCR amplifications

The conditions for general PCR and inverse-PCR amplifications are described in Sections 5.2.3 and 5.2.4.

2.14 DNA sequencing, computer analyses and database searches

DNA sequencing in this study was performed by an automated DNA sequencer at the Waikato DNA Sequencing Centre, University of Waikato. The principle of sequencing was based on the method first described by Barnes (1987) using dideoxyribonucleotides as chain terminators. Double stranded DNA was provided at 50-200 ng/µl concentration in TE, pH 8.0. Data was processed on a Macintosh 7300 computer. The software programmes for sequencing analysis included BLAST, Mac

Vector, version 4.0 and DNA strider (version 1.2). The data base searches were conducted using BLASTP, BLASTX with the SWISS-PROT, EMBL (with daily) update and GenBank (with daily update). Phylogenetic trees were generated with the CLUSTAL (W) program (version 1.6, Altschul et al., 1997).

2.15 Gene expression

2.15.1 Construction of expression clones

The open reading frames (ORFs) representing the full-length sequences of the PFK gene from *D. thermophilum* or *T. maritima* were amplified by PCR using appropriate genomic DNA with the appropriate primers (containing restriction sites) corresponding to the N-termini and C-termini. The primers with restriction sites are described in Chapter 5 for the *Dictyoglomus pfp* and in Chapter 7 for the *Thermotoga pfp* and *pfk* genes.

The single PCR-generated DNA fragments were sequenced before being cloned into either the pKK223-3 or pPROEX HTb expression vectors after restriction digestion of the ends with appropriate restriction enzymes at 37°C overnight followed by ligation with T_4 DNA ligation (Section 2.12.7). The ligation mixtures with expression plasmid and restricted PCR product containing the PFK genes were used to transform *E. coli* strains (either JM109 or DH5α) by electroporation, which were prepared according to the manufacturer's instructions (Gene Pulser, Bio-Rad). The detailed procedures are described in Chapters 5 and 7.

2.15.2 Small-scale isolation of cloned vectors and induction of expressed enzymes

Screening of the clones for those with inserts (in small-scale) was carried out using the alkaline lysis miniprep plasmid isolation (Ronimus, 1993). Potential clones with inserts were identified by restriction digests. The selected clones were then transferred to 10 ml LB broths with ampicillin (100 μg/ml) and IPTG (1.0 mM) was added when the A_{600} reached approximately 0.6 after incubation with vigorous shaking (200 rpm) at 30°C. The cells were harvested by centrifugation at 4,000 x *g* at RT for 20 min, then resuspended in the buffer described in Section 2.4. After sonication, cell-free extracts were heated at either 70°C (for the *Dictyoglomus* PP_i-PFK) or at 80°C (for both the *Thermotoga* PP_i- and ATP-PFK) for 40 min. The heat-treated supernatants were centrifuged and the enzyme activities checked at 1.0, 2.0, 3.0, 4.0 and 5.0 h after induction.

2.15.3 Large-scale induction of recombinant enzymes

The transformant selected after screening was grown at 30°C in 700-800 ml of LB broth with 100 μg/ml ampicillin. When the A_{600} reached 0.6-1.0, the expression of the protein was induced by addition of IPTG to 1.0 mM. After 5 h of induction, the cells were harvested by centrifugation at 4,000 x *g* at 4°C for 20 min. Approximately 5.0 g of cell pellet (wet weight) was resuspended in 250 ml of lysis buffer (Section 2.4) and sonicated. The cell debris was removed by centrifugation at 15,000 x *g* for 40 min at 4°C and the cell lysate incubated with the same conditions as described in Section 2.15.2. The denatured *E. coli* proteins were removed by centrifugation at 15,000 x *g* at 4°C for 40 min.

2.15.4 Purification and characterisation of recombinant enzymes

The conditions of the purification and characterisation for the recombinant *Dictyoglomus* PP_i-PFK were essentially the same as those described for the native enzyme described in Chapter 4. A nickel nitrilotriacetic acid (Ni-NTA) column was employed for the purification of both the recombinant PP_i- and ATP-PFKs from *T. maritima*. The protocol followed the manufacturer's instructions (Life Technologies). The characterisation of the native and recombinant enzymes from *D. thermophilum* and the recombinant enzymes from *T. maritima* are described in Chapters 4, 5 and 7.

2.16 Crystallography studies

Crystallisation is important in at least four areas: as a method of purification; as a confirmation of homogeneity; as a method of stable storage and of most importance to this study, for determining the tertiary structure by diffraction techniques of the thermal stable PFKs (Dixon and Webb, 1979). The purified recombinant *Dictyoglomus* PFK crystals were grown by the hanging drop vapour diffusion technique. The protein is first concentrated in a Centricon 30 microconcentrator and the buffer exchanged with a solution of 2.0 mM sodium pyrophosphate, 1.0 mM $MgCl_2$, 1.0 mM DTT, and 10 mM Tris-HCl, buffered to pH 7.5. The final protein concentration was approximately 5.0 mg/ml. The crystallisation and X-ray diffraction of the PP_i-PFK from *D. thermophilum* is being undertaken by Dr. Stanley Moore at Massey University, Palmerston North, New Zealand.

CHAPTER THREE

SCREENING OF PHOSPHOFRUCTOKINASE ACTIVITY FROM BACTERIA AND ARCHAEA

3.1 INTRODUCTION

The 'classic' ATP-PFK (EC 2.7.1.11) is found in almost all higher eukaryotes and most bacteria and generally catalyses the rate-limiting step in the glycolytic direction. Although most highly evolved organisms use ATP, other phosphoryl donors such as PP_i or ADP are also used, especially amongst thermophiles and anaerobes (Reeves et al., 1974; Mertens et al., 1991; Janssen and Morgan, 1992). Since the first PP_i-PFK was reported by Reeves et al. (1974) in *E. histolytica*, a large number of PP_i-PFKs have been reported in higher plants, primitive eukaryotes and bacteria (O'Brien et al., 1975; Yan and Tao, 1984; Pollack and Williams, 1986; Mertens et al., 1989; Janssen and Morgan, 1992; Peng and Mansour, 1992; Phillips and Li, 1995). In addition, a single example of a PP_i-PFK has also been characterised, cloned and sequenced from the thermophilic archaeon *T. tenax* (Siebers et al., 1998). A unique ADP-PFK activity has been identified in the euryarchaeal genera *Pyrococcus* and *Thermococcus* (Kengen et al.,1994; Selig et al., 1997).

Several descriptions of anaerobic and thermophilic bacterial PFKs have also shown that PP_i-PFKs catalyse a nearly reversible reaction (Morgan and Ronimus, 1998). The PP_i-PFKs have been classified into different types based on their molecular size, pH optima for activity, sensitivity to F-2,6-P_2 and their apparent K_m values for F-6-P. For example, the Type I enzyme is found mostly among the amitochondriate protists and anaerobic bacteria, is not activated by F-2,6-P_2 and usually consists of homodimers with an individual subunit size of 36-50 kDa (Morgan and Ronimus,

1998; Phillips and Li, 1995). The Type II PP_i-PFKs usually have a heterotetrameric structure with the α and β subunits having sizes between 60-65 kDa, is activated by F-2,6-P_2 and are present in plants (Todd et al., 1995) and some protists (Mertens et al., 1989b; Miyatake et al., 1986).

S. thermophila is able to degrade a diverse array of saccharides but is not able to utilise a variety of alcohols, Krebs cycle intermediates, organic acids or amino acids suggesting that the glycolytic direction is predominant during normal growth conditions (Janssen and Morgan, 1992; Aksenova et al., 1992). A PP_i-PFK has been identified from this organism (Janssen and Morgan, 1992), which suggested that if other *Spirochaeta* strains either the thermophilic or the nonthermophilic also had a PP_i-PFK. Because parasitic spirochete PP_i-PFKs may represent promising enzymes to target with pharmaceutically-appropriate inhibitors, it was of interest to determine if the presence of this PP_i-PFK simply reflected its thermophilic habitat or whether it was common throughout the genus.

Thermophilic microorganisms include bacteria and archaea, which are considered to be deeply rooted in the 16S rRNA phylogenetic tree (Woese et al., 1990). In the bacterial domain, it was still not known if these thermophilic bacteria possessed the same phosphoryl donor type of enzyme (ATP-PFK) as their mesophilic counterparts or perhaps another subtype of PFK. Since PP_i is regarded as a potential energy source from geothermal and/or prebiotic environments (Baltscheffsky, 1996; Yamagata et al., 1991), it was of particular interest to determine if these organisms possessed PP_i-PFKs.

The main goal of the work presented in this chapter was to screen the PFK subtypes, with respect to their phosphoryl donors, from cell-free extracts of both the

thermophilic and nonthermophilic members of the order Spirochaetales and deeply rooted members of the bacteria and archaea, which are all either extreme thermophiles or hyperthermophiles.

3.2 MATERIALS AND METHODS

All strains cultivated, together with the temperature for growth and cultivation medium are listed in Table 2.1. Media compositions and their methods of preparations are shown in Appendix A. Bovine rumen fluid was kindly supply by Dairying Research Corporation Ltd., Hamilton, New Zealand. The fresh bovine rumen fluid was centrifuged at 11,000 x *g* for 20 min and the resulting supernatant used for media preparation. Rabbit serum was purchased from Rapid Rabbits, Christchurch, New Zealand. The rabbit serum was filter-sterilised through a 0.2 μm filter and stored at 4°C in a sterilised universal for long-term storage. Other chemicals were prepared following the protocol described by either ATCC, DSM or TRUCC. Cells from different organisms were harvested by centrifugation (11,000 x *g* for 20 min at 4°C) when the A_{600} was over 0.6. The cell pellets were washed twice with 50 mM imidazole buffer (pH 7.4) containing 6.0 mM $MgCl_2$, 0.1 M KCl, 7 mM β-mercaptoethanol, 10% glycerol (v/v), and then the cell pellets were resuspended in an appropriate volume of the washing buffer above. Harvested cells were sonicated at full power (Heat Systems-Ultrasonics Inc.), checked microscopically to ensure that near-complete lysis had been achieved, then centrifuged at 11,000 x *g* at 4°C for 20 min. The supernatant obtained after centrifugation were used for enzyme assays. The Sources of reagents and determination of enzyme activities are described in Sections 2.2 and 2.3.

3.3 RESULTS

3.3.1 Strain cultivation

All the organisms shown in Table 3.1 were successfully grown to an A_{600} of at least 0.6 using the described conditions. Cell-free extracts were obtained for analysis of their dependence with varying phosphoryl donors and the PFK activities. Results are summarised in Table 3.1.

Table 3.1 PFK activities in cell-free extracts of representative thermophilic and nonthermophilic bacteria and archaea

	Specific Activity (U/mg)			
Strains	PP_i	ATP	ADP	PFK Type
Non-thermophilic bacteria				
Spirochaeta sp.				
S. alkalica	5.25	0.73	ND	PP_i
S. asiatica	2.27	0.63	ND	PP_i
S. halophila	3.02	0.06	0.09	PP_i
S. isovalerica	3.23	1.04	ND	PP_i
S. litoralis	0.1	0.01	0.02	PP_i
S. zuelzerae	1.4	0.19	0.16	PP_i
Treponema sp.				
T. bryantii	0.15	6.29	0.46	ATP
T. denticola	ND	0.86	0.29	ATP
Leptospira sp.				
L. biflexa	0.38	0.90	0.16	ATP
Thermophilic bacteria				
C. saccharolyticum	ND	0.29	0.15	PP_i
C. fervidus	0.01	0.005	0.008	PP_i
D. thermophilum	0.104	0.024	0.01	PP_i
F. nodosum	0.104	0.26	ND	ATP/ PP_i
S. thermophila	1.47	0.31	0.19	PP_i
S. thermophila Rt 118. B.1	3.90	0.18	ND	PP_i
S. thermophila GAB 76	1.10	0.02	0.02	PP_i
T. maritima	0.025	0.098	ND	ATP
Thermophilic archaea				
D. amylolyticus	ND	0.043	0.01	ATP

Table 3.1 (continued)

T. zilligii AN1	0.161	ND	0.20	ADP
Thermococcus Wai 21 S1	ND	0.01	0.03	ADP

ND: not detected

3.3.2 PFK activities within the Spirochaetales

Glucose metabolism in *Spirochaeta* strains was studied by Hespell and Canale-Parola (1973). The results from this study (in Table 3.1), showed that all six mesophilic *Spirochaeta* species, *S. thermophila* and the two thermophilic strains of *Spirochaeta* were found to possess primarily a PP_i-PFK activity. The specific activities for eight of the nine *Spirochaeta* samples varied between 5.25 and 1.1 U/mg while that of *S. litoralis* was significantly lower (0.1 U/mg). A low level of ATP-dependent background activity was present in some *Spirochaeta* cell-free extracts but in all cases the PP_i-based activity was a minimum of 3.1-fold (*S. isovalerica*) and up to between 50-fold (*S. halophila*) and 55-fold higher (strain GAB 76).

A similar low-level background activities with ATP (0.31 U/mg) and ADP (0.19 U/mg) was seen with extracts of *S. thermophila* and these were almost entirely removed upon further purification of the PP_i-PFK (Ronimus et al., 1999b). ADP-dependent activity (likely due to adenylate kinase activity within the extracts) was either absent or a maximum of 20% (*S. litoralis*) of the PP_i-based activity when the *Spirochaeta* cell-free extracts were screened, and is most likely not of physiological significance.

In contrast to the findings, members of the genus *Spirochaeta*, *T. bryantii*, *T. denticola* and *L. biflexa* possessed primarily an ATP-dependent PFK activity, whereas no PFK activity (either ATP-, ADP- or PP_i-based) was detected in either *T.*

pectinovorum or *L. interrogans,* despite repeated attempts. Similar to the results obtained with *Spirochaeta* strains, some background activities (either PP_i- or ADP-dependent or both) was present in some cases but the ATP-based activity was 13.7-fold higher than the next highest activity (ADP-) in the case of *T. bryantii*, nearly 3-fold higher than the ADP-based activity for *T. denticola* and 2.4-fold higher than that of the PP_i-based *L. biflexa* activity.

3.3.3 PFK activities within thermophilic bacteria

The PFK activity from six species of thermophilic bacteria was investigated. *D. thermophilum* was found to possess a PP_i-PFK activity. This result is of interest as *D. thermophilum* is phylogenetically related to *T. maritima*, which represents one of the most ancient lineage within the bacterial domain based on 16S rRNA sequence analysis (Love et al., 1993). In addition, a PP_i-dependent PFK activity was also identified in *C. fervidus*, although with a much lower specific activity of 0.01 U/mg compared to the *Dictyoglomus* enzyme (0.104 U/mg).

Interestingly, the hyperthermophilic bacterium *T. maritima* cell-free extract possessed both an ATP- (0.098 U/mg) and a very low PP_i-PFK activity (0.025 U/mg). ATP-PFK activity was also found in *C. saccharolyticum* and *F. nodosum* (Table 3.1), with the latter organism having a surprisingly high PP_i-PFK activity as well. These results suggested that both PP_i- and ATP-PFK activities might be present in *T. maritima* and *F. nodosum.*

3.3.4 PFK activities within thermophilic archaea

An ATP-PFK activity (0.043 U/mg) was found in the cell-free extract from crenarchaeon *D. amylolyticus*, which supports the previous report of Selig et al.

(1997). Even though a relatively high level background of ADP-dependent enzyme activity (0.01 U/mg) was present in the cell-free extract, it was entirely removed upon further purification and is described in Chapter 6.

ADP-PFK activity was detected from two strains of the euryarchaea, *T. zilligii* AN1 and *Thermococcus* strain Wai 21 S1. Kengen et al. (1994) first reported the presence of an ADP-PFK activity in *Pyrococcus* and so the presence of an ADP-PFK in the closely related genus *Thermococcus* was not unexpected. Surprisingly, a PP_i-PFK activity (nearly 80% of the ADP-PFK activity) was also found with cell-free extracts in *T. zilligii* AN1 (Table 3.1).

3.4 DISCUSSION

3.4.1 Cultivation of microorganisms

The organisms listed in Table 2.1 have been successfully grown during this study. It was extremely difficult to grow some of these organisms with their broad diversity of phenotypic and physiological characteristics. For example, some organisms required very strict conditions for their growth, i.e. alkaline or anaerobic media with different combination of gases and/or rare nutrients (rabbit serum, rumen fluid, volatile fatty acids, and hemin). To obtain successful growth of the organisms, it was important to follow exactly the protocols for media preparation described by either ATCC, DSM or TRUCC. In addition, the volume of medium for initial inoculation appeared to be crucial, with the smaller the medium volume, the more successful the cultivation. Finally, it was also important to use as fresh media as possible for the cultivation of the organisms.

3.4.2 Distribution of PFK subtypes within the Spirochaetales

The first goal of this study was to investigate the distribution of PFK subtypes within the Spirochaetales and in particular, whether all *Spirochaeta* species contained a PP_i-PFK. The spirochete group of bacteria is of interest with respect to the origins and evolution of PFK due to their phylogenetic cohesiveness, as judged by 16S rRNA gene sequence analysis (Paster et al., 1991). Despite their phylogenetic clustering, they contain a diverse array of phenotypes, which include: strictly anaerobic, facultatively anaerobic, halophilic, alkaliphilic, mesophilic and thermophilic, free-living and obligately parasitic (Hespell et al., 1973; Greenberg and Canale-Parola, 1976; Canale-Parola, 1977; Harwood et al., 1982; Harwood and Canale-Parola, 1984; Zhilina et al., 1996a, 1996b).

The fact that PP_i-PFK activity was the dominant activity type in all of the *Spirochaeta* tested in this study, and has also been found in *S. stenostrepta* (Morgan and Ronimus, 1998), suggests that the presence of a PP_i-PFK as the dominant PFK activity may represent a reliable phenotypic marker for the genus, which is apparently not related to their diverse physiologies (Morgan and Ronimus, 1998).

The presence of other subtypes of PFK cannot be totally excluded, as exemplified by the sequence analysis from *T. maritima* which has shown it to possess both an ATP- and a PP_i-PFK encoding genes (Nelson et al., 1999). The small but significant portions of those background activities in other organisms might also be due to the inherent substrate specificities of their dominant PP_i-PFKs, as even the highly purified PFK from *S. thermophila* still possessed some activity with either ATP or ADP. In addition, the possibility of the formation of PP_i from added ATP which

would then drive the PP_i-PFK reaction cannot be ruled out when working with cell-free extracts.

One potential reason for why an ATP-dependent activity was found in *T. bryantii* and *T. denticola* (which are host-associated spirochetes) and *L. biflexa* may be that the normal supply of growth substrates (i.e. rabbit serum) for these organisms may not be as limiting or random as would presumably be the case for free-living spirochetes. A reduction in the net ATP produced per glucose by using an ATP-PFK may not be so critical for these organisms. An ATP-PFK activity was detected in the cell-free extracts from two *Treponema* species, and this is somewhat surprising as the recently sequenced *T. pallidum* and *B. burgdorferi* genomes each putatively contain two PP_i-PFK encoding genes based on sequence comparisons (Fraser et al., 1997; Fraser et al., 1998). These two PFKs, which represent potentially promising enzyme activities to target with inhibitors, were cloned and expressed later by Zheng et al. (1999).

Interestingly, no PFK activity was found with either the *T. pectinovorum* or *L. interrogans* extracts despite repeated attempts at detection. It is possible that these organisms may degrade sugars via the ED pathway, as has been suggested for *T. saccharophilum* (Paster et al., 1985). Thus, the overall results presented here suggest that significant differences do exist in how species within genera of the order Spirochaetales degrade saccharides.

3.4.3 PFK activity in extremely thermophilic bacteria and archaea

A PP_i-PFK activity was found to be the dominant PFK activity in the extremely thermophilic bacterium *D. thermophilum.* This was particularly interesting, and provides some support for PP_i to have been a prebiotic energy source for some

ancient and extremely thermophilic enzymes in the bacterial domain. It was deemed that the study of the biochemical properties, gene sequence and cloning of this subtype of PFK would supply additional insights into the evolution of PFKs, and aid in the understanding of the early evolution of glycolysis.

The hyperthermophilic bacterium *T. maritima* represents the second most ancient lineage (after *Aquifex aeolicus*) within the bacterial domain. The results from cell-free extracts suggested that the ATP-PFK represented perhaps the only PFK activity in this organism because the PP_i-PFK activity was about 25% of the ATP-PFK level and might well be considered as a background level. However, when Nelson et al. (1999) published the genome sequence of *T. maritima*, both PP_i- and ATP-PFK genes were identified. The cloning and expression of both PP_i- and ATP-PFK from this organism is described in Chapter 7.

C. fervidus was isolated from a fresh water hot spring in New Zealand (Patel et al., 1987). This organism is a strictly anaerobic, Gram negative, spore forming and sluggishly motile rod. This organism could use trypticase peptone or yeast extract, which was also essential for the fermentation of carbohydrates including glucose, maltose, mannose, xylan, starch and pyruvate (Patel et al., 1987). The PP_i-PFK activity identified in *C. fervidus* extracts suggested that this extremely thermophilic bacterium utilises the EM pathway for its carbohydrate metabolism. The PPi-PFK could function for gluconeogenesis within this organism. The extremely thermophilic bacterium *C. saccharolyticus* is a straight rod, Gram negative, is nonsporeforming and grows strictly anaerobically within the temperature range 45-80°C (Rainey et al., 1994). The organism can utilise monosaccharides as substrates and acid is produced from arabinose, amorphous cellulose, avicell, cellobiose, fructose, galactose, glucose and glycogen (Rainey et al., 1994). The predominant

PFK activity detected from this organism was ATP-based and the results indicated that the EM pathway was also present in this organism.

Phylogenetic studies based on 16S rRNA suggests that the extremely thermophilic bacterium *F. nodosum* is highly related to *T. maritima*, which had an ATP-PFK as the dominant activity. Interestingly, although *F. nodosum* possessed predominately an ATP-PFK activity its PP_i-PFK activity was still about 50% as high as that of its ATP-PFK activity (Table 3.1). It would also be of interest to see if this organism, like *T. maritima*, possesses genes encoding for both a PP_i- and an ATP-PFK. This was particularly interesting in light of our hypothesis that PP_i could have served as prebiotic energy source for the PFK reaction. This organism is one of the most deeply rooted in the bacterial domain and has evolved from the universal ancestor.

The results of the study of the thermophilic microorganisms showed that *D. amylolyticus* possessed an ATP-PFK. An ADP-PFK activity was found in both *T. zilligii* AN1 and *Thermococcus* strain Wai 21 S1. Interestingly, the *T. zilligii* AN1 predominantly possessed an ADP-PFK but its PP_i-PFK was about 80% as high as that of its ADP-PFK, which suggests that this organism might contain genes encoding for both an ADP- and PP_i-PFK. Surprisingly, the enzyme purification from *T. zilligii* AN1 indicated that no an extra PP_i-PFK was present in this archaeon (Ronimus et al., 1999). The high PP_i-PFK activity obtained within this archaeal extract might have been caused by a high level of contaminating adenylate kinase activity.

Archaea, therefore, is the only domain of life containing three subtypes of PFK; an ATP-PFK from crenarchaeon *D. amylolyticus* (Selig et al., 1997), a PP_i-PFK from *T. tenax* (Siebers et al., 1998) and an ADP-PFK from euryarchaeon *T. zilligii* AN1 and *P. furiosus* (Kengen et al., 1994, 1996; Ronimus et al., 1999). The biochemical study

of the first archaeal ATP-PFK would be particularly interesting, and would also provide further insight for understanding this key enzyme in the evolution of glycolysis. In Chapter 6, the purification and characterisation of this archaeal ATP-PFK is described. The purification and properties of the PP_i-PFK from extremely thermophilic bacterium *D. thermophilum* is described in Chapter 4 and the sequencing, gene cloning and expression of this interesting enzyme is described in Chapter 5.

CHAPTER FOUR

PURIFICATION AND CHARACTERISATION OF A PP_i-PFK FROM AN EXTREMELY THERMOPHILIC BACTERIUM *DICTYOGLOMUS THERMOPHILUM*

4.1 INTRODUCTION

Since the first report of a PP_i-PFK activity in the amoebic parasite *E. histolytica* (Reeves et al., 1974), a number of PP_i-PFKs have been identified in many anaerobic bacteria, primitive eukaryotes and some plants (O'Brien et al. 1975; Yan and Tao, 1984; Mertens et al., 1993). In addition, Janssen and Morgan (1992) found that a thermophilic *Spirochaeta* strain possessed PP_i-PFK activity. Siebers et al. (1998) and Morgan and Ronimus (1998) have suggested that a PP_i-PFK which utilises pyrophosphate as the phosphoryl donor for PFK activity might represent the ancestral form of the enzyme, although others (e.g. Mertens, 1991) have proposed it represents only an adaptation to anaerobic metabolism and thus would have presumably arisen on multiple occasions from ATP-PFKs.

To determine if PP_i-PFK activity was also distributed in other extremely thermophilic bacteria and hyperthermophilic archaea, PFK activity from several genera was screened (described in Chapter 3). The discovery of a PP_i-PFK activity from *D. thermophilum* Rt46 B.1 is significant as the genus *Dictyoglomus* represents a deeply rooted bacterial hyperthermophilic lineage (Love et al., 1993), which is phylogenetically related to the hyperthermophilic bacterium *T. maritima* previously shown to possess an ATP-PFK (Schröder et al., 1994). The purification and characterisation of a PP_i-PFK from this organism would be of interest since it would

represent the first PP_i-PFK from an extremely thermophilic bacterium with an enzyme of this type.

4.2 MATERIALS AND METHODS

4.2.1 Bacteria and culture conditions

D. thermophilum Rt46 B.1 was obtained from the TRUCC which is equivalent to ATCC 35947 and was cultured anaerobically in the medium described by Patel et al. (1987). Large-scale cultures (80 litre) for protein purification were carried out in 20 litre polypropylene containers. The medium was filter-sterilised (0.2 μm) and gassed with 80% N_2 + 20% CO_2 for 60 min prior to inoculation with 0.5 litre of log phase culture per 20 litre fresh medium at 73°C. Cells were harvested by diafiltration with a 0.1 μm hollow fiber system (Amicon, USA), recovered by centrifugation at 11,000 x *g* for 40 min at 4°C, and the resulting cell pellet then stored at -70°C prior to initiating the purification.

4.2.2 Determination of PFK reaction rates and protein concentrations

All chemicals and reagents used for purification and characterisation are described in Section 2.2. The standard methods for determining the forward and reverse PFK reaction rates are described in Section 2.3. The protein concentration was assayed as described in Section 2.8.

4.2.3 Purification of the *Dictyoglomus* PP_i-PFK

A flow chart of the procedure utilised for purifying the *Dictyoglomus* PP_i-PFK is shown in Figure 4.1.

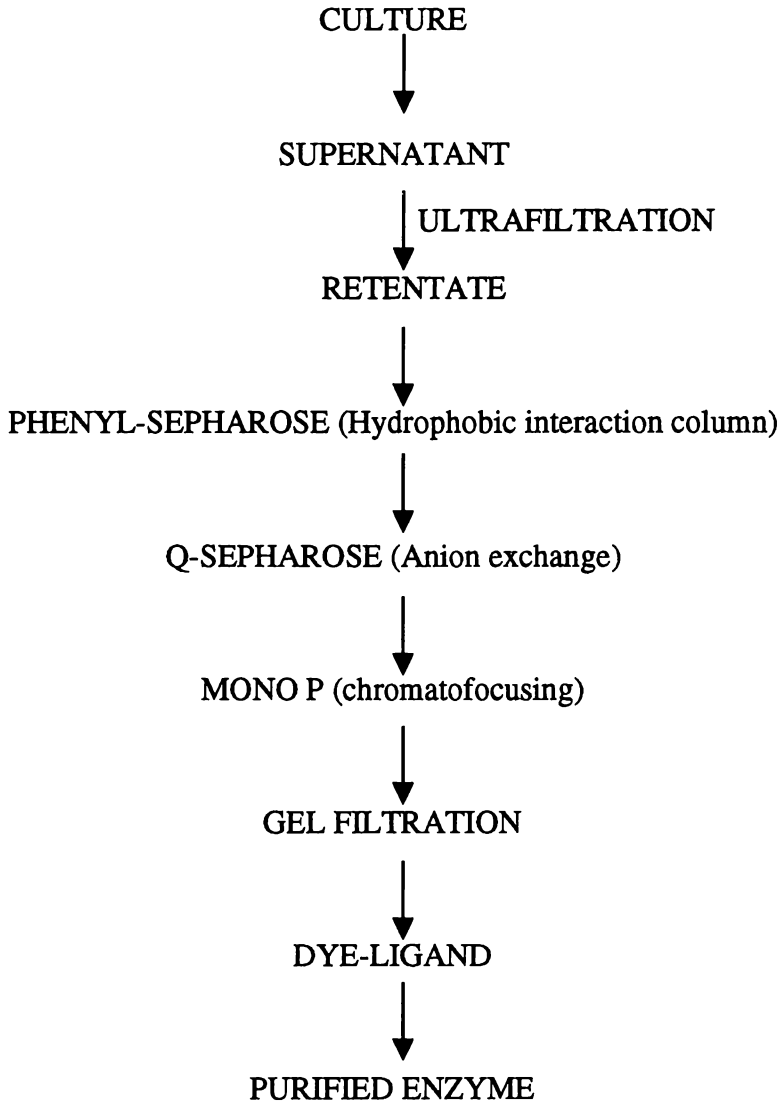


Figure 4.1 Purification procedure used with the *Dictyoglomus* PP_i-PFK

4.2.3.1 Preparation of cell-free extract

Dictyoglomus cells (120 g wet weight) were suspended in 1.2 litre of cell suspension solution (Section 3.2.1) and lysed by sonication in 6 x 200 ml aliquots for three periods of 2 min each at full power. Cell debris was removed by centrifugation at 4°C at 11,000 x *g* for 40 min. Ammonium sulfate was added to the supernatant to a final concentration of 1.0 M. An aliquot (1.0 ml) of the supernatant was transferred

to a 1.5 ml Eppendorf tube and stored at –70°C for determination of protein concentration and enzyme specific activity.

4.2.3.2 Phenyl-Sepharose hydrophobic interaction chromatography

The cell-free extract (1.0 litre) was applied to a 700 ml phenyl-Sepharose column (Pharmacia Biotech) at the flow rate of 20 ml/min pre-equilibrated with 3 litres of starting buffer A: 50 mM Tris-HCl, 1.0 mM $MgCl_2$, 1.0 M $(NH_4)_2SO_4$, pH 7.5. The fractions were eluted with a linear gradient (4.0 litre) with buffer B: 50 mM Tris-HCl, 1.0 mM $MgCl_2$, pH 7.5, and further eluted with a linear gradient (2.0 litre) of buffer C: 50 mM Tris-HCl, 1.0 mM $MgCl_2$, 50% ethanediol, pH 7.5. Fractions (20 ml for each tube) were collected and assayed for PP_i-PFK activity. The active fractions were pooled and concentrated with a YM 30 ultrafiltration membrane (Amicon) and dialysed against buffer B at 4°C overnight.

4.2.3.3 Q-Sepharose anion exchange chromatography

Initially, a 55 ml of Q-Sepharose column 26/10 (Pharmacia Biotech) was cleaned with one column volume of 1.0 N NaOH, then washed with MilliQ water until the pH was close to neutral and the column recharged with 1 M NaCl. After being equilibrated with the starting buffer (buffer B above), the dialysed enzyme (120 ml with 927 mg of protein) from the phenyl-Sepharose step was applied to the column. The column was washed with three volumes of the starting buffer and the enzyme was eluted with a linear gradient (400 ml) of buffer C containing 50 mM Tris-HCl, 1.0 mM $MgCl_2$, 1.0 M NaCl, pH 7.5 at a flow rate of 4 ml/min. Active fractions were pooled, concentrated by ultrafiltration with a YM30 membrane (Amicon) and dialysed against buffer B at 4°C overnight.

4.2.3.4 Chromatofocusing

The recovered fractions (100 ml with 633 mg of protein) from the Q-Sepharose step were split into six batches and loaded onto a Mono P HR 5/20 column (Pharmacia Biotech). Proteins were eluted with a 40 ml pH linear gradient (6.5-5.5) in buffer D and E with a flow rate of 1.0 ml/min. Buffer D contained 25 mM Bis-Tris, 1.0 mM $MgCl_2$, pH 6.5, and buffer E contained 25 mM Bis-Tris, 1.0 mM $MgCl_2$, 4.0 ml Polybuffer 96 (Pharmacia), 6.0 ml Polybuffer 74 in a total volume of 100 ml (Pharmacia Biotech), pH 5.5. Active fractions were pooled and concentrated (10.5 ml with 58.7 mg protein) by ultrafiltration and dialysed at 4°C overnight against buffer F: 25 mM N-[2-hydroxylethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), 50 mM NaCl, 1.0 mM $MgCl_2$, pH 7.8.

4.2.3.5 Gel filtration chromatography

The concentrated and dialysed sample (0.5 ml for each run) was applied to a BIOSEP-SEC3000 column (60 x 0.78 cm, Phenomenex, USA) which was equilibrated with buffer F as the mobile phase at 0.75 ml/min. Active fractions were pooled, concentrated (30 mg of protein) and dialysed at 4°C overnight against buffer G containing 25 mM Bis-Tris, 5 mM $MgCl_2$, pH 6.0.

4.2.3.6 Reactive red 120 dye-ligand chromatography

Dialysed active fractions following gel filtration were loaded onto a 20 ml reactive red 120 dye-ligand column which was equilibrated with buffer G. After washing of the unbound protein, the enzyme was eluted by three linear gradients of $MgCl_2$, pH and PP_i utilising buffers G and H. Buffer H contained 25 mM Tris-HCl, 1.0 mM $MgCl_2$, 10 mM PP_i, pH 7.5 at a flow rate 3.0 ml/min. The purified PFK was

concentrated (10 ml with 3.37 mg of protein) and extensively dialysed against 20 mM Bis-Tris, 1.0 mM $MgCl_2$, pH 6.8, to remove PP_i from the final purification step. Aliquots of the purified enzyme were then stored in 50% glycerol, 7.0 mM β-mercaptoethanol, 0.1 mM EDTA and 1.0 mM $MgCl_2$ at –70°C.

4.2.4 SDS-PAGE gel electrophoresis

Determination of the subunit molecular weight was carried out using 10-15% SDS Phast Gels (Pharmacia Biotech). The *p*I was derived from comparison of the purified enzyme on pH 3-9 IEF gels (Pharmacia Biotech) with a pH 3.0-10.0 isoelectric focusing standard (Pharmacia Biotech). The protocols for SDS-PAGE and IEF separations and subsequent silver staining are listed and described in Sections 2.9.2 to 2.9.4.

4.2.5 Determination of the native molecular mass and thermostability

The molecular mass of the purified PP_i-PFK was determined by either the gel filtration using a BIOSEP-SEC3000 column (Phenomenex, USA) equilibrated with buffer described in Section 2.6 or mass spectrometry (Section 2.7). The standard proteins used are listed in Section 2.6. All injections for gel filtration were of a constant size (100 µl) and the proteins were eluted at a flow rate of 1.0 ml/min in the buffer used for equilibration. The thermostability of the purified *Dictyoglomus* PFK was investigated using the protocol described in Section 2.10.3.

4.2.6 N-terminal sequencing and BLASTP searches

The first 21 residues were sequenced and used for homology searching and incorporated the BLASTP search programme at the National Centre for

Biotechnology Information, National Library of Medicine, National Institute of Health, Bethesda, Maryland, USA (Altschul et al., 1997).

4.2.7 Determination of kinetic parameters and activator/inhibitor effects

Forward reaction kinetic parameters were determined at 50°C and pH 6.0 by either varying the concentration of F-6-P or PP_i (or other phosphoryl donors) in the presence of saturating quantities of PP_i (1.0 mM) or F-6-P (5.0 mM), respectively. Reverse reaction parameters were determined at 50°C and pH 7.2 by either varying the concentration of F-1,6-P_2 or phosphate (P_i) in the presence of saturating quantities of P_i (10 mM) or F-1,6-P_2 (10 mM), respectively. Possible activators or inhibitors of PP_i-PFK were added separately (each pH adjusted to 7.0) to the assay mixture with the purified enzyme, using near-apparent K_m concentrations of the substrates F-6-P (0.5 mM) and PP_i (0.3 mM).

4.3 RESULTS

4.3.1 Purification of the *Dictyoglomus* PP_i-PFK

The elution profiles for the *Dictyoglomus* PP_i-PFK purification are presented in Figures 4.2-4.6.

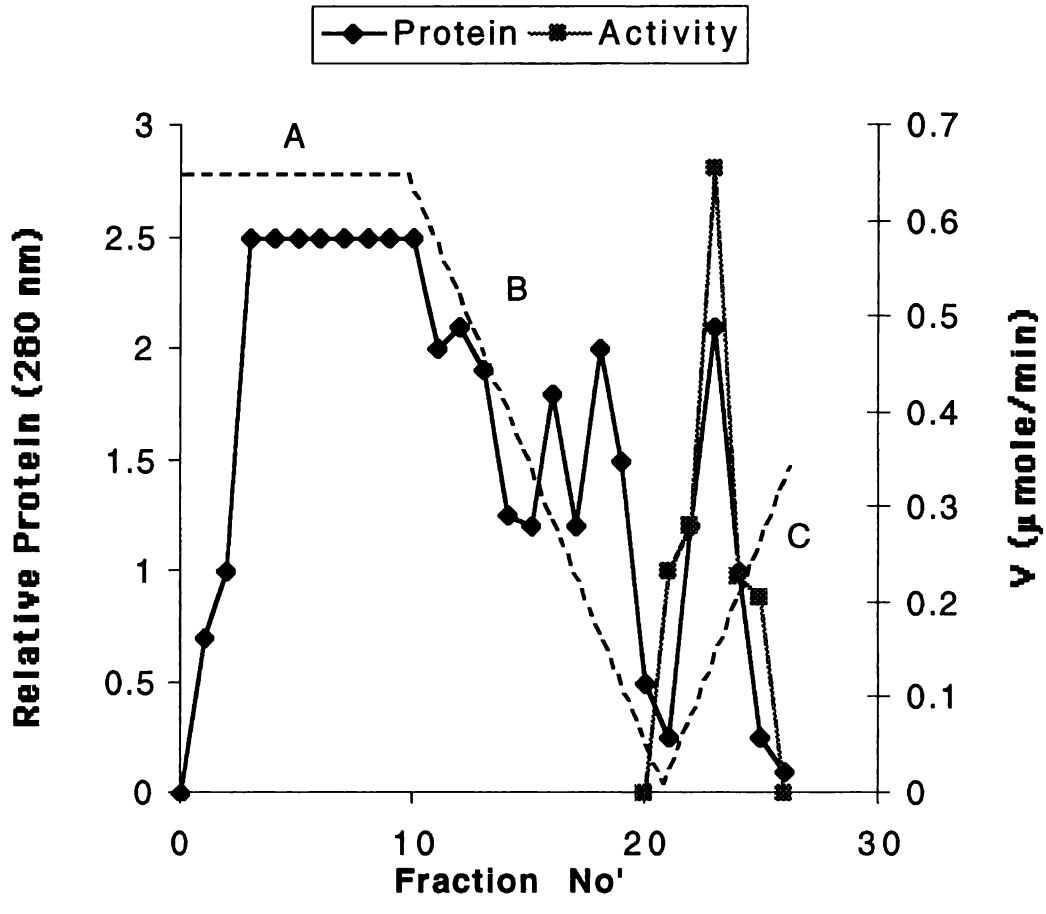


Figure 4.2 The elution profile of the *Dictyoglomus* PP_i-PFK from a phenyl-Sepharose column. Unbound protein was eluted from the column between fractions 1 and 10. A linear gradient from 1.0 M to 0.0 M of ammonium sulfate was carried out between fractions 11 and 21. An ethanediol gradient from 0% to 50% (v/v) was used for fractions 21 to 27. A: 50 mM Tris-HCl buffer (pH 7.5) containing 1.0 M $(NH_4)_2SO_4$, 5.0 mM $MgCl_2$; B: a decreasing linear gradient from 1.0 M to 0.0 M $(NH_4)_2SO_4$ in the Tris-HCl buffer; C: an increasing linear gradient from 0% to 50% ethanediol in the Tris-HCl buffer, pH 7.5. The PFK eluted at the end of the ammonium sulfate gradient and at the start of the ethanediol gradient between fractions 21 and 25. The 280 nm absorbance (A_{280}) plot indicates the relative amount of protein eluted from the column. The reaction velocity (µmole/min) was monitored and is based on assay A_{340}. Fraction No': Fraction number.

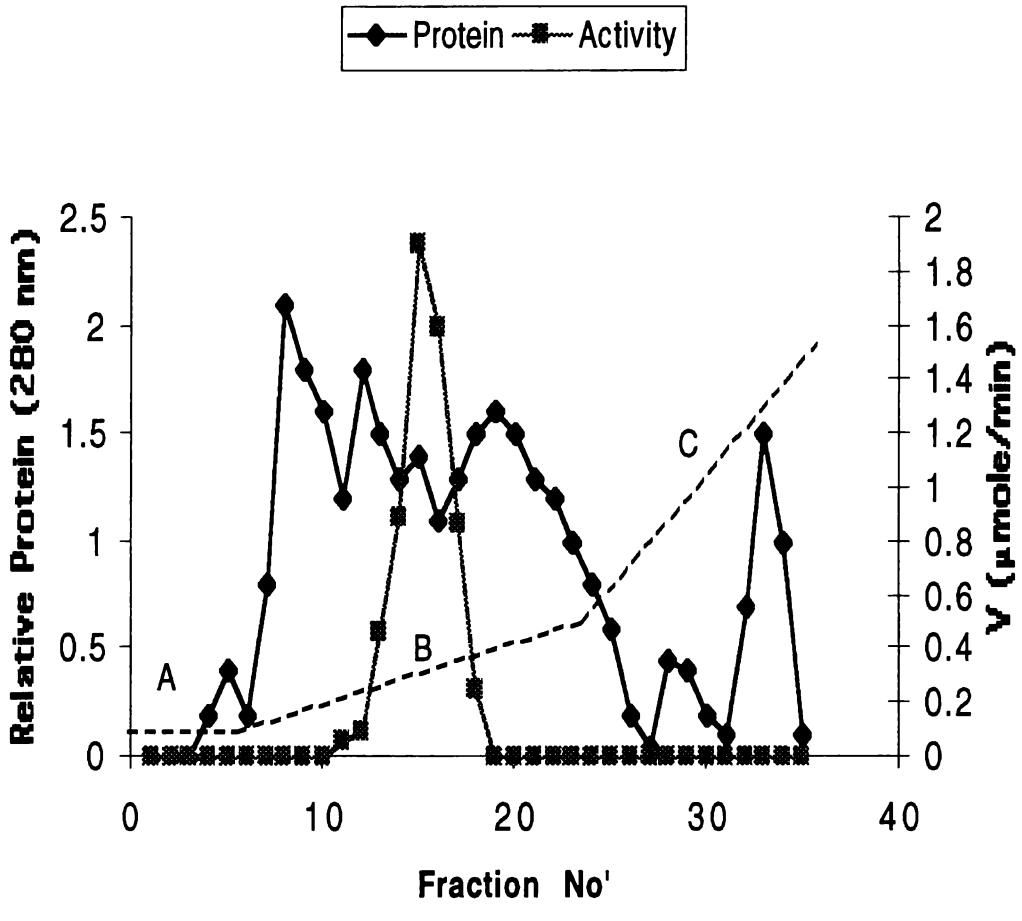


Figure 4.3 The elution profile of the *Dictyoglomus* PP_i-PFK from a Q-Sepharose column. Unbound protein was eluted from the column between fractions 4 and 7. The elution profile indicated that most of the proteins were bound onto a Q-Sepharose column. The PFK was eluted within approximately 200-400 mM of NaCl between fractions 12 and 18. A: 50 mM Tris-HCl buffer (pH 7.5) containing 1.0 mM $MgCl_2$; B: a linear gradient of NaCl from 0 to 500 mM in the Tris-HCl buffer; C: A linear gradient of NaCl from 0.5 M to 1.0 M in the Tris-HCl buffer. The 280 nm absorbance (A_{280}) plot indicates the relative amount of protein eluted from the column. The reaction velocity (μmole/min) was monitored and is based on assay A_{340}. Fraction No': Fraction number.

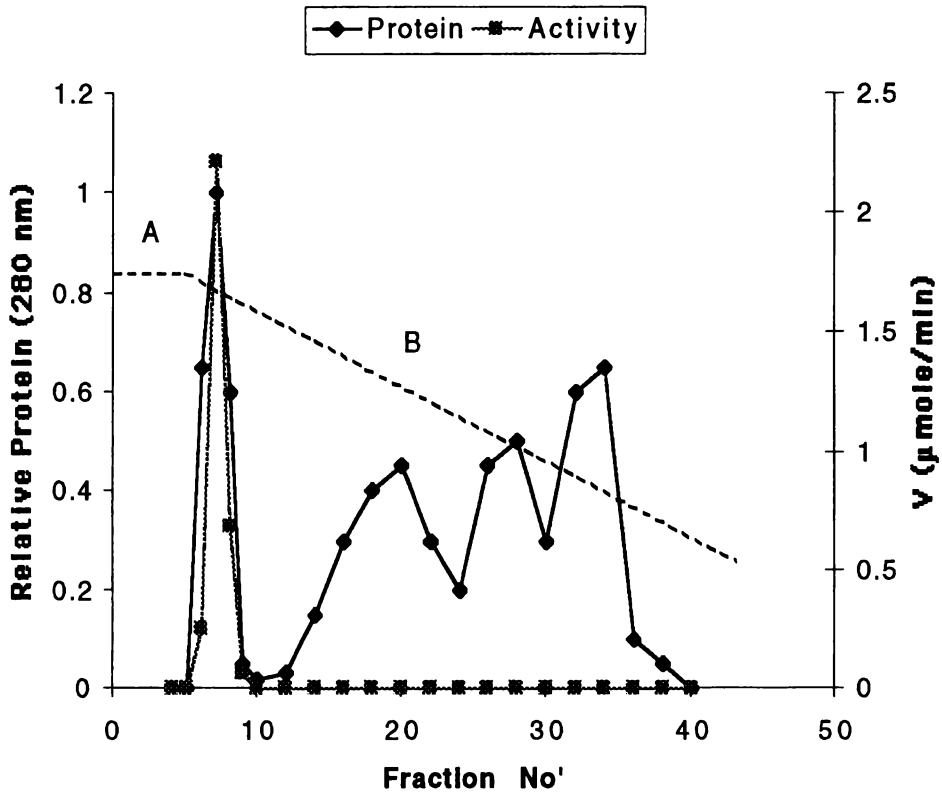


Figure 4.4 The elution profile of the *Dictyoglomus* PP_i-PFK from a Mono-P column. A linear pH gradient from pH 6.1 to 5.0 was employed in Mono-P chromatofocusing. The *Dictyoglomus* PP_i-PFK was eluted between the pH of 6.0-5.8. A: 25 mM Bis-Tris (pH 6.5) containing 1.0 mM $MgCl_2$; B: 25 mM Bis-Tris, 1.0 mM $MgCl_2$, 4.0 ml Polybuffer 96, 6.0 ml Polybuffer 74 in a total volume of 100 ml (Pharmacia Biotech), pH 5.5. A 1.5-fold purification was achieved in this step. The 280 nm absorbance (A_{280}) plot indicates the relative amount of protein eluted from the column. The reaction velocity (µmole/min) was monitored and is based on assay A_{340}. Fraction No': Fraction number.

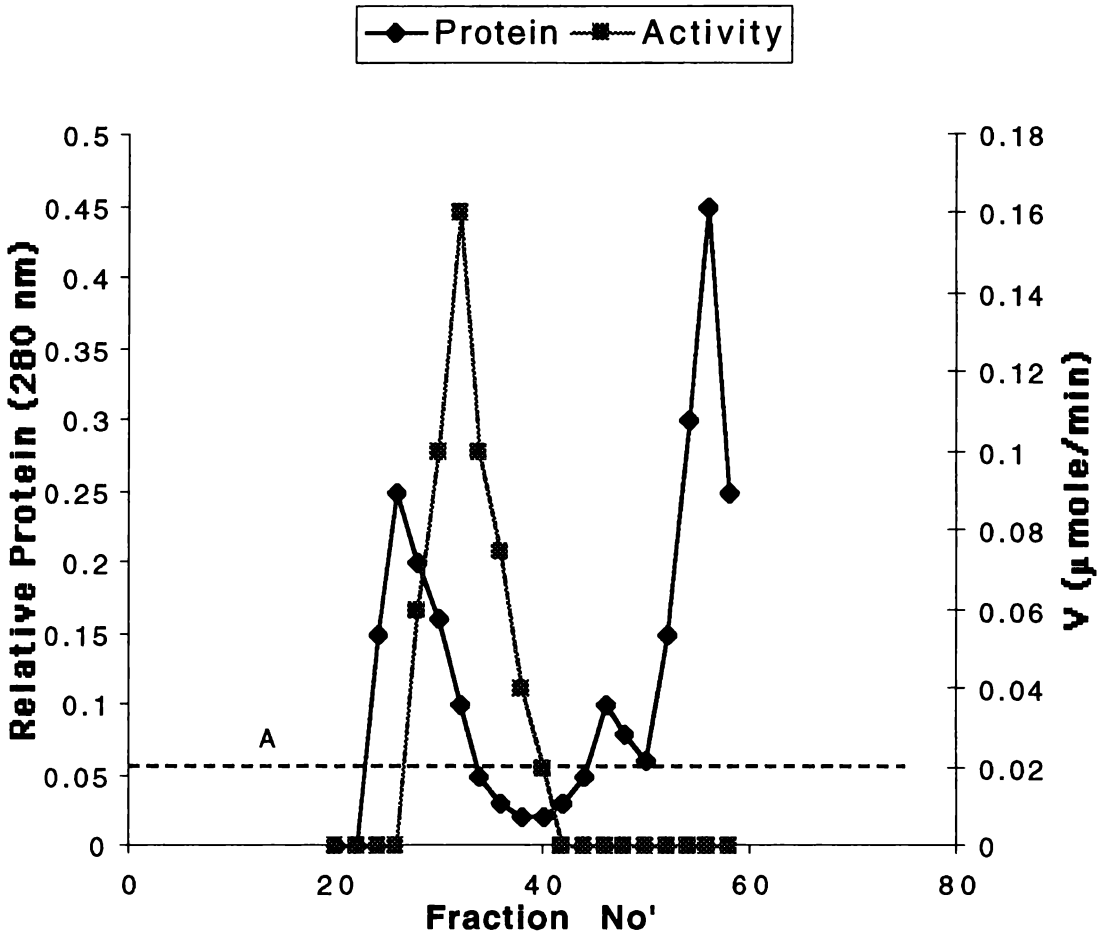


Figure 4.5 The elution profile of the *Dictyoglomus* PP_i-PFK during gel filtration. The active fractions with the PP_i-PFK activity between fractions 25 to 40 were pooled. A: 25 mM HEPES buffer with 50 mM NaCl, pH 7.8 was used as elution buffer. The 280 nm absorbance (A_{280}) plot indicates the relative amount of protein eluted from the column. The reaction velocity (μmole/min) was monitored and is based on assay A_{340}. Fraction No': Fraction number.

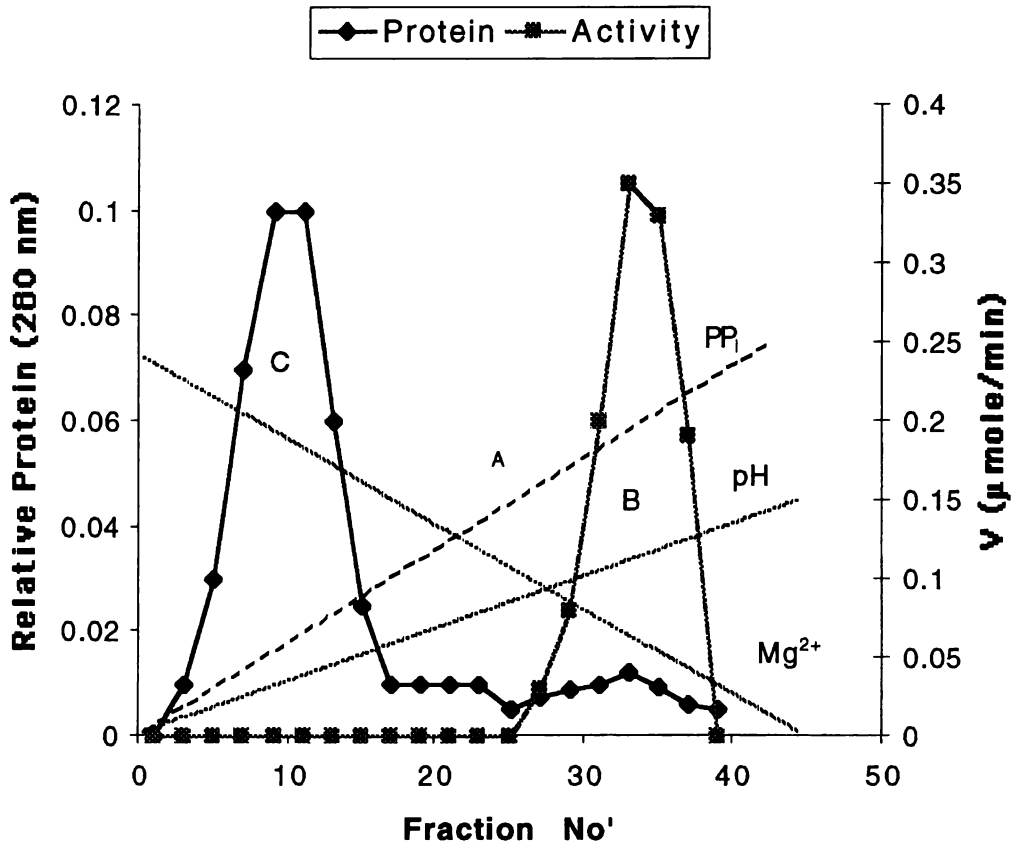


Figure 4.6 The elution profile of the *Dictyoglomus* PP_i-PFK from a reactive red 120 dye-column. Three gradients were employed in this step. A: a linear gradient of PP_i from 0.0 mM to 5.0 mM; B: a linear gradient of pH from 6.0 to 7.0; C: a linear gradient of $MgCl_2$ from 5.0 to 2.0 mM. A large protein peak without enzyme activity was eluted between fractions 1 and 18. The *Dictyoglomus* PP_i-PFK was eluted strongly with PP_i from 2.5 and 5.0 mM. The strategies of low pH (6.0) and high Mg^{2+} (5.0 mM) in starting buffer was used to enhance enzyme binding onto the reactive red 120 dye-column (Scopes, 1994). The 280 nm absorbance (A_{280}) plot indicates the relative amount of protein eluted from the column. The reaction velocity (μmole/min) was monitored and is based on assay A_{340}. Fraction No': Fraction number.

The PP_i-PFK was purified 231-fold to homogeneity with a final yield of 2.4% of the activity in the cell-free extract as indicated by SDS-PAGE (Fig.4.7), after elution from the reactive red 120 dye-column and had a specific activity of 9.7 U/mg protein

(purification summarised in Table 4.1). The enzyme was apparently not sensitive to oxygen during the purification. Based on the % yield of activity and the quantity of enzyme recovery after the reactive red 120 step, the PFK would represent approximately 0.4% of the total soluble cell protein.

Table 4. 1 Purification of the PP_i-PFK from the *D. thermophilum* Rt46 B.1

Step	Total activity(U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Cell-free extract	1,374	13,230	0.1	1	100
Phenyl-Sepharose	955	927	1.0	10	70
Q-Sepharose	730	633	1.1	11	53
Mono P	103	58.7	1.7	17	7.5
Gel filtration	72.8	30.3	2.4	23	5.3
Reactive red 120	32.9	3.37	9.7	97	2.4

4.3.2 Subunit structure, *p*I and thermostability of the enzyme

The subunit molecular weight of the PP_i-PFK was estimated to be approximately 37.000 by SDS-PAGE (Fig. 4.7). Mass spectrometry data indicated that the *Dictyoglomus* enzyme had a subunit size of 37.3 kDa (Appendix B.5). Gel filtration showed that the native enzyme had a molecular mass of approximately 65 kDa (Fig. 4.8). Thus, the PP_i-PFK from *D. thermophilum* Rt46 B.1 is indicated to be a homodimer. The half-life for the enzyme was determined to be approximately 10 min at 90°C and 160 min at 80°C when 50 mM MOPS buffer was used.

The *Dictyoglomus* PP_i-PFK had a molecular mass of approximately 65 kDa (Fig. 4.8) which was determined by gel filtration with the standard markers described in Section 2.6. The *p*I of the purified native *Dictyoglomus* PP_i-PFK was 4.4. The *p*I standard markers are described in Section 2.9.3.

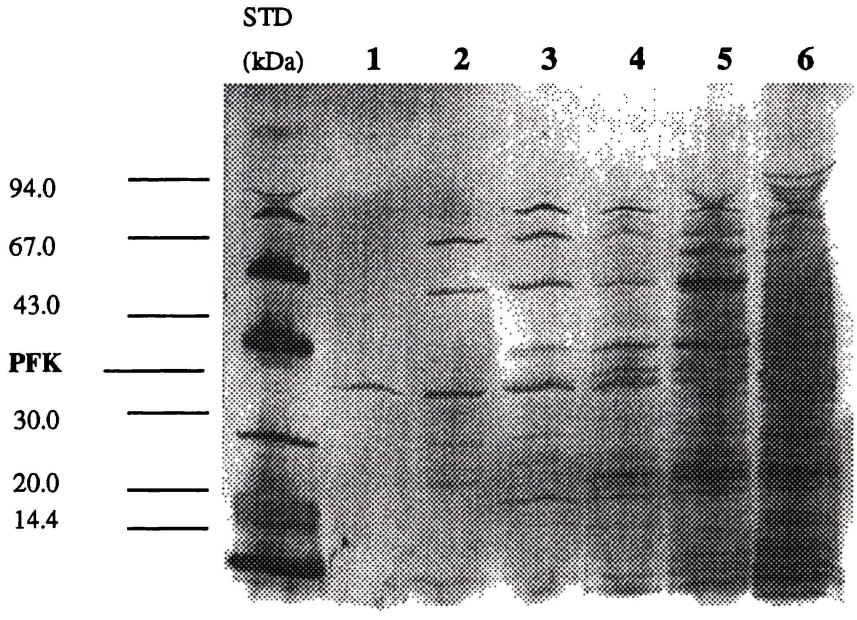


Figure 4.7 SDS-PAGE of the purified *Dictyoglomus* PP_i-PFK and purification steps. SDS-PAGE gel (silver-stained) of fractions obtained during purification of the PP_i-PFK. Lane 1 contained 0.3 μg of purified native *Dictyoglomus* PP_i-PFK and the remaining lanes contained (purification step, quantity in μg): lane 2 (step 5, 1.0); lane 3 (step 4, 1.0); lane 5 (step 2, 4.0); and lane 6 (step 1, 8.0). Lane STD (standard marker) contained the standard markers described in Section 2.9.2.

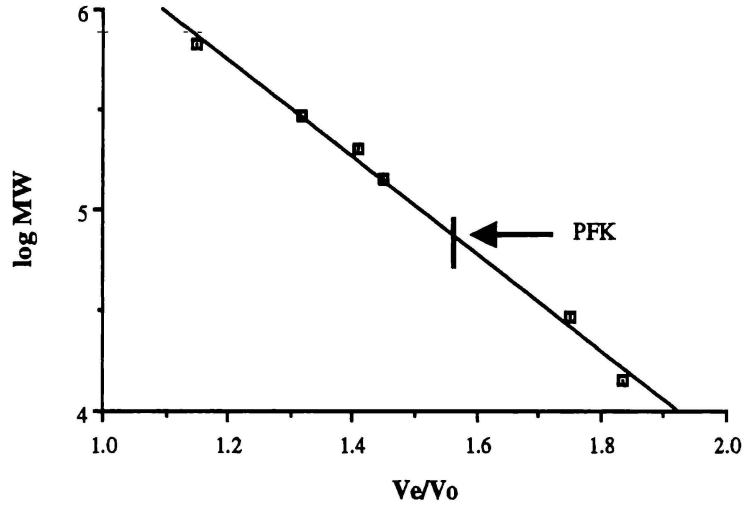


Figure 4.8 Determination of native molecular mass of the *Dictyoglomus* PP_i-PFK by gel filtration chromatography

4.3.3 Substrate specificity, cation effects and kinetic properties

The enzyme had optimal activity in the forward reaction at pH 5.7-6.3 at 50°C. Various phosphoryl donors were tested and it was found the enzyme could utilise PP_i, PPP_i and polyP (n=15±3). The purified *Dictyoglomus* enzyme could not use any of following as the phosphoryl donor: ATP, ADP, TTP, TDP, GTP, GDP, CTP, CDP, UTP, UDP, PEP, acetyl phosphate, phosphoarginine, D-glycerate phosphate, D-ribose-5-phosphate, D-glucose-6-phosphate and β-erythrose-4-phosphate. Interestingly, when the concentration of $MgCl_2$ was increased from 3.5 to 30 mM, the activity on polyP (n=15±3) was nearly as great as that on PP_i (Table 4.2). To our knowledge, this is the first report indicating polyP (n=15±3) based PFK activity in an extremely thermophilic bacterium.

Table 4.2 Phosphoryl donors for the *D. thermophilum* PP_i-PFK

Phosphoryl donor	mM	% Activity (3.5 mM $MgCl_2$)	% Activity (30 mM $MgCl_2$)
PP_i	1.0	100	100
PPP_i	1.0	82	57
polyP (n=15±3)	1.0	75	98
ATP	1.0	0	ND
ADP	1.0	0	ND

20 mM Bis-Tris, 5.0 mM F-6-P, 3.0 mM $MgCl_2$, pH 6.0 (at 50°C)
ND: not detected

The pH optimum for the reverse reaction was 7.0-7.5. Optimal activity for the forward reaction was obtained with 0.5-3.5 mM $MgCl_2$ (with a PP_i concentration of 1.0 mM; not shown). The apparent K_m and V_{max} values for the forward and reverse reactions, as determined by double-reciprocal plots, are summarised in Table 4.3. The apparent K_m values for both substrates for the reverse reaction were much higher than the substrates for the forward reaction and the V_{max} values much lower. For

example, the K_m for F-1,6-P_2 was 23-fold higher than the K_m for F-6-P and the ratio of K_m (P_i) to K_m (PP_i) was 195-fold. In addition, the ratios of V_{max}/K_m for PP_i and F-6-P for the forward reaction were much higher than the V_{max}/K_m ratios for P_i and F-1,6-P_2 for the reverse reaction. The apparent K_m and V_{max} values for PPP_i were an order of magnitude higher and approximately half that determined for PP_i, respectively. In addition to the enzyme using PP_i as the phosphoryl donor, the enzyme could also utilise PPP_i and polyP (n=15±3) at 57% and 98% of the control reaction rates, respectively, when sufficient Mg^{2+} (30 mM) was present.

Table 4.3 Kinetic parameters of the *D. thermophilum* PP_i-PFK

Variable substrate	K_m (mM)	V_{max} (U/min)	V_{max}/K_m
F-6-P[a]	0.228	4.62	20.3
PP_i[b]	0.022	5.30	241
PPP_i[b]	0.220	2.86	13.1
F-1, 6-P_2[c]	2.900	0.15	0.05
P_i[d]	4.300	0.60	0.14

[a] With 3 mM PP_i and 3.5 mM $MgCl_2$
[b] With 5 mM F-6-P and 3.5 mM $MgCl_2$
[c] With 20 mM P_i and 3.5 mM $MgCl_2$
[d] With 10 mM F-1,6-P_2 and 3.5 mM $MgCl_2$

The activity o f the *Dictyoglomus* PP_i-PFK was not affected by the traditional allosteric effectors, i.e. ATP, ADP, AMP, GDP, PEP or citrate (all at 1.0 mM). In addition, the activity of *Dictyoglomus* PP_i-PFK was not affected by either 0.1 or 1.0 mM F-2,6-P_2 (not shown).

Experiments were performed by adding the concentration of cation listed to the standard reaction mix described in Section 2.3. The enzyme activity was strongly inhibited by Cu^{2+}, Se^{4+}, Mn^{2+} and Fe^{3+} (Table 4.4). For example, the addition of only 1.0 μM Cu^{2+} resulted in a decrease in activity of 62% (compared to the control value)

whereas in control reactions only a slight inhibition of the coupling enzyme system was found at a Cu^{2+} concentration of 100 μM. In contrast to the inhibitory effects of Cu^{2+}, the enzyme was partially activated by K^+ (10 mM, 138%), Na^+ (10 mM, 135%), Fe^{2+} (0.1 mM, 120%) and Co^{2+} (0.1 mM, 120%). Higher concentrations (100 mM) of K^+ and Na^+ had no significant effect on the enzyme activity. Interestingly, there was not rate-limiting for PFK assay found even when the activity of coupling enzyme system was decreased to 32% (as the absolute A_{340} is over 1.2; not shown).

Table 4.4 Effect of some cations on the *Dictyoglomus* PP_i-PFK activity

Cations	mM	V (U/min)	% Relative Activity	% Relative Activity (CS)[a]
$MgCl_2$[b]	3.5	0.96	100	100
KCl	10	1.24	138	119
KCl	100	0.99	103	89
NaCl	10	1.3	135	152
NaCl	100	0.88	92	125
$FeCl_2$	0.1	1.16	121	32
$CoCl_2$	0.1	1.16	121	66
$MnCl_2$	0.1	0.53	54	87
$FeCl_3$	0.1	0.46	48	69
$ZnCl_2$	0.1	0.46	48	84
$SeCl_4$	0.1	0.24	25	75
$SeCl_4$	0.01	0.36	38	NT
$CuCl_2$	0.1	0	0	71
$CuCl_2$	0.01	0.08	8	NT
$CuCl_2$	0.001	0.36	38	NT
$CuCl_2$	0.0001	0.77	80	NT

[a]CS: coupling system

[b]Control assay: 30 mM Bis-Tris (pH 6.0) containing 3.0 mM $MgCl_2$, 5.0 mM F-1,6-P_2, 0.2 mM NADH

NT: not tested

4.3.4 Determination of N-terminal sequence

The first 21 amino acid residues of the *Dictyoglomus* PP_i-PFK were SKMRIGVLTGGGDCPGLNPAI. From Table 4.5, the N-terminal sequence is 89%, 80% and 78% identical to the N-termini of the PP_i-PFKs from *Mycobacterium tuberculosis, A. methanolica* and *T. tenax*, respectively, but only 58% identical to the PP_i-PFK of the primitive eukaryote, *T. vaginalis* (Altschul et al., 1997).

Table 4.5 N-Terminal sequencing of the *D. thermophilum* PP_i-PFK from BLAST search results

Organism	Identities (%)	Positives (%)	PFK
M. tuberculosis	89	89	PP_i
M. leprae	84	89	PP_i
A. methanolica	80	90	PP_i
S. coelicolar	78	89	ATP
T. tenax	78	89	PP_i
T. thermophilus	77	83	ATP
B. stearothermophilus	77	83	ATP
B. subtilis	72	83	ATP
E. coli	72	83	ATP
A. aeolicus	68	78	ATP
L. delbrueckii	66	83	ATP
T. brucei	64	76	ATP
T. vaginalis	58	70	PP_i
N. fowleri	58	70	PP_i

4.4 DISCUSSION

The *D. thermophilum* PP_i-PFK was strongly retained on the phenyl-Sepharose column, eluting partially at the end of gradient B and required buffer B with 50% ethanediol for complete elution (Fig. 4.2). The chromatographic profile shows a large unbound peak without activity was removed during elution from the phenyl-

Sepharose column, which gave a 10-fold level of purification. The most successful purification step in this work involved the used of the reactive red 120 dye-column (Procion Red H-E3B). The low pH (6.0) and high $MgCl_2$ (5.0 mM) in the starting buffer were used to enhance protein binding onto the red dye which possesses nine aromatic rings, six residues of sulphonic acid groups, two chloride and two hydroxyl groups (Scopes, 1994). These groups might interact with proteins by hydrophobic, electrostatic and/or hydrogen bonding. The results of the chromatography show that nearly all the protein bound onto the resin. The PP_i-PFK was eluted by PP_i combined with a relative low concentration (3.0-2.0 mM) of Mg^{2+} and a near neutral pH (6.8-7.0). In addition, other types of dye-ligand matrices were screened (Hondmann and Visser, 1990) and the results showed that the *D. thermophilum* PP_i-PFK could also bind onto a green A column and the enzyme could be eluted with PP_i from the green A column (not shown).

The *D. thermophilum* PP_i-PFK is indicated from the SDS-PAGE (37 kDa), mass spectrometry (37.3, Appendix B.5) and gel filtration results (65 kDa) to be a homodimer. The optimum pH was 5.7-6.3 for the forward reaction and 7.0-7.5 for the reverse reaction. The traditional allosteric effectors of bacterial ATP-PFK, i.e. F-2,6-P_2, AMP, GDP, PEP succinate or citrate, do not significantly effect the *Dictyoglomus* enzyme activity. The lack of effect of traditional eukaryal or bacterial allosteric ATP-PFK effectors (PEP, ADP, GDP, succinate, AMP, cAMP and citrate) on the *Dictyoglomus* enzyme, or by F-2,6-P_2 which is a potent glycolytic effector for most PP_i-PFKs from higher eukaryotes but not in either prokaryotes or most anaerobic protozoal PP_i-PFKs, suggests that one of the major control points of the EM pathway is not operational (Mertens, 1991). The lack of allosteric control is a general feature of PP_i-PFKs (Mertens, 1991) and implies that the rate of the PP_i-PFK catalysed reaction must instead be controlled by a combination of the level of activity

of the enzyme, its kinetic properties and the cellular concentrations of reactants and products. In support of the kinetic parameters having a significant effect on controlling the glycolytic flux are the much lower apparent K_m values for the forward reaction, the lower V_{max} values in the reverse reaction and the much higher V_{max}/K_m ratios seen with either PP_i or F-6-P for the reaction. These results taken together suggest that the glycolytic direction of the reaction would be favoured in this organism.

A number of PP_i-PFKs have been identified from bacteria, primitive eukaryotes and higher plants. For example, a dimeric PP_i-PFK with a subunit size of 37,000 was partially purified from *A. laidlawii* (Pollack and Williams, 1986). The acidic pH optimum for the forward reaction and neutral to slightly alkaline optimum for the reverse reaction are similar to the PP_i-PFKs from some bacteria and primitive eukaryal organisms, e.g. *A. methanolica* (Alves et al., 1996), *R. rubrum* (Pfleiderer and Klemme, 1980), and in the anaerobic protozoa *I. prostoma*, *T. gondii* and *T. vaginalis* (Mertens et al., 1989b). *T. foetus* (Mertens et al., 1989b), *T. gondii* (Peng and Mansour, 1982), *E. tenella* (Denton et al., 1994) and *N. fowleri* (Mertens et al., 1993). In contrast, the pH optima of PP_i-PFKs from the bacterial *P. freudenreichii*, primitive eukaryotes *G. lamblia* (Li and Phillips, 1995) and *E. histolytica* and those of higher plants are near-neutral for the forward and the reverse reactions (Reeve et al., 1976; Yan and Tao, 1984; O'Brien et al., 1991). The *Achloeplasma* enzyme required a pH of 7.4 for maximum activity, which was also different from the *Dictyoglomus* enzyme (Pollack and Williams, 1986).

The relative thermostability of the *Dictyoglomus* PFK could be related to increased hydrophobicity, chain rigidity or the overall tightness of intramolecular packing (Creighton, 1990; Jaenicke et al., 1996). Alternatively, the thermostability could be

due to unique tertiary structure interactions, as the thermostability of the *Thermotoga* EF-Tu is critically dependent upon unique tertiary structure of interactions involving certain N-terminal residues of the molecule (Sanagelantoni et al., 1996).

The *Dictyoglomus* PP_i-PFK has a requirement for Mg^{2+} ions for optimal activity and was partially activated by some cations, i.e. K^+ (10 mM), Na^+ (10 mM), Fe^{2+} (0.1 mM) and Co^{2+} (0.1 mM). The enzyme was sensitive to Zn^{2+}, Se^{4+} and Fe^{3+} and, interestingly, extremely sensitive to Cu^{2+}. The extreme sensitivity of the enzyme to Cu^{2+} ions could have a significant role in determining the ecological distribution of *D. thermophilum* strains. The organism must have cellular detoxifying mechanisms for maintaining the intracellular concentration of Cu^{2+} at extremely low levels.

The ability of the *D. thermophilum* PP_i-PFK to utilise PP_i, PPP_i and polyP (n=15±3) is potentially relevant with regard to the origins of PFK activity. This is because PP_i and PPP_i are known to be formed continuously under hydrothermal conditions (Yamagata et al., 1991) and are considered as relics of ancient metabolism (Kornberg, 1995). These substrates may have provided a continuing energy source to drive metabolism during primeval conditions (Baltscheffsky, 1996). In such a scenario, the earliest life forms would be expected to have possessed a PP_i-PFK activity (Morgan and Ronimus, 1998). The reversible nature of the reaction with PP_i as phosphoryl donor with its $\Delta G^{\circ\prime}$ value of -2.08 kcal/mol (-8.7 kJ/mol) would be compatible with either a glycolytic or gluconeogenic origin of the EM pathway (Mertens, 1991). In support of this scenario, Siebers et al. (1998) have recently inferred that the PP_i-PFKs from the hyperthermophilic crenarchaeon *T. tenax* and the mesophilic bacterium *A. methanolica* represent a unique lineage of PFK, and possibly the most ancient form of the PFK enzyme. The PP_i-PFK from *D. thermophilum* Rt46 B.1 is similar to the *T.*

tenax enzyme in respect to its nonallosteric nature, subunit size and N-terminal amino acid sequence. It was an unexpected finding to discover a PP_i-PFK in *Dictyoglomus* as this organism branches at the base of the phylum Thermotogales (Love et al., 1993). Both *T. maritima* (Selig et al., 1997) and *F. nodosum*, which also branches within the Thermotogales (Love et al., 1993), predominantly possess an ATP-PFK activity, but *F. nodosum* also had a relatively high PP_i-PFK activity (Section 3.3.1).

Surprisingly, *A. aeolicus*, the deepest branching thermophilic bacterium, has also been indicated by sequence analysis to contain a putative ATP-PFK (Deckert et al., 1998) and this enzyme clusters with the ATP-PFKs of *T. thermophilus* and *B. stearothermophilus* (Section 5.3.4). It will be interesting to see if the full sequence of the *Dictyoglomus* PP_i-PFK encoding gene confirms a phylogenetic linkage with the *T. tenax* enzyme or whether it is more closely linked to the ATP-PFKs of *Aquifex*, *Thermotoga* and/or *Fervidobacterium*.

CHAPTER FIVE

SEQUENCING, CLONING AND HIGH-LEVEL EXPRESSION OF THE *pfp* GENE ENCODING A PP_i-PFK FROM THE EXTREMELY THERMOPHILIC BACTERIUM *DICTYOGLOMUS THERMOPHILUM*

5.1 INTRODUCTION

A large number of PFKs from the three domains of life have been sequenced, cloned and expressed during the last 20 years. For example, sequenced, cloned and expressed ATP-PFKs have been described from human muscle and platelet cells (Nakajima et al., 1987; Simpson and Fothergill-Gilmore, 1991), rabbit muscle (Lee et al., 1987), animals (Currie and Sullivan, 1994), Plants (Kruger and Hammond, 1988); primitive eukaryote (Michels et al., 1997), mesophilic bacteria (Hellinga and Evans, 1985; Chevalier et al., 1990; Kretschmer et al., 1991; Wu et al., 1991; Xiao and Moore, 1993; Branny et al., 1993, 1996; Ding et al., 1994; Alves et al., 1997) and thermophilic bacteria (Kolb et al., 1980; French et al., 1987; Belouski et al., 1998; Xu et al., 1991) and yeast (Heinisch, 1993). PP_i-PFKs from plants (Carlisle et al., 1990; Todd et al., 1995), primitive eukaryotes (Huang et al., 1995; Rozario et al., 1995; Wessberg et al., 1995; Bruchhaus et al., 1996; Mertens et al., 1998), bacteria (Ladror et al., 1991; Alves et al., 1996; Branny et al., 1996) and archaea (Siebers et al., 1998) have also been sequenced and cloned. In addition, ADP-PFKs have been sequenced and cloned from *P. furiosus* and *T. zilligii* (Tuiniga et al., 1999; Ronimus et al., 2000). At the start of this thesis, no PP_i-PFK from an extremely thermophilic bacterium had yet been sequenced, cloned or expressed.

The biochemical study of the purified *Dictyoglomus* PP_i-PFK suggested that this enzyme possessed characteristics common to most bacterial and primitive eukaryal

PP_i-PFKs. From the N-terminal sequence comparison, it was found to be closely related to the PP_i-PFKs from *A. methanolica*, *M. tuberculosis* and *T. tenax* (Section 4.3.4). Because of the deep phylogenetic origin of this organism, the nucleotide sequence of the PP_i-PFK was of interest. The crystal structure of the enzyme would also aid in the understanding of its relationship to ATP-PFKs. For both of these purposes, the cloning, sequencing and overexpression of the *pfp* gene from *Dictyoglomus* was undertaken and the results are described in this chapter.

5.2 MATERIALS AND METHODS

5.2.1 Bacterial strains and plasmids

D. thermophilum Rt46 B.1 was obtained from the TRUCC and grown in the *Dictyoglomus* medium described by Patel et al. (1987). The *E. coli* strain JM109 was used for cloning and expression (Promega Life Science). It was grown at 30°C with vigorous aeration (200 rpm) in LB broth supplemented with ampicillin (100 μg/ml). The expression plasmid pKK223-3 was obtained from Pharmacia Biotech.

5.2.2 Protocols used for gene cloning, sequencing and expression

Chemicals and reagents used in this chapter are listed in Section 2.2. The standard conditions for enzyme assays are described in Section 2.3. Two protocols (colony lifting and inverse-PCR techniques) were used for the cloning, sequencing and expression of the *Dictyoglomus pfp* gene. Genomic DNA from *D. thermophilum* was prepared as described in Section 2.11.3. Large-scale plasmid DNA was purified from *E. coli* by using the alkaline lysis method combined with caesium chloride gradient purification (Section 2.11.4.2). DNA fragments produced by PCR and restriction digests, subsequently used for sequencing and cloning experiments, were

separated on 1% TAE preparative gels using the "freeze and squeeze" technique (Section 2.12.5) and phenol:chloroform extraction, ethanol precipitation according to the methods described by Ronimus (1993) and Sambrook et al. (1989).

Table 5.1 The main strategies used for sequencing, cloning and expression of the *pfp* gene from *D. thermophilum* Rt46 B.1

Method 5.1 **Colony lifting**	**Method 5.2** **Inverse-PCR**
Probe preparations	Probe preparations
↓	↓
Genomic DNA preparations	Genomic DNA preparations
↓	↓
Restriction digest	Restriction digests
↓	↓
Southern blotting	Southern blotting
↓	↓
De-phosphorylation of vectors	Inverse-PCR
↓	↓
Ligation	Full gene sequencing
↓	↓
Transformation	Gene expression
↓	↓
Colony lifting	Enzyme purification and properties

5.2.3 Gene cloning by colony lifting method (Method 5.1)

The general procedures and protocols used for colony lifting are described in Section 2.12.

5.2.3.1 PCR amplification of a 350 bp region of the gene encoding the PP_i-PFK of *D. thermophilum*

A region of the gene encoding the PP_i-PFK of *D. thermophilum*, approximately 350 bp long, was amplified by PCR, using 40 ng of DNA template in 50 µl (final volume) of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 4.0 mM $MgCl_2$, 0.001% (w/v) gelatin, 200 µM each deoxyribonucleotide triphosphate, 50 pmol of sense and antisense primers and 2.5 U of Ampli *Taq* Gold polymerase (Perkin-Elmer Cetus). The sense and antisense degenerate primers (5'-ACW GGW GGW GGW GAT TGT C-3') and (5'-ATC ATT ATC WAT WGT TTT WGG-3') where W=A or T, were designed according to the N-terminal sequence SKMRIGV and the internal F-6-P binding conserved amino acid sequence PKTIDND, respectively. The latter amino acid sequence is highly conserved and encodes a F-6-P binding site found in most ATP- and PP_i-PFKs (see Fig. 5.13). Potential degeneracy was reduced by considering the low G+C% (29.5%) content of the *Dictyoglomus* genomic DNA (Patel et al., 1987). PCR was carried out for 35 cycles in a Mastercycler Gradient PCR thermocycler (Eppendorf, Germany) as follows: one cycle of "hot start" at 94°C for 9 min followed by 34 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 1 min and elongation at 72°C for 2 min with a 6 min final extension step.

5.2.3.2 Analysis of the sequence data in the 350 bp fragment

The 350 bp fragment by PCR amplification was purified, sequenced and the data used to search the nonredundant GenBank database with the BLASTX algorithm to ensure that the correct gene had been amplified (Table 5.2, Altschul et al., 1997).

5.2.3.3 Genomic DNA restriction digests and Southern hybridisation

Restriction digests were carried out at 37°C overnight using 2.0 μg of genomic DNA with 10 U of appropriate restriction enzymes (Section 2.12.2). The buffers used for the combination digests were chosen using the following strategies: for example, buffer H (Table 2.7) was used for all *Eco*R I/*Eco*R V, *Eco*R I/*Hin*d III, *Eco*R I/*Pst* I, *Eco*R I/*Sau*3A I digests. Buffer D was used for all of *Eco*R V/*Hin*d III, *Eco*R V/*Pst* I, *Eco*R V/*Sau*3A I digests. Digested DNAs originating from either genomic or plasmid sources were electrophoresed through 1.0% (w/v) agarose gels in 1 x TBE buffer, then processed for Southern blotting (Section 2.12.4). Southern blot transfer was conducted according to the recommendations provided by the Hybond-N^+ membrane manufacturer (Amersham Life Sciences). The DNA was fixed to the membrane by either exposure to UV light (120 joules/cm^2, 30 sec; BLX-254, Life Technology) or by baking at 80°C for 2 h. The 350 bp probe was labelled with ^{32}P-(dCTP) by the random hexamer labelling method described in Section 2.12.8 (Boehringer Mannheim). Using the 350 bp of DNA fragment as a probe labelled with ^{32}P-dCTP, hybridisation was conducted at 60°C for 16 h (Sections 2.12.8 and 2.12.9).

5.2.3.4 De-phosphorylation of vectors

The cloning vector pGEM-7Zf(+) was digested (20 μg) with *Eco*R I (3.0 U/μg) at 37°C for 6 h (Section 2.12.6). Electrophoresis was carried out to check the digest, then the digested DNA was purified for the freeze and squeeze method (Section 2.12.5). This was followed by phenol:chloroform extraction ethanol precipitation and quantification using the "spot test" method (Section 2.11.3).

5.2.3.5 Ligation, transformation and colony lifting

The *Dictyoglomus* genomic DNA was digested with *Eco*R I (3.0 U/μg) at 37°C for 6 h. The reaction mixture was loaded onto a 1.0% TAE preparative gel and run at 20-40 volts for 6-16 h. An appropriate area of the gel corresponding to the Southern hybridisation data was excised and wrapped with a sheet of Parafilm for "freezing and squeezing". After purification and quantification, the DNA was used for ligation with the de-phosphorylated cloning vector. The protocols for ligation, transformation, insert screening and colony lifting hybridisation are described in Sections 2.12.7 to 2.12.12.

5.2.4 Sequencing and cloning with inverse-PCR amplification

5.2.4.1 Restriction digests for inverse-PCR

On the basis of the data from Section 5.2.4, additional 11 new restriction enzymes (*Ava* I, *Ban* II, *Bgl* II. *Dde* I, *Kpn* I, *Msp* I, *Rsa* I, *Sau*96 I, *Sca* I, *Sty* I and *Tru*9 I) were used to screen for suitable enzymes for the inverse-PCR method.

5.2.4.2 Southern hybridisation for inverse-PCR

Southern hybridisation for inverse-PCR was performed as described in Sections 2.12.4 and 2.12.9.

5.2.4.3 Inverse-PCR amplification of the *D. thermophilum pfp* gene

The inverse-PCR technique (Collins and Weissman, 1984; Howard et al., 1988) for obtaining the sequence of the *Dictyoglomus pfp* gene was also employed in this study. The core region (350 bp), corresponding to the region between the N-terminus and

conserved F-6-P binding site, was analysed by restriction enzyme analysis (Fig. 5.2). The *Dictyoglomus* genomic DNA was digested with appropriate restriction enzymes and processed by Southern blotting. The regions adjacent to the amplified fragment were obtained using inverse-PCR (Collins and Weissman, 1984; Howard et al., 1988). Genomic DNA (1.0 μg) was completely digested at 37°C overnight with 10 U of *Sau*3A I, *Rsa* I and *Sau*96 I, respectively, then ligated separately under conditions that favoured self-circularisation (4-16 ng/μl in either 10 or 20 μl total reaction). These experiments for ligation were carried out using genomic DNA, i.e. without purifying the suitable size of DNA fragments from agarose gels. The ligation reactions were initiated by the addition of 1.0 U of T_4 DNA ligase for 40-160 ng of genomic DNA (Promega) at RT overnight. The ligation mixtures were heat-denatured at 65°C for 15 min prior to PCR and used as templates in inverse-PCR amplifications. A summary of the primers used for inverse-PCR to find the full-length *Dictyoglomus pfp* gene is as follows (shown diagrammatically in Fig. 5.11): 1> forward primer (for *Sau*3A I) 5'-AGA CAC TCT AGG AGT TGC-3'; the reverse primer used was 5'-ACC TAT AAC TTC GTC TCC-3'. 2> forward primer (for *Rsa* I) 5'-TTC CAA TGA TCG GAG TTC-3'; the reverse primer used was 5'-ACC TAT AAC TTC GTC TCC-3'. 3> forward primer (for *Sau*96 I) 5'-TCA CAT AAG AAA ACT ATA CGC-3'; the reverse primer used was 5'-ACC TAT AAC TTC GTC TCC-3'. The PCR products with the appropriate size were identified according to the data from Southern blotting, purified from 1% TAE agarose gels and prepared for DNA sequencing, computational analysis and phylogenetic comparisons (Section 2.14).

5.2.5 Construction of the *Dictyoglomus* PP_i-PFK expression clone

The open reading frame (ORF) representing the full-length sequence of the *D. thermophilum pfp* gene was amplified using genomic DNA from *D. thermophilum* by

PCR with the following primer set: a forward primer corresponding to the N-terminus containing the first 7 codons and an upstream *Eco*R I site (in bold) and 5' end-spacer (5'-GGA **GAA TTC** ATG AGT AAA ATG CGT ATT GGT G-3'); and a reverse primer corresponding to the C-terminus containing the last 7 codons and flanking *Hin*d III site (in bold) and a 5' end-spacer (5'-GC GAG **AAG CTT** TAC TTA TTA AAG AAA GTT TTT ACG-3'). The single PCR-generated DNA fragment of approximately 1.0 kb was cloned into pKK223-3 expression vector after restriction digestion of the ends with *Eco*R I and *Hin*d III at 37°C overnight, followed by ligation with T_4 DNA ligase using standard protocols (Sambrook et al., 1989).

5.2.6 Expression, purification and characterisation of the recombinant *Dictyoglomus* PP_i-PFK

The ligation mixture with plasmid pKK223-3 and restricted PCR product containing the *Dictyoglomus pfp* gene was used to transform *E. coli* strain JM109 by electroporation, which was prepared according to instructions (Bio-Rad, Gene Pulser). Screening of the clones for those with inserts was carried out through alkaline lysis miniprep plasmid isolation (Sambrook et al., 1989). The potential clones with inserts were examined by restriction digest with *Eco*R I and *Hin*d III, then transferred to 10 ml LB broth with ampicillin (100 μg/ml). In-frame ligation of the amplified DNA into pKK223-3 plasmid was confirmed by DNA sequencing (Appendix B.4). IPTG (1.0 mM) was added when the culture density reached approximately 0.6-1.0 at 600 nm. The enzyme activities were checked at 1.0, 2.0, 3.0, 4.0 and 5.0 h after IPTG induction.

The transformant selected after screening was grown at 30°C in large-scale (800 ml) of LB broth containing 100 μg/ml ampicillin with agitation (200 rpm). When the

optical density of the culture reached 0.6-1.0, the expression of the protein was induced by addition of IPTG to 1.0 mM. After 5 h of induction, the cells were harvested by centrifugation at 4,000 x *g* for 20 min at 4°C. Approximately 5.0 g of cell pellet (wet weight) was resuspended in 250 ml of lysis buffer (Section 4.2.3.1) and sonicated. The cell debris was removed by centrifugation at 11,000 x *g* for 40 min at 4°C and the cell lysate incubated in a waterbath for 30 min at 70°C. The denatured *E. coli* proteins were removed by centrifugation at 11,000 x *g* for 40 min at 4°C. The conditions for purification and characterisation of the expressed enzyme were essentially the same as described for the purification of the native enzyme (Section 4.2.3). The molecular mass of the native and the recombinant enzyme was also measured using mass spectrometry (Sections 2.6 to 2.9). Determination of protein concentrations is described in Section 2.8.

5.3 RESULTS

5.3.1 Cloning of the *D. thermophilum pfp* gene by colony lifting

5.3.1.1 Amplification of the 350 bp fragment by PCR

A single 350 base PCR fragment (the size estimated initially from gel electrophoresis; Fig. 5.1) was produced by using degenerate primers corresponding to the N-terminal sequence of the purified protein and to a conserved F-6-P binding consensus sequence common to PFKs described in Section 5.2.3.1. This fragment was purified, quantified and sequenced. The data from the sequence and restriction map of this PCR product was later used for probe preparation and Southern hybridisation.

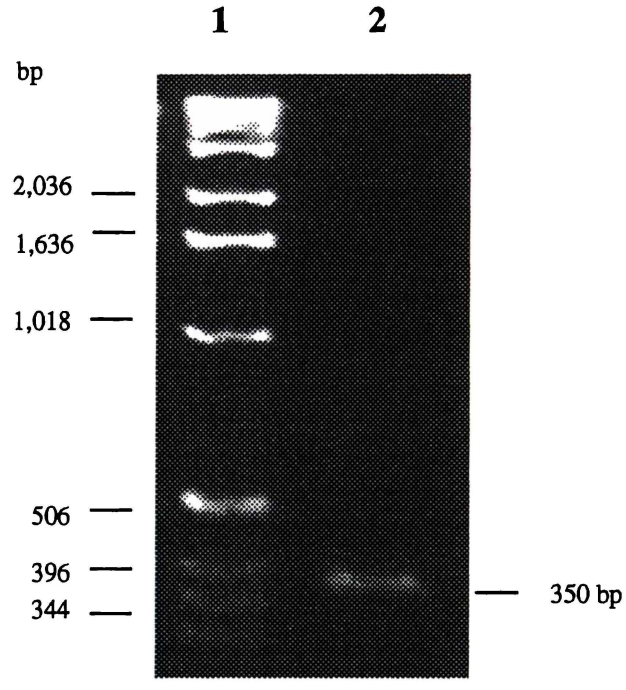


Figure 5.1 PCR amplification of a 350 bp fragment to be used for probe. Lane 1. Standard DNA (1 kb marker); Lane 2. PCR product (350 bp).

5.3.1.2 Sequence, restriction map and BLAST search of the 350 bp

The sequence and restriction map of a 350 bp PCR product obtained and corresponded to the N-terminal sequence and a consensus F-6-P binding site is shown as follows ABI sequence output (Appendix B.4).

5'-TCCCAGCTATCCGTGGTATTGTCATGAGAGCATTAGATTATGGAGACGAAGTTATAGGT
TTGAAGTATGGATGGGCTGGTCTTCTTAAGGCAGATACTATGCCTTTATCCTTAGAAATGGTAGAAGA
TATTCTTGAAATCGGCGGAACAATTCTTGGAAGTTCTAGAACAAACCCATTCAAAAAAGAAGAAGATG
TTCAAAAATGTGTTGAGAACTTCAAAAAGTTAAACTTAGATGCCTTAATCGCCATAGGTGGAGAAGAC
ACTCTAGGAGTTGCATCAAAATTTAGCAAACTTGGTCTTCCAATGATCGGAGTTCCAAAAACTATTGA
TAATGAT-3'

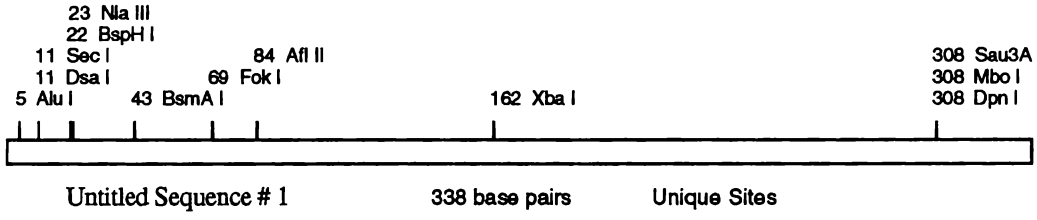


Figure 5.2 Restriction map of the 350 bp PCR fragment

A *Sau*3A I site was found at 308 bp. No restriction site for either *Eco*R I, *Eco*R V or *Hin*d III within this fragment was found. The related experiments of Southern hybridisation were related to these restriction enzymes (Sections 5.3.1.2 and 5.3.1.3). Following the purification of the 350 bp fragment and subsequent sequencing, a BLASTX search showed close homology with most ATP- and PP_i-PFKs, and particularly with group III PFKs including the crenarchaeon *T. tenax* and the bacterial *M. tuberculosis*, *A. methanolica* and *S. coelicolor* (Table 5.2).

Table 5.2 Homologues of the 350 bp fragment identified with BLAST X

Type of PFK	Organism	(bits)Value (Altschul et al., 1997)
PP_i-PFK	*A. methanolica*	117
ATP-PFK	*S. coelicolor*	113
ATP-PFK	*B. stearothermophilus*	91
ATP-PFK	*E. coli*	91
ATP-PFK	*Enterobacter cloacae*	90
PP_i-PFK	*T. tenax*	89
ATP-PFK	*A. aeolicus*	87
ATP-PFK	*B. subtilis*	85
ATP-PFK	*T. thermophilus*	85
ATP-PFK	*L. delbrueckii*	83
ATP-PFK	*T. maritima*	83
ATP-PFK	*M. tuberculosis*	75

5.3.1.3 Restriction digests and Southern hybridisations for colony lifting

The *Dictyoglomus* genomic DNA was digested by 5 restriction enzymes and 10 different double-enzyme combinations (Fig. 5.3). Each digest contained 3.6 μg of genomic DNA and was cut with either 10 U in single enzyme digests or if two enzymes were present with 5 U each enzyme and the reactions carried out at 37°C for 7.5 h.

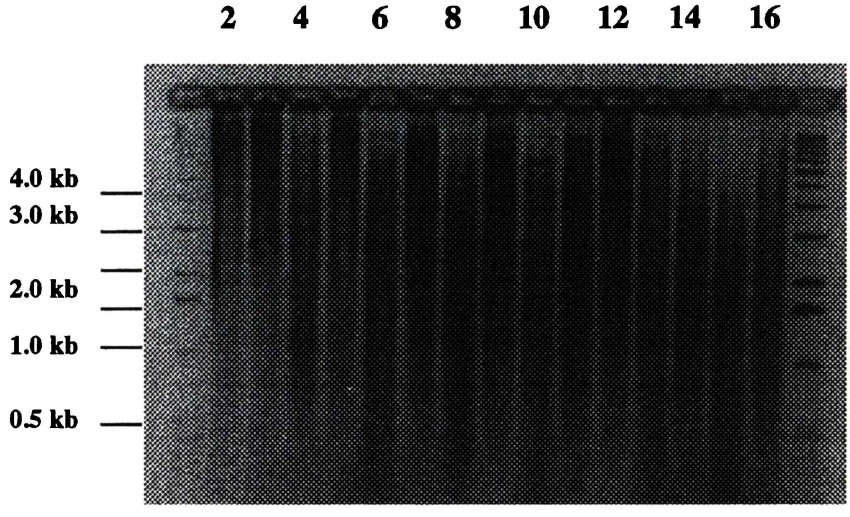


Figure 5.3 Restriction digestions of *Dictyoglomus* genomic DNA with 5 different enzymes and 10 double-enzyme combinations. Lanes 1 and 17 are 1 kb ladder; lanes from 2 to 16 were digested with the following restriction enzymes and their combinations (lane number is parentheses): *Eco*R I (2), *Eco*R V(3), *Hin*d III (4), *Pst* I (5), *Sau*3A I (6), *Eco*R I/*Eco*R V (7), *Eco*R I/*Hin*d III (8), *Eco*R I/*Pst* I (9), *Eco*R I/*Sau*3A I (10), *Eco*R V/*Hin*d III (11), *Eco*R V/*Pst* I (12), *Eco*R V/*Sau*3A I (13), *Hin*d III/*Pst* I (14), *Hin*d III/*Sau*3A I (15) and *Pst* I/*Sau*3A I (16), respectively.

The Southern hybridisation data indicated that *Eco*R I, *Eco*R V and *Hin*d III digests contained fragments of 4.5, 4.6 and 3.9 kb fragments, respectively (Fig. 5.4). A band of approximately 0.7 kb was identified in lanes 6, 10, 13, 15 and 16, which were digested with *Sau*3A I, and its combinations with other 4 restriction enzymes. Based

on the data from Fig. 5.3, *Eco*R I, *Eco*R V and *Hin*d III were used again for follow-up experiments which are described in Section 5.3.1.4 (Figs. 5.5 and 5.6).

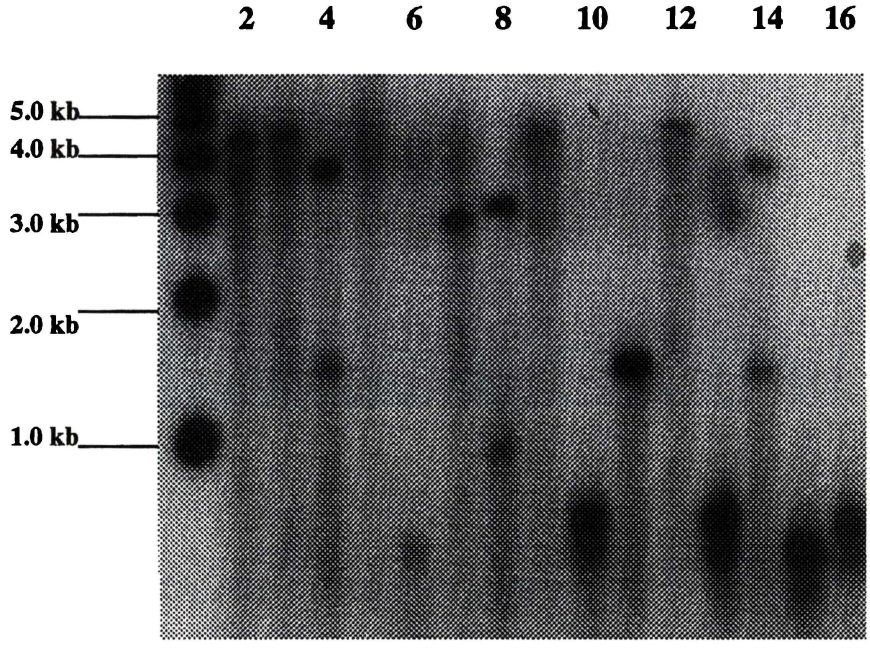


Figure 5.4 Autoradiograph after Southern hybridisation (described in Section 5.3.1.3. and Figure 5.3) using the 350 bp *pfp* fragment combined with the 1 kb ladder as probe.

5.3.1.4 Examination of restriction digestion conditions and incubation times for Southern hybridisation

To optimise the conditions of restriction digestion conditions for Southern blotting, the incubation times for restriction enzymes and some combinations of these were varied from 2 to 22 h at 37°C (Fig. 5.5). After electrophoresis, the results indicated that the digests were nearly completed after 4 h of incubation.

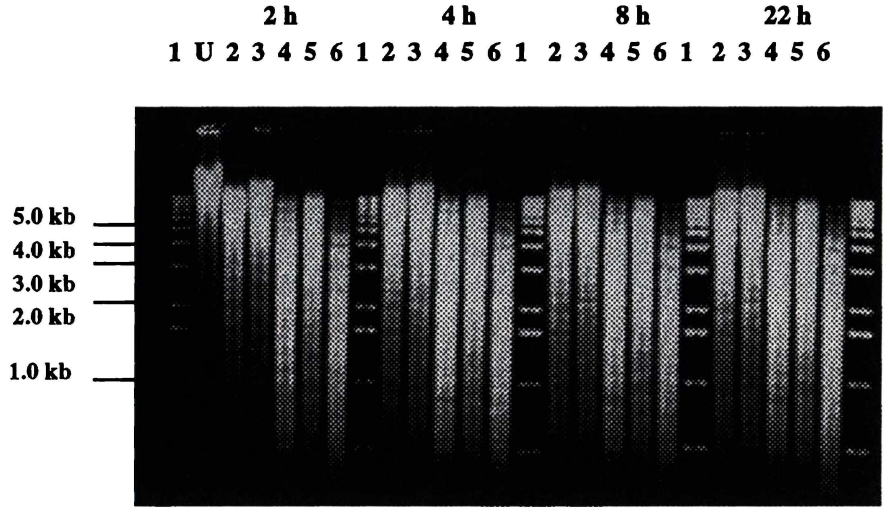


Figure 5.5 Digestions of the *Dictyoglomus* DNA with 3 restriction enzymes and with some different combinations of these. Lane 1: 1 kb ladder. Lane U: control with uncut *Dictyoglomus* DNA; lanes 2 to 6 were digested with *Eco*R I, *Eco*R V, *Hin*d III, *Eco*R I/*Eco*R V, *Eco*R I/*Hin*d III, respectively.

5.3.1.5 Analysis of Southern hybridisation

The corresponding gel for Southern hybridisation is shown in Fig. 5.5. The Southern hybridisation data was obtained from the restriction digests (Fig. 5.6) which was carried out using a high stringency protocol for hybridisation as described in Section 2.12.9. The results indicated that there were strong hybrids after 4 h of digestion and yet the bands were still getting darker and stronger as the incubation time of the restriction digests increased from 4 to 22 h, which suggested that optimal length of time of restriction digest for this experiment was about 22 h (Fig. 5.6).

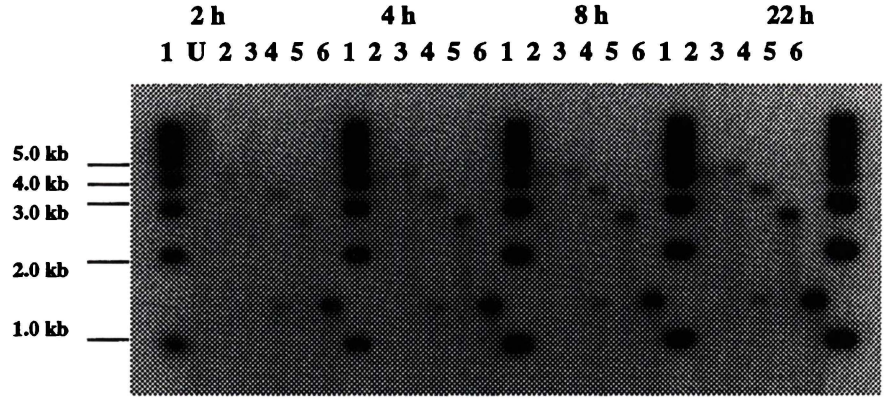


Figure 5.6 Examination of restriction digestions using different times.

5.3.1.6 Preparative digestion of genomic DNA with *Eco*R I

Based on the results from Southern hybridisation, *Dictyoglomus* genomic DNA (40 μg) was digested with *Eco*R I (120 U) at 37°C overnight. An area around 4.5-5.0 kb was excised from the gel and the DNA fragments were isolated, purified and quantified in preparation for ligation.

5.3.1.7 Screenings of clones with inserts

After the ligation of 4.5-5.0 kb fragments (digested with *Eco*R I) into vector pGEM 7Zf(+) and electroporation, colony lifting hybridisation was carried out as described in Section 2.12. White colonies were selected from LBA plates containing IPTG and X-gal, incubated at 37°C overnight, then transferred to Hybond-N^+ for hybridization. One positive hybridisation signal was identified from more than 600 white colonies (Fig. 5.7). The conditions for hybridisation are described in Section 2.12.9. The protocol for identification of bacterial colonies which contained recombinant plasmids is described in Section 2.12.10.

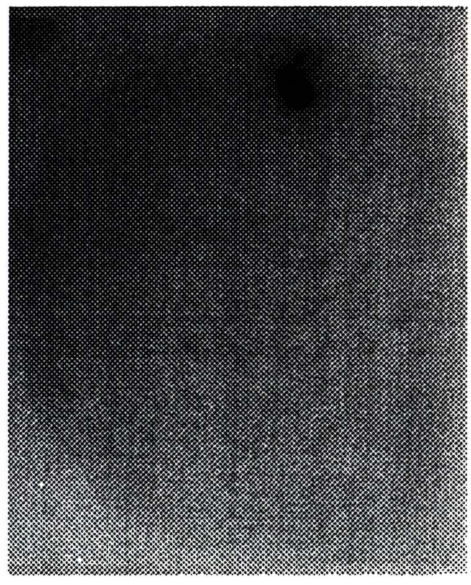


Figure 5.7 Colony lifting experiment for the *Dictyoglomus pfp* gene cloning

5.3.2 Sequencing and cloning with inverse-PCR

As a potential toxin gene might be within the 4.5 kb *Eco*R I fragment (Fig. 5.7), the *E. coli* could not be recovered after several attempts. Therefore, the 350 bp PCR product was also labelled to be used as a probe with inverse-PCR method for the *Dictyoglomus pfp* gene sequencing.

5.3.2.1 Restriction digests and Southern hybridisation for inverse-PCR amplification

The *Dictyoglomus* DNA (2.0 μg per lane) was digested with 15 U of each enzyme at 37°C overnight. This result reflected the low G+C % (29.5%) of the *Dictyoglomus* genomic DNA.

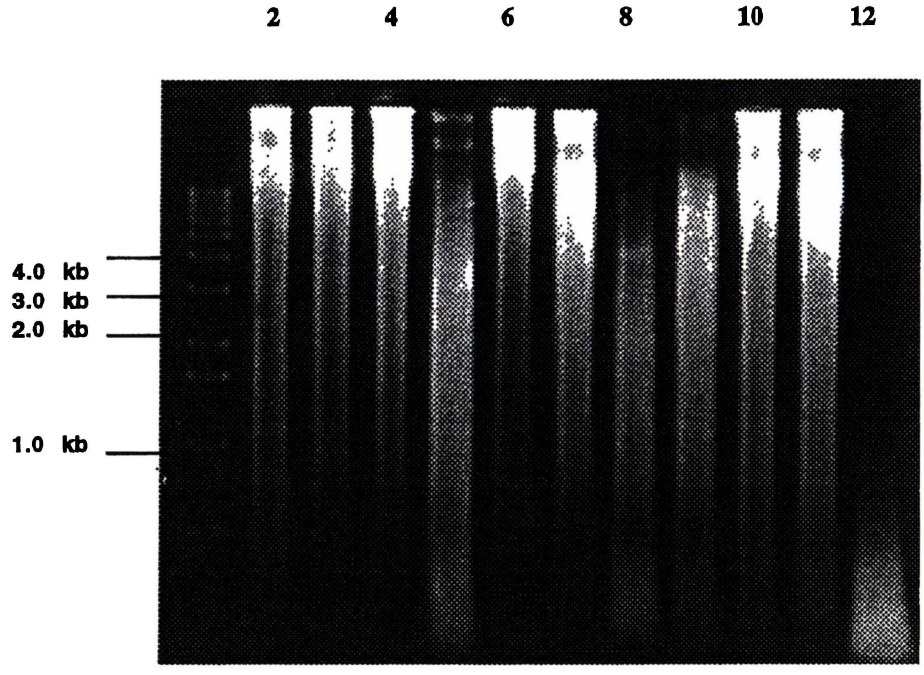


Figure 5.8 Restriction digests for inverse-PCR amplification. Lane 1: 1 kb ladder; lanes 2 to 12 were restriction digests with the following enzymes (number of lane): *Ava* I (2), *Ban* II (3), *Bgl* II (4), *Dde* I (5), *Kpn* I (6), *Msp* I (7), *Rsa* I (8), *Sau*96 I (9), *Sca* I (10), *Sty* I (11) and *Tru*9 I (12), respectively (Fig. 5.8).

The hybrids from lanes 8 and 9 digested with *Rsa* I (1.0 kb) and *Sau*96 I (2.5 kb) were chosen for inverse-PCR based on the analysis of the restriction map from the core region of 350 bp fragment (Figs. 5.2 and 5.9).

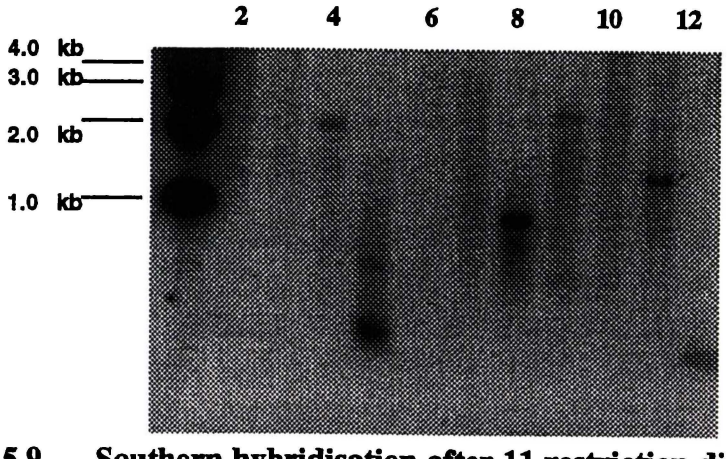


Figure 5.9 Southern hybridisation after 11 restriction digests for inverse-PCR amplification. Lane 1: 1 kb ladder; lanes 2 to 12 used restriction enzymes with the same enzymes used in Figure 5.8.

Finally, those restriction digests with *Sau*3A I were chosen for inverse-PCR amplifications and a single fragment with each enzyme (360, 700 and 2,100 bp) was amplified (Fig. 5.10).

5.3.2.2 Inverse-PCR amplification of the regions encompassing the *Dictyoglomus pfp* gene

Using the sequence obtained in the core region of the 350 bp fragment, specific primers were designed for amplification of the upstream and downstream flanking regions of the gene. The largest inverse-PCR fragment amplified in this work was 2.1 kb, which is at the upper limit for inverse-PCR amplifications in Figure 5.9 (Collins and Weissman, 1984; Howard et al., 1988).

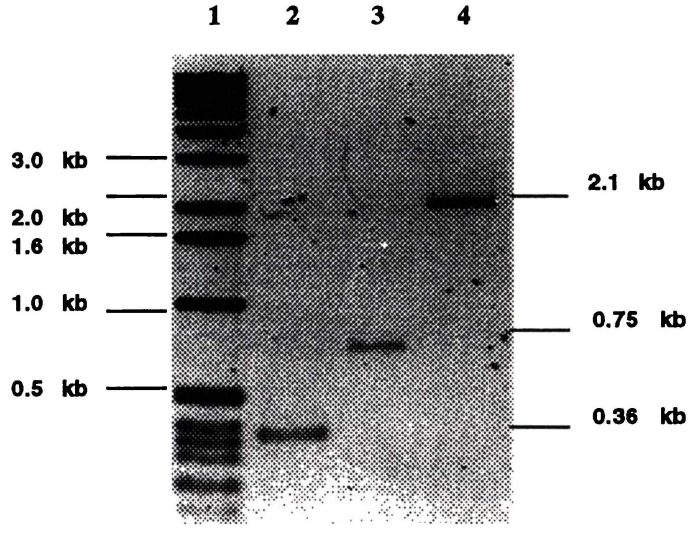


Figure 5.10 Agarose-electrophoresis (reverse image) of inverse-PCR products with *Sau*3A I, *Rsa* I and *Sau*96 I digested *Dictyoglomus* DNA. Lane 1: 1 kb marker; lane 2: a 360 bp PCR fragment from the DNA completed digested with *Sau*3A I; lane 3: a 750 bp PCR fragment from *Rsa* I digest; lane 4: a 2,100 bp PCR fragment from *Sau*96 I digest (Fig. 5.10). The sequences from these inverse-PCR amplifications are presented in Appendix B.4.

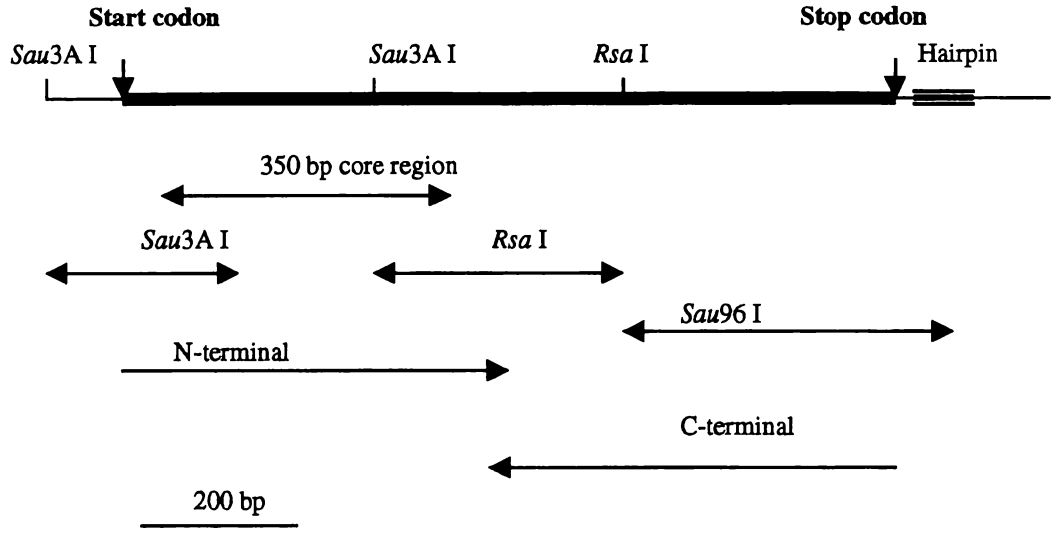


Figure 5. 11 Inverse-PCR amplification map of the *Dictyoglomus pfp* gene

5.3.2.3 Identification of the open reading frame encoding the *pfp* gene

The sequence of the full-length *pfp* gene from *Dictyoglomus* was obtained by combining the overlapping sequences (Fig. 5.11) of PCR fragments from the core region (350 bp), in addition to those resulting from the restriction digests with *Sau*3A I (360 bp), *Rsa* I (750 bp) and *Sau*96 I (2,100 bp). The complete sequence of 1,416 nucleotides contains an ORF of 346 codons (1,041 bp; Fig. 5.12) beginning with an ATG start and ending with a TAA stop codon. One hairpin sequence downstream of the stop codon was found which could act as a transcriptional terminator signal similar to those described in *E. coli* (Hellinga and Evans, 1985). Potential promoter sites at the 5'-end of the coding region were also identified. For example, a Pribnow-like box sequence (TAAAAT) that is located 41 nucleotides upstream from the ATG start codon and is similar to the -10 (TATAAT) promoter sequence. In addition, the TTGTCA sequence located 17 bases upstream from the TAAAAT sequence is similar to the -35 (TTGACA) promoter (Kim et al., 1999). Finally, a potential ribosome binding site (AGGAGG) was also identified and is located four nucleotides upstream of the start codon.

GATCTTACCCTCAAATATTATTAATCTACGACTATAAAGCTAACAATCCTTAAAAAGC**TTGTCAAT**

TTTTACTCTAAATA**AT****AAAAT**CATAATTAACCAAAAACAAACTCTAAGAAGG**AGGAGG**AAAA

```
                                                                      60
ATG AGT AAA ATG CGT ATT GGT GTA TTA ACA GGT GGA GGC GAT TGC CCA GGT CTA AAC CCA
M   S   K   M   R   I   G   V   L   T   G   G   G   D   C   P   G   L   N   P
                                                                     120
GCT ATC CGT GGT ATT GTC ATG AGA GCA TTA GAT TAT GGA GAC GAA GTT ATA GGT TTG AAG
A   I   R   G   I   V   M   R   A   L   D   Y   G   D   E   V   I   G   L   K
                                                                     180
TAT GGA TGG GCT GGT CTT CTT AAG GCA GAT ACT ATG CCT TTA TCC TTA GAA ATG GTA GAA
Y   G   W   A   G   L   L   K   A   D   T   M   P   L   S   L   E   M   V   E
                                                                     240
GAT ATT CTT GAA ATC GGC GGA ACA ATT CTT GGA AGT TCT AGA ACA AAC CCA TTC AAA AAA
D   I   L   E   I   G   G   T   I   L   G   S   S   R   T   N   P   F   K   K
                                                                     300
GAA GAA GAT GTT CAA AAA TGT GTT GAG AAC TTC AAA AAG TTA AAC TTA GAT GCC TTA ATC
E   E   D   V   Q   K   C   V   E   N   F   K   K   L   N   L   D   A   L   I
                                                                     360
GCC ATA GGT GGA GAA GAC ACT CTA GGA GTT GCA TCA AAA TTT AGC AAA CTT GGT CTT CCA
A   I   G   G   E   D   T   L   G   V   A   S   K   F   S   K   L   G   L   P
                                                                     420
ATG ATC GGA GTT CCA AAA ACT ATT GAT AAA GAT TTA GAA GAA ACT GAC TAT ACT CTT GGA
M   I   G   V   P   K   T   I   D   K   D   L   E   E   T   D   Y   T   L   G
                                                                     480
TTT GAC ACT GCT GTT GAA GTA GTG GTA GAT GCA ATT AAA AGA CTT AGA GAT ACT GCA AGA
F   D   T   A   V   E   V   V   V   D   A   I   K   R   L   R   D   T   A   R
                                                                     540
TCT CAT GCA AGA GTT ATC GTA GTA GAA ATA ATG GGA AGA CAT GCA GGA TGG TTA GCT CTT
S   H   A   R   V   I   V   V   E   I   M   G   R   H   A   G   W   L   A   L
                                                                     600
TAT GGT GGG CTT GCA GGA GGA GCA GAT TAT ATC TTA ATC CCT GAA GTA GAA CCT AAT CTT
Y   G   G   L   A   G   G   A   D   Y   I   L   I   P   E   V   E   P   N   L
                                                                     660
GAG GAT CTT TAC AAT CAC ATA AGA AAA CTA TAC GCA AGA GGA AGA AAT CAC GCA GTT GTA
E   D   L   Y   N   H   I   R   K   L   Y   A   R   G   R   N   H   A   V   V
                                                                     720
GCC ATC GCT GAG GGA GTA CAA CTA CCA GGA TTT ACT TAT CAA AAA GGA CAA GAA GGA ATG
A   I   A   E   G   V   Q   L   P   G   F   T   Y   Q   K   G   Q   E   G   M
                                                                     780
GTA GAT GCC TTT GGT CAC ATT CGC TTA GGT GGT GTA GGT AAT GTA CTA GCC GAA GAG ATA
V   D   A   F   G   H   I   R   L   G   G   V   G   N   V   L   A   E   E   I
                                                                     840
CAG AAG AAC TTG GGA ATT GAA ACC AGA GCC GTA ATC TTA AGC CAC CTA CAA AGG GGA GGA
Q   K   N   L   G   I   E   T   R   A   V   I   L   S   H   L   Q   R   G   G
                                                                     900
AGT CCA TCA ATA AGA GAT AGA ATC ATG GGG CTT CTC CTT GGT AAG AAG GCT GTA GAC TTA
S   P   S   I   R   D   R   I   M   G   L   L   L   G   K   K   A   V   D   L
                                                                     960
GTA CAT GAA GGA AAA TCT GGA TTA TTT GTT GCT GTA AAA GGA AAC GAA TTA GTA CCA GTA
V   H   E   G   K   S   G   L   F   V   A   V   K   G   N   E   L   V   P   V
                                                                    1020
GAT ATA ACT TTA ATT GAA GGG AAA ACA AAG AAT GTT GAT CCT GCC TTT TAT GAG AGC GTA
D   I   T   L   I   E   G   K   T   K   N   V   D   P   A   F   Y   E   S   V
                        1041
AAA ACT TTC TTT AAT AAG TAA
K   T   F   F   N   K   *
GAAAACTCTCAAAAAGAGGAAGGCGTTTGCCTTCCTCTTTTTTTTCTTTTTCTATAAGCTATTA
          --------------------------------
```

Figure 5.12 Nucleotide and deduced amino acid sequence of the cloned *pfp* gene from *D. thermophilum* Rt46 B.1. The putative promoter (-35 and –10 regions) and ribosomal binding site are bolded in the 5'-flanking region. The stop codon is marked by an asterisk and the transcriptional hairpin terminator is

indicated by broken lines. The *Dictyoglomus pfp* gene sequence data has been submitted to GenBank under accession number AF268276.

5.3.2.4 Active site residues, amino acid composition and codon usage for the *Dictyoglomus* PP_i-PFK

The amino acid residues in the active sites of eleven different PFKs were analysed and are summarised in Table 5.3. F-6-P binding sites are highly conserved in all of the PFKs and the *Dictyoglomus* PFK has the same amino acids as those from *A. methanolica, T. tenax, S. coelicolor, B. stearothermophilus* and *E. coli* (Evans and Hudson, 1979; Schirmer and Evans, 1990).

Table 5.3 Amino acid residues in the active sites of selected PFKs

Site and Position*	*D.t* PP_i-PFK	*A.c* PP_i-PFK	*T.t* PP_i-PFK	*T.m* PP_i-PFK	*E.h* PP_i-PFK	*S.c* ATP-PFK	*T.b* ATP-PFK	*T.m* ATP-PFK	*B.s* ATP-PFK	*E.c* ATP-PFK
F-6-P binding										
125 (124)	**T**	**T**	**T**	T	T	**T**	T	T	**T**	**T**
127 (126)	**D**	**D**	**D**	D	D	**D**	D	D	**D**	**D**
129 (128)	**D**	**D**	**D**	D	D	**D**	D	D	**D**	**D**
162 (161)	**R**	**R**	**R**	R	N	**R**	Y	R	**R**	**R**
169 (168)	**M**	**M**	**M**	M	M	**M**	M	M	**M**	**M**
170 (169)	**G**	**G**	**G**	G	G	**G**	G	G	**G**	**G**
171 (170)	**R**	**R**	**R**	R	R	**R**	R	R	**R**	**R**
222 (220)	**E**	**E**	**E**	E	E	**E**	E	E	**E**	**E**
ATP or PP_i binding										
11 (10)[a]	**G**	**G**	**G**	G	G	**G**	G	G	**G**	**G**
41 (39)[b]	**W**	**W**	**W**	F	Y	**W**	Y	Y	Y	Y
77 (75)[c]	**K**	**K**	**K**	K	S	**K**	D	K	R	R
103 (101)[d]	**E**	**E**	D	D	D	**E**	D	E	G	G
104 (102)[a]	**D**	**D**	**D**	D	G	**D**	G	G	G	G
105 (103)[a]	**T**	**T**	**T**	T	T	**T**	T	S	S	S
107 (105)[c]	**G**	**G**	**G**	F	R	**G**	R	T	M	M
108 (106)[c]	**V**	**V**	A	S	G	**V**	G	G	G	G

*The residues involved in F-6-P and ATP or PP_i binding were assigned based on the crystallographic structures of the *E. coli* and *B. stearothermophilus* PFKs (Evans and Hudson, 1979; Schirmer and Evans, 1990). Residues amongst the PP_i-PFKs from *D. thermophilum, T. tenax, A. methanolica, T. maritima, E.*

histolytica and ATP-PFK from *S. coelicolor*, *S. coelicor*, *T. brucei*, *T. maritima*, E. *coli* and *B. stearothermophilus* which are the same are in boldface type.

[a] involved in binding to β-phosphate

[b] involved in binding ribose

[c] binds to adenosine

[d] binds to Mg^{2+}

Table 5.4 Amino acid composition of the *D. thermophilum* PP_i-PFK

Amino acid	No./molecule	% of total (mol%)
Non-polar		
Ala (A)	25	7.2
Val (V)	31	9.0
Leu (L)	39	11.3
Ile (I)	26	7.5
Pro (P)	12	3.5
Met (M)	9	2.6
Phe (F)	10	2.9
Trp (W)	2	0.6
Polar		
Gly (G)	43	12.4
Ser (S)	12	3.5
Thr (T)	15	4.3
Cys (C)	2	0.6
Tyr (Y)	9	2.6
Asn (N)	12	3.5
Gln (Q)	6	1.7
Acidic		
Asp (D)	21	6.1
Glu (E)	24	7.0
Basic		
Lys (K)	24	7.0
Arg (R)	17	5.0
His (H)	7	2.0

The codon usage for the *Dictyoglomus* PFK (Table 5.5), as expected, reflected the G+C mole % content of the genomic DNA (29.5%). For example, among the 43 glycine codons, only 2 had a terminal C and 3 with a G. In addition, all codons for phenylalanine, proline and threonine were terminated with either an A or a U.

Table 5. 5 Codon usage for the *D. thermophilum* PP_i-PFK

1st	2nd				base				3rd
base	U	No.	C	No.	A	No.	G	No.	base
U	Phe	5	Ser	3	Tyr	7	Cys	1	U
	Phe	0	Ser	1	Tyr	2	Cys	1	C
	Leu	11	Ser	2	*	0	*	0	A
	Leu	0	Ser	0	*	0	Trp	2	G
C	Leu	14	Pro	4	His	3	Arg	2	U
	Leu	1	Pro	0	His	4	Arg	1	C
	Leu	6	Pro	8	Gln	5	Arg	0	A
	Leu	0	Pro	0	Gln	1	Arg	0	G
A	Ile	9	Thr	7	Asn	6	Ser	3	U
	Ile	10	Thr	0	Asn	6	Ser	3	C
	Ile	7	Thr	16	Lys	16	Arg	13	A
	Met	9	Thr	0	Lys	8	Arg	1	G
G	Val	10	Ala	7	Asp	16	Gly	14	U
	Val	1	Ala	7	Asp	5	Gly	2	C
	Val	19	Ala	11	Glu	19	Gly	24	A
	Val	1	Ala	0	Glu	5	Gly	3	G

5.3.3 Amino acid alignment for some PFKs

Eleven representative amino acid sequences of PFKs from eukaryotes, bacteria and the crenarchaeon *T. tenax* were retrieved from sequence data bases and aligned (Altschul et al., 1997) with the PP_i-PFK sequence from *D. thermophilum* (Fig. 5.13). The F-6-P binding sites are conserved in all PFKs examined. The *Dictyoglomus* PFK has almost the same amino acid residues for F-6-P binding as those from *A. methanolica, T. tenax, S. coelicolor, B. stearothermophilus* and *E. coli* (Alves et al., 1996, 1997; Siebers et al., 1998).

```
                   A                         A
                  10        20        30        40        50
D.t (pfp^a)  MSKMRIGVLTGGGDCPGLNPAIRGIVMR-A-LDYGDEVIGLKYGWAGLLKADT---MPLS
A. m(pfp)    --QMRVGVLTGGGDCPGLNAVIRAVVRKGI-EAHGWEIVGFRSGWRGPLTGDS---RPLG
T. t(pfp)    ---MKIGVLTGGGDAPGLNIAVYTFVKL---AERKHEVYAIYHGWRGLLNKEV---KRVS
T. m(pfp)    -MAERLGILVGGGPAPGINSVISSVTIEA--INNGLEVIGIYDGFKHLVEGKTNMVKKLS
E. h(pfp)    --TTKVAIVTCGGLCPGLNNVIRGLVLNLYNRYHVNNIFGLRWGYEGLVPELS-EVQRLT
S. c(pfk^b)  ---MKVGVLTGGGDCPGLNAVIRAVVRKGV-QEYGYDFTGFRDGWRGPLEGDT---VPLD
T. b(pfk)    --ETTIGIVTCGGICPGLNDVIRSITLTGINVYNVKRVIGFRFGYWGLSKKGSQTAIELH
T. p(pfp)    ---VHAGIVTCGGLCPGLNDVIRAIVRCLWGRYGVKRISGIRFGYKGLLPDYNFDILPLT
T. m(pfk)    -MKKIAVLTSGGDAPGMNAAVRAVVRY-G-VRQGLEVIGVRRGYSGLIDGDF---VKLE
E. c(pfk)    -MIKKIGVLTSGGDAPGMNAAIRGVVRS-A-LTEGLEVMGIYDGYLGLYEDRM---VQLD
B. s(pfk)    --MKRIGVLTSGGDSPGMNAAIRSVVRK-A-IYHGVEVYGVYHGYAGLIAGNI---KKLE
                       ..  ** .**.*  .                    *             .

                               AA   A                      AAA AA
                    60        70        80        90       100       110
D.t(pfp)     LEMVEDILEIGGTILGSSRTNPFKKEEDVQKCVENFKKLNLDALIAIGGEDTLGVASKFS
A.m(pfp)     LDDVEEILIRGGTILGSSRTNPYKEEGGVEKIRAVLADQGVDALIAIGGEDTLGVAKKLT
T.t(pfp)     SRDLLDFAFSGGTYIRTSRTNPFKDEERARLLESNVKELGLDVVVAIGGDDTLGAAGEAQ
T.m(pfp)     IEDVSRIHIEGGSILRTSRVNPAKSEETLEKTVQTLKKLGIKYLVTIGGDDTAFSASKVC
E.h(pfp)     PEIVSDIHQKGGSILGTSRGAQS-----PEVMAQFLIDNNFNILFTLGGDGTLRGANAIN
S.c(pfk)     IPAVRGILPRGGTVLGSSRTNPLKQRDGIRRIKDNLAALGVEALITIGGEDTLGVATRLA
T.b(pfk)     RGRVTNIHHYGGTILGSSRGPQD-----PKEMVDTLERLGVNILFTVGGDGTQRGALVIS
T.p(pfp)     PEVIDNCHKTGGSLLGTSRGGGNR----VVDIVDGIERLNLHILFIIGGDGSQKGAKEIA
T.m(pfk)     YKDVAGITEKGGTILRTSRCEEFKTEEGRELAAKQIKKHGIEGLVVIGGEGSLTGAHLLY
E.c(pfk)     RYSVSDMINRGGTFLGSARCPEFRDENIRAVAIENLKKRGIDALVVIGDGGSYMGAMRLT
B.s(pfk)     VGDVGDIIHRGGTILYTARCPEFKTEEGQKKGIEQLKKHGIEGLVVIGGDGSYQGAKKLT
               .         **. . ..*                      .   .  .*   .   *

                          F F F                     F
                        120       130        140       150       160
D.t(pfp)     KL-G-----LPMIGVPKTIDKDLE--ETDYTLGFDTAVEVVVDAIKRLRDTARSHAR-VI
A.m(pfp)     DD-G-----IGVVGVPKTIDNDLA--ATDYTFGFDTAVHIATEAIDRLRTTAESHYR-AM
T.t(pfp)     RRGI-----LDAVGIPKTIDNDVY--GTDYTIGFDSAVNAAIEATESFKTTLISHER-IG
T.m(pfp)     ERSKG---EIKVVHVPKTIDNDLPLPENMPTFGFETARHVATELVYNLMQDSRTTNR-WY
E.h(pfp)     KELRRRKVPITVVGIPKTIDNDC---YTDSTFGFQTAVGLSQEAINAVHSEAKSAKNGIG
S.c(pfk)     DEYG-----VPCVGVPKTIDNDLS--ATDYTFGFDTAVGIATEAIDRLHTTAESHMR-VL
T.b(pfk)     QEAKRRGVDISVFGVPKTIDNDLS--FSHRTFGFQTAVEKAVQAIRAAYAEAVSANYGVG
T.p(pfp)     DEIKHRNLKISIIGIPKTVDNDIS--FVQKSFGFDTAIVKATEAVAAAHMEARSQINGIG
T.m(pfk)     EEHK-----IPVVGIPATIDNDIG--LTDMCIGVDTCLNTVMDAVQKLKDTASSHER-AF
E.c(pfk)     EMG------FPCIGLPGTIDNDIK--GTDYTIGFFTALSTVVEAIDRLRDTSSSHQP-IS
B.s(pfk)     EHG------FPCVGVPGTIDNDIP--GTDFTIGFDTALNTVIDAIDKIRDTATSHER-TY
                              .* *.* *         . *   ..                .

                 FFF           E
                 170       180       190        200        210
D.t(pfp)     VVEIMGRHAGWLALYGGLAGG-ADYILIPEV---EPNLED----LYNHIRKLYARGRNHA
A.m(pfp)     VVEVMGRHAGWIALHAGLAGG-ANVILVPER---PFSVEQ-----VVEWVERRFEKMYAP
T.t(pfp)     VVEVMGREAGWIALFTGLSTM-ADAVLIPER---PASWDS----VAKRVKEAYNE-RRWA
T.m(pfp)     FVAMMGREAGHLALGVGKAAS-ATITIIPEEFKEGVTLEEVCDVLDGAILKRKLMGRDDG
E.h(pfp)     IVRLMGRDAGFIALYASLANGDANLVLIPEI---DIPITQ------ICEFVGKRIMSKGV
S.c(pfk)     VVEVMGRHAGWIALHSGLAGG-ANVILIPEQ---RFDVEQ-----VCSWVTSRFRASYAP
T.b(pfk)     VVKLMGRDSGFIAAQAAVASAQANICLVPEN---PISEQE-----VMSLLERRFCHSRSC
T.p(pfp)     LVKLMGRESGFIATYTAIASHETNFVLIPEV---SFDLDGPN--GLLAHLEKRIALRKHA
T.m(pfk)     IVEVMGRHSGYIALMAGLVTG-AEAIIVPEI---PVDYSQ----LADRILEERRRGKINS
E.c(pfk)     VVEVMGRYCGDLTLAAAIAGG-CEFVVVPEV---EFSRED----LVNEIKAGIAKGKKHA
B.s(pfk)     VIEVMGRHAGDIALWSGLAGG-AETILIPEA---DYDMND----VIARLKRGHERGKKHS
             . .*** .* ..              .   ..**
```

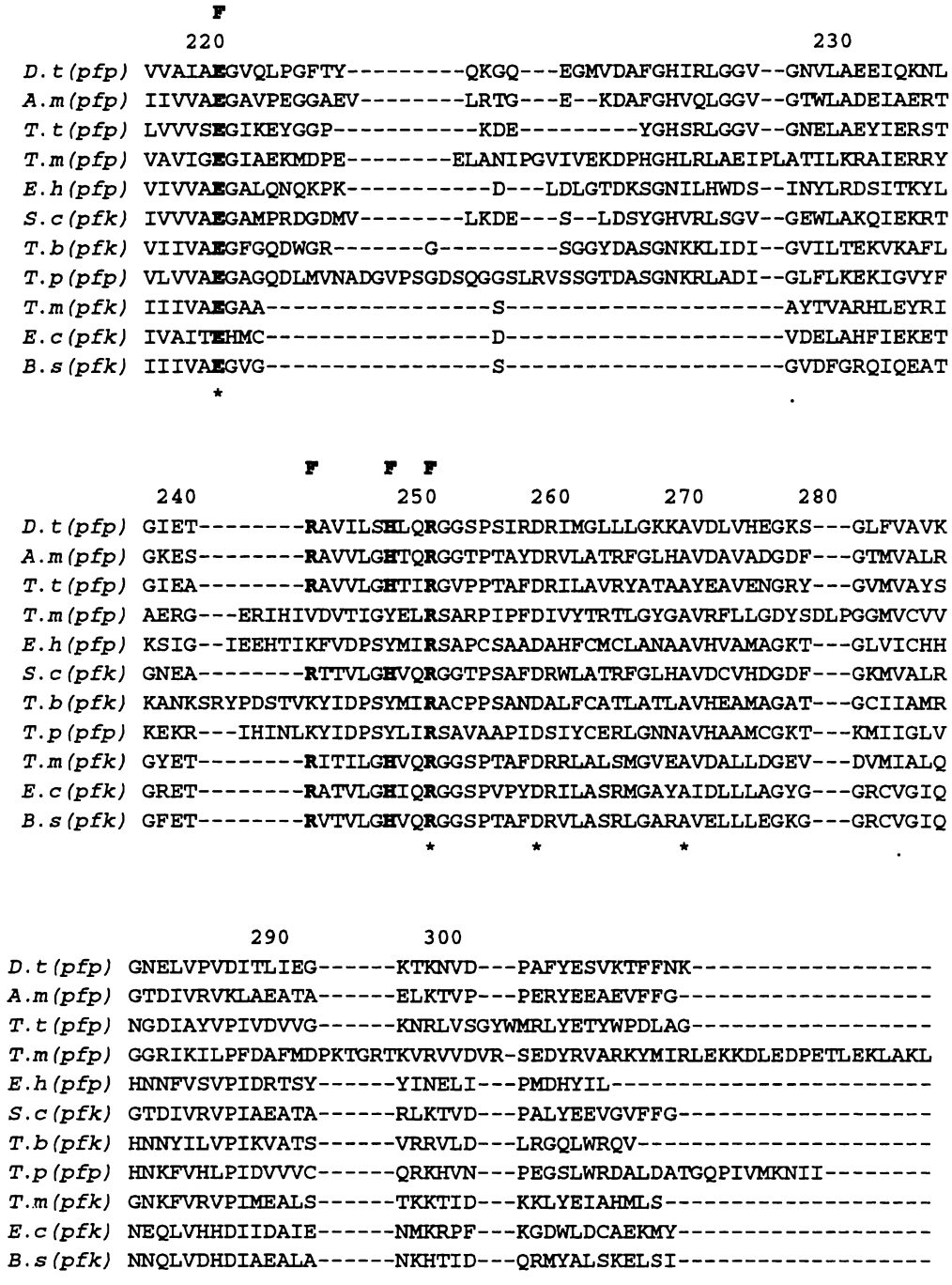


Figure 5.13 Multiple alignment of PFKs with the *Dictyoglomus* PP_i-PFK amino acid sequence. The alignment was generated using the *Dictyoglomus* PP_i-PFK and 10 amino acid sequences of PP_i-PFKs and ATP-PFKs from *A. methanolica*, *T. tenax*, *T. maritima*, *E. histolytica*, *S. coelicolor*, *T. brucei*, *T. pallidum*, *E. coli* and *B. stearothermophilus*. A, F and E refer to residues involved in binding either ATP, F-6-P or PEP, respectively, based on the *E. coli* ATP-PFK crystallographic structure (Hellinga and Evans, 1985). [a]: PP_i-PFK gene; [b]: ATP-PFK gene; asterisks: identical residues; dashes: gaps; dots: highly conserved residues.

5.3.4 Tree construction and phylogenetic comparison

Twenty-one representative amino acid sequences of PFKs from eukaryotes, bacteria and the crenarchaeon *T. tenax* were retrieved from sequence data bases and aligned with the PP_i-PFK sequence from *D. thermophilum* (Appendix F). A phylogenetic tree was generated from the above alignment comparison (Fig. 5.14). One of the main findings is that the *D. thermophilum* sequence is indicated to be most homologous to group III PFKs, e.g. the PP_i-PFKs from *T. tenax* and *A. methanolica* and the ATP-PFK from *S. coelicolor.*

5.3.5 Purification and characterisation of the recombinant *D. thermophilum* PP_i-PFK

The purification steps utilised in the purification of the *Dictyoglomus* enzyme included heat-treatment, phenyl-Sepharose, Q-Sepharose and reactive red 120 dye-ligand chromatography. The final yield of the highly purified recombinant enzyme was 53 mg protein from 800 ml of induced culture (Table 5.6), which indicated that a high-level expression of the enzyme was achieved.

Table 5.6 Purification of the recombinant PP_i-PFK from *D. thermophilum* Rt46 B.1

Step	Total activity (U)	Total protein (mg)	Sp act (U/mg)	Purification (fold)	Yield (%)
Heated cell-free extract	757	6,600	0.12	1	100
Phenyl-Sepharose	516	329	1.6	13	68
Q-Sepharose	363	79	4.6	38	48
Red dye-120	328	53	6.2	52	43

The purity after Q-Sepharose after SDS-PAGE was estimated by densitometry to be approximately 97%. To ensure purification to homogeneity, the enzyme was purified further on a red dye-ligand 120 column, using 5.0 mM of PP_i to elute the enzyme (Section 4.2.4.6 lane 5, Fig. 5.15).

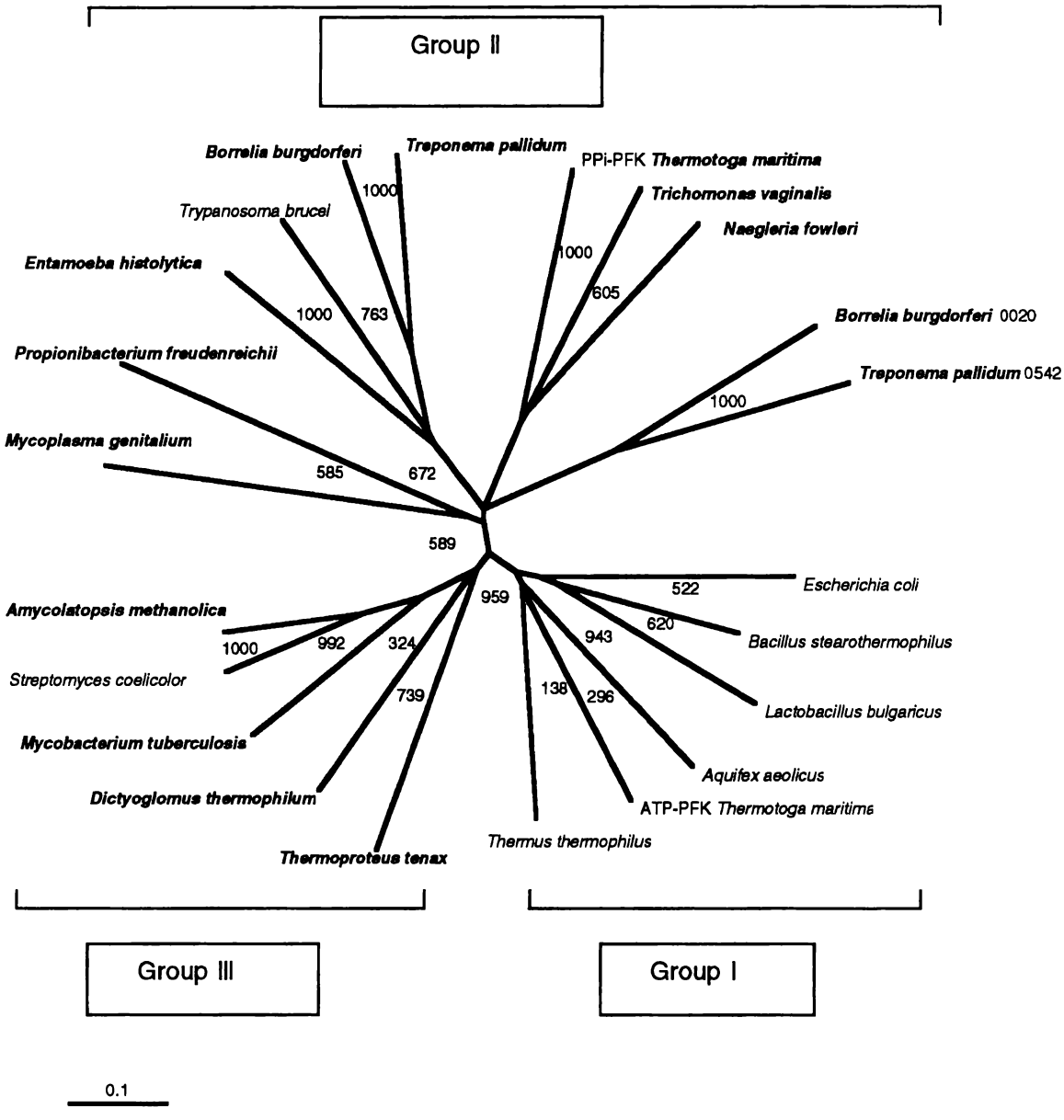


Figure 5.14 Phylogenetic relationships of PP_i- and ATP-PFKs from some organisms. The tree is based on distance analysis (neighbor-joining method) of full amino acid sequences of PP_i- and ATP-PFKs in the EMBL and Swiss Prot data banks and GenBank. Bootstrap values are based on 1,000 replicates and are given at each node (Fig. 5.14). All of the PP_i-PFKs are bolded. Group I includes: *T. thermophilus*, *T. maritima* (ATP-PFK), *A. aeolicus*, *L. bulgaricus*, *B. stearothermophilus* and *E. coli*. Group II includes: *T. pallidum* 0542, *B. burgdorferi* 0020, *N. fowleri*, *T. vaginalis*, *T. maritima* (PP_i-PFK), *T. pallidum*, *B. burgdorferi*, *T. brucei*, *E. histolytica*, *P. freudenreichii* and *M. genitalium*. Group III includes: *T. tenax*, *D. thermophilum*, *M. tuberculosis*, *A. methanolica* and *S. coelicolor*.

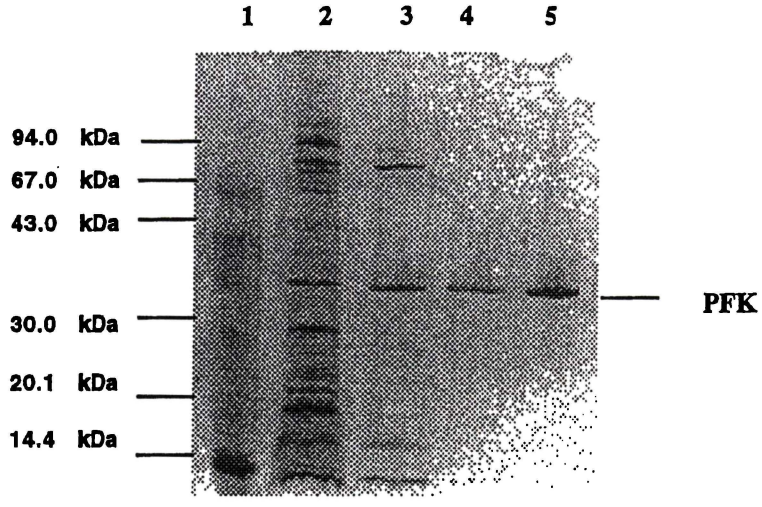


Figure 5.15 - SDS-PAGE for the recombinant *Dictyoglomus* PP_i-PFK. Lane 1: standard molecular marker of SDS-PAGE; lane 2: cell-free extract following heat treatment; lanes 3 through 5: fractions obtained after purification through phenyl-Sepharose, Q-Sepharose, reactive red 120 dye-ligand, respectively. Lanes 2, 3, 4, and 5 contained 3.0, 1.0, 0.75 and 0.75 μg of protein, respectively.

The recombinant and native enzymes had the same estimated molecular mass (37 kDa, Fig. 5.15) when both enzymes were run on the same SDS-PAGE gel (not shown) and the protein possessed a high specific activity with PP_i as the phosphoryl donor. The enzyme is likely to be a homodimer in comparison with the size estimate obtained by SDS-PAGE as a molecular mass of 74,181 Da was found for the expressed protein using a large secondary peak in the mass spectrometry data (Appendix B.5) and 76.7 kDa by gel filtration.

5.3.6 Comparative study between *Dictyoglomus* native and recombinant enzymes

Most of the biochemical and the kinetic properties of the recombinant enzyme were very similar to those of the native enzyme including *p*I values (Table 5.7), optimum Mg^{2+} concentration for activity, thermostability, extreme sensitivity to

of response to traditional allosteric modulators, e.g. ADP, GDP, cAMP, AMP, PEP, citrate, succinate or F-2,6-P_2.

Table 5.7 Comparative data between the native and the expressed *D. thermophilum* PP_i-PFK

Properties	Native enzyme	recombinant enzyme
K_m (μM) F-6-P	127	136
K_m (μM) PP_i	82	116
Specific activity (U/mg)	9.6	6.2
Optimum Mg^{2+} (mM)	1-3	1-3
Optimum pH	5.8-6.2	6.0-6.8
*p*I	4.4	4.3
SDS-PAGE (mol. wt., Da)	37,000	37,000
Mass spectrometry (mol. wt., Da)	37,349	38,057
Gel filtration mol wt (Da)	65	76
Thermostability (min) @ 90 °C	10	10
Thermostability (min) @ 80 °C	170	165
% activity with Cu^{2+} (1 μM)	38	40

mol. wt: molecular weight

5.3.7 Crystallisation of the recombinant *Dictyoglomus* PP_i-PFK

Crystallisation conditions were found using the Hampton Crystal Screen Kits. The best crystals grew from 18% PEG-8000, 100 mM KCl, and 100 mM Bis-Tris, pH 6.5 (Fig. 5.17; Dr. Stanley Moore, personal communication). The crystals are flattened prisms and diffract to beyond 3 Angstrons resolution using a rotating anode generator. The space group is C2221 (number 20, International Tables) and there are three PFK monomers per asymmetric unit. The unit cell dimensions are a=82.2 b=142.4 c=192.8 Angstroms giving Vm=2.7 Angstroms per Dalton.

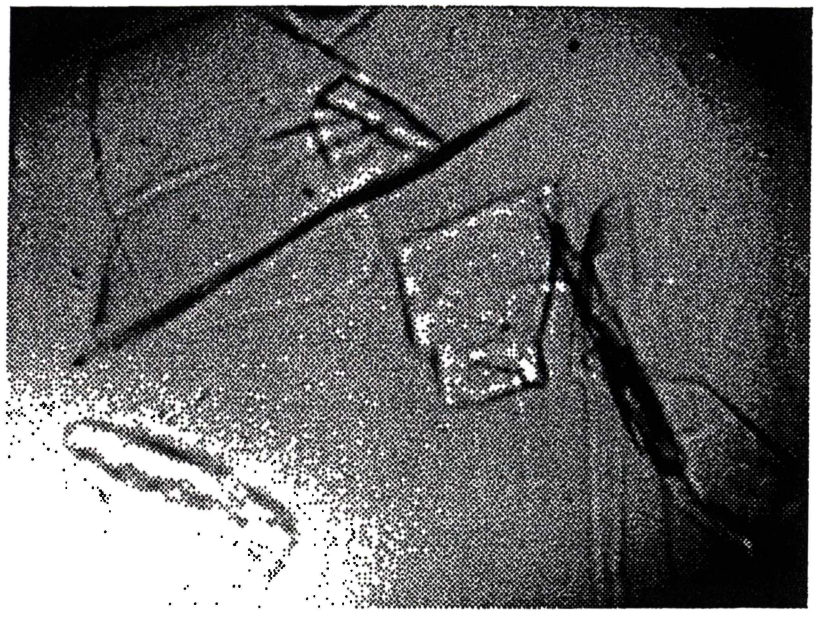


Figure 5.16 The crystallisation of the recombinant *D. thermophilum* PP_i-PFK

5.4 DISCUSSION

The sequencing and cloning of the first extremely thermophilic PP_i-PFK encoding gene from *D. thermophilum* and overexpression of the enzyme have been described. The initial studies using colony lifting techniques suggested that a potentially toxic gene might be contained within the 4.5 kb *Eco*R I fragment (Fig. 5.7) as the clone in *E. coli* JM109 could not, unfortunately, be recovered. However, in parallel with this strategy, the inverse-PCR technique was being used for cloning of the *pfp* gene and ultimately proved to be successful.

The data from the alignment of 11 sequences and the phylogenetic tree (Figs. 5.13 and 5.14) supports the contention that the central carbohydrate metabolism of glycolysis was established before the segregation of the three domains of life. The fact that all of the sequences presented in the phylogenetic tree can be easily

aligned, despite their varying phosphoryl donor specificities, demonstrates their homology, and therefore that they must have evolved from a single common ancestral sequence. However, the PFK-2 of *E. coli* (Daldal, 1984) and the ADP-PFK found in *T. zilligii* and *P. furiosus* show no apparent sequence homology with group I, II or III of ATP- and PP_i-PFKs and, therefore, represent independently evolved PFK lines of descent (Tuininga et al., 1999; Ronimus et al., 2000). The group III enzymes are suggested to be more ancient than the group II PFKs (Siebers et al., 1998), so it then follows that the PP_i-PFKs from the archaeon *T. tenax* and extremely thermophilic bacteria may represent more ancient lineages of this enzyme than the other enzymes in mesophilic bacteria, primitive eukaryotes and higher plants (Siebers et al., 1998). In support of this latter contention, in two cases that of *S. coelicolor* and *T. brucei*, there is strong evidence for the evolution of ATP-PFKs from PP_i-PFKs (Alves et al., 1997; Michels et al., 1997).

A number of reports using X-ray crystallography and site-directed mutagenesis have demonstrated the relative importance to substrate binding, homotropic cooperatively and catalytic role of the PFK amino acids residues listed in Table 5.3 (Poorman et al., 1984; Lau et al., 1987; Shirakihara and Evans, 1988; Rypniewski and Evans, 1989; Berger and Evans, 1992; Auzat et al., 1994a; Zheng and Kemp, 1994a; Byington et al., 1997). In addition, many other residues related to allosteric properties have also been studied (Valdez et al., 1989; Berger and Evans, 1990, 1992; Serre et al., 1990; Auzat et al., 1995). For example, when the Glu^{-187} of *E. coli* PFK is replaced by Asp or Leu, the allosteric transition is abolished. The Glu^{-187} of *E. coli* PFK is necessary for the protein to undergo the change from the active into the inactive state induced by PEP (Auzat et al., 1994). Valdez et al. (1988) reported that Arg^{-25} and Arg^{-211} are involved in the direct binding of PEP and GDP. Sequence analysis of the *Dictyoglomus* PP_i-PFK also shows an Arg^{-211} but the biochemical studies have indicated that both the native

and recombinant *Dictyoglomus* PFK are nonallosteric (Ding et al., 1999, 2000a). It is unknown if this residue is specifically related to GDP binding in other PFKs as a Glu^{-211} has been identified in *B. stearothermophilus* PFK (Ding et al., 2000a; Fig. 5.13). Another PP_i-PFK from the hyperthermophilic bacterium *T. maritima* has a The^{-187}, an Ile^{-25} and Met^{-211} and characterisation of the recombinant enzyme demonstrate that this enzyme is also nonallosteric (Ding et al., 2000b).

The PFK of *D. thermophilum* Rt46 B.1 is the first biochemically characterised extremely thermophilic bacterial PP_i-dependent enzyme to become available for phylogenetic studies. As seen with the enzyme from *T. tenax*, the *Dictyoglomus* enzyme offers additional opportunity to gain improved insight into the differentiation of PFK substrate specificities and the characteristics of the phenotype of the original ancestral PFK precursor. As seen in Figure 5.13, the N-terminal halves of PFKs are somewhat more conserved than the C-terminal halves and this is reflected in the N-terminal half possessing most of the catalytically important sites. A higher proportion of catalytically important conserved sites within the N-terminal halves, interestingly, is also true for several enzymes of glycolysis; for example, glyceraldehyde 3-phosphate dehydrogenase, 3-phosphoglycerate kinase and enolase (Fothergill-Gilmore and Michels, 1979). This is only a general trend, however, as conserved binding sites are also found in the C-terminal portions, e.g. the F-6-P binding site from 222 to 250 in PFKs (Fig. 5.13). Serre and Garel (1990) found that C-terminal sequences of *E. coli* PFK-1 appear to contribute to the stability of the interactions between subunits. When the gene corresponding to position Tyr^{-279} was changed into TAA (Ochre), the mutated gene was not expressed as an active enzyme as the enzyme lost 41 C-terminal residues and could not apparently fold and/or assemble under the intracellular conditions. However, when the gene was mutated corresponding to position 311 causing the Try^{-311} codon to be replaced by TAG (Amber) it was expressed as an active enzyme. They suggested that the last nine residues were

not important for either substrate binding, homotropic cooperativity, catalytic efficiency or stability, but the affinity for the allosteric effectors was reduced (Serre and Garel, 1990). Serre and Garel (1990) concluded that the residues between position 279 and 310 were crucial for the formation of the folded monomeric or the dimeric intermediates involved in the assembly of active *E. coli* PFK-1.

A comparison of the native and recombinant *Dictyoglomus* PP_i-PFKs enzymes demonstrates that they possess a high degree of similarity (Ding et al., 1999, 2000a). For example, the optimum pH and Mg^{2+} concentration for activity, lack of allosteric response, thermostability, sensitivity to Cu^{2+} and the apparent K_m values for substrates of the forward reaction are not significantly different. The calculated molecular mass for the recombinant enzyme from the gene sequence was 37,445 Da, which is similar to the estimated size of 37 kDa for both the native and recombinant proteins obtained by analysis with SDS-PAGE. The enzyme is indicated to be a homodimer based on a molecular mass of 74.2 kDa obtained from mass spectrometry and comparison of the sizes obtained by SDS-PAGE and gel filtration (Hans et al., 1998). The estimated molecular masses of the native (65 kDa) and the recombinant (76 kDa) *Dictyoglomus* enzymes were slightly different which might be caused by intracellular condition in host (*E. coli* or *D. thermophilum*) or by experimental error. In addition, the specific activity of recombinant enzyme from *D. thermophilum* was slightly lower than that of native enzyme (Table 4.1 and Table 5.7).

The expressed enzyme possessed a similar half-life of 2.5 h at 80°C and 10 min at 90°C compared to the native enzyme. These results support the general result that proteins from extremely thermophilic microorganisms maintain their heat resistance upon expression in mesophilic hosts, indicating that heat stability is primarily due to the intrinsic features of the molecules rather than to extrinsic

factors (Sanagelantoni et al., 1992, 1996). The alignment of amino acid sequences of PFKs shows that no unique amino acid substitution schemes, or exclusive sequence motifs, distinguish the thermophile enzymes, i.e. PP_i-PFK and ATP-PFK from *T. tenax* and *D. thermophilum*, *A. aeolicus* and *B. stearothermophilus* from mesophilic species.

A more stringent analysis of PFK functions and phylogeny will become possible after X-ray crystallography data becomes available. As PFKs are present in most organisms, its conserved and slowly evolving nature make it a valuable genotypic marker enabling the exploration of the origins of glycolysis and of life. It is hoped that the X-ray crystallographic determination of the structure of PP_i-PFK from *D. thermophilum* Rt46 B.1 will help clarify the phylogenetic origins of PFKs. Initial attempts at crystallisation with the purified recombinant *Dictyoglomus* enzyme have been successful with a crystal diffracting to 3 Å having been obtained. Experiments are underway to improve this.

CHAPTER SIX

PURIFICATION AND CHARACTERISATION OF A HYPERTHERMOPHILIC ARCHAEAL ATP-PFK FROM *DESULFUROCOCCUS AMYLOLYTICUS*

6.1 INTRODUCTION

The pathways of central metabolism in most organisms provide the metabolic links between the catabolic (degradative) and anabolic (biosynthetic) routes in living organisms (Danson et al., 1998). In thermophilic archaea that are able to grow on hexose sugars, it was initially thought no PFK was employed in the central pathway (Danson, 1989). Tomlinson et al. (1974) reported that an extremely halophilic M6 strain could use glucose by a modification of the ED pathway in which oxidation of glucose precedes phosphorylation. Interestingly, the glycolytic pathway, or modifications of it, has now also been found throughout a number of archaea (Altekar and Rangaswamy 1990; 1991; Schäfer and Schönheit, 1991, 1993; Yu et al., 1994; Kengen et al., 1994, 1995, 1996; Selig et al., 1997). An ADP-PFK activity was also found in *P. furiosus* and *Thermococcus* (Kengen et al., 1994; Selig et al., 1997; Ronimus et al., 1999a).

In addition, a PP_i-PFK has been found in *T. tenax* (Siebers et al., 1998). This archaeal PP_i-PFK has been purified, cloned and sequenced, and it was suggested that the archaeal enzyme may represent the most ancient lineage in group III PFKs as described by Siebers et al. (1998). Being a hyperthermophilic archaea, *D. amylolyticus*, like other thermophilic archaea, possesses numerous differences from the nonthermophilic archaea. For example, its modes of division tend to be unusual and varied, its ribosomal subunits have an unusual shape; it seems to be insensitive to most antibiotics and both its tRNAs and rRNAs are highly modified (Danson et al., 1998). Strikingly, in these organisms, the conversion of

glyceraldehyde 3-phosphate to 3-phosphoglycerate is catalysed by a ferredoxin oxidoreductase, without the intermediate 1,3-diphosphoglycerate that would otherwise lead to the formation of ATP (Kengen et al., 1994).

The finding of an ATP-PFK in *D. amylolyticus* by Selig et al. (1997) was of particular interest because it established that sugar metabolism could proceed in crenarchaeota via glycolysis utilising the EM pathway with the conventional phosphoryl donor, ATP. The biochemical properties and phylogeny of an archaeal ATP-PFK are of obvious interest in elucidating the origin of this enzyme and providing additional insights into the understanding of the evolution of the glycolytic pathway. Therefore, the purification and characterisation of the first hyperthermophilic archaeal ATP-PFK was undertaken and is described in this chapter.

6.2 MATERIALS AND METHODS

6.2.1 Source of strains and large-scale culture conditions

D. amylolyticus was obtained from the TRUCC which is equivalent to DSM 3822. Large-scale cultures (400 litre) for protein purification were carried out in a 600-litre fermenter at 80°C for 48 h. The *Desulfurococcus* medium (Appendix A) was filter-sterilised (0.2 μm) and inoculated with 40 litre of a log phase culture. The cells were harvested by diafiltration with a 0.1 μm hollow fibre system (Amicon), centrifuged at 11,000 x *g* for 30 min at 4°C and the resulting cell pellet then stored at -70°C prior to initiating purification of the enzyme.

6.2.2 Determination of ATP-PFK reaction rates

All the chemicals and reagents are listed in Section 2.2. The PFK assay protocol mainly used for *Desulfurococcus* ATP-PFK is described in Section 2.3. In

addition, the another method of ADP formation protocol (Fig. 2.1b) was also employed to confirm the enzyme specificity. The final concentrations of this standard reaction mixture, in a total volume of 100 µl, were 5.0 mM F-6-P; 1.0 mM ATP; 5.0 mM $MgCl_2$; 0.2 mM NADH; 2.0 mM DTT; 1.0 mM PEP; 50 mM KCl; 0.04 U pyruvate kinase; 0.1 U lactate dehydrogenase and 5.0 U triosephosphate isomerase at optimal pH at 50°C.

6.2.3 Purification of the *D. amylolyticus* ATP-PFK

A flow chart summarising procedure used for purifying the *Desulfurococcus* ATP-PFK is shown in Fig. 6.1

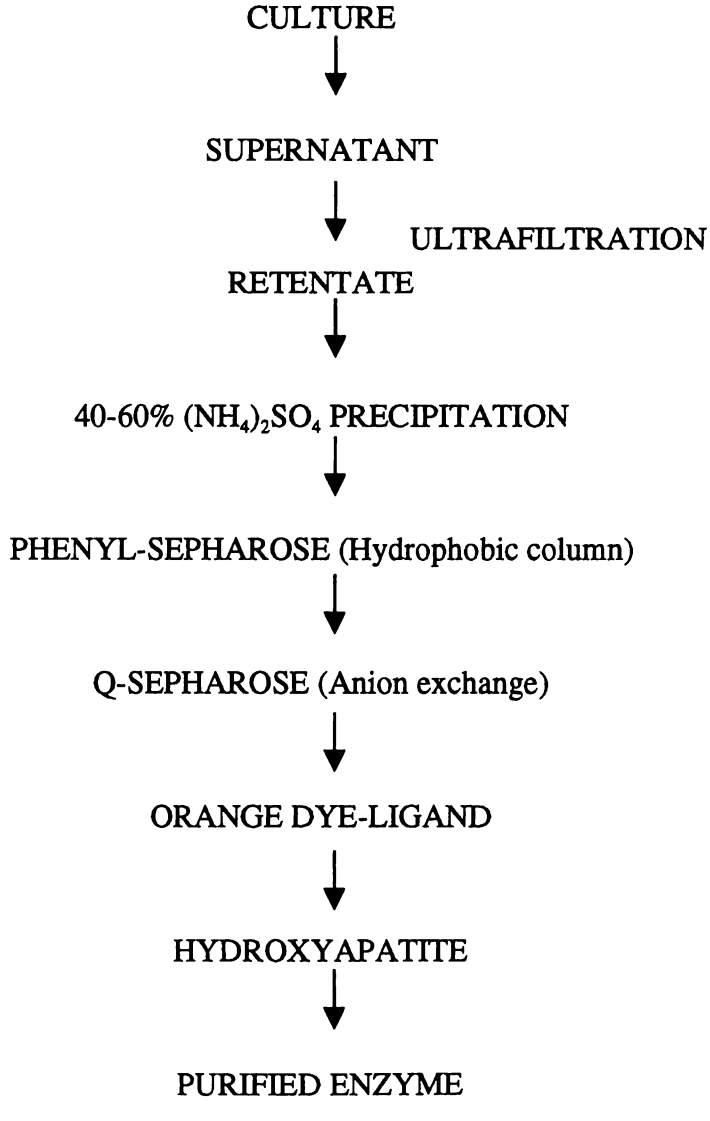


Figure 6.1 Purification procedure of the *Dictyoglomus* PP_i-PFK

6.2.3.1 Preparation of cell-free extract

Cells (100 g wet weight) were resuspended in 1.2 litre of cell suspension solution described in Section 4.2.4.1 and lysed by sonication at full power (Heat Systems-Ultrasonics Inc.) in 6 x 200 ml aliquots for 3 periods of 15 min each in an ice-water bath. Cell debris was removed by centrifugation at 4°C at 11,000 x *g* for 40 min.

6.2.3.2 Ammonium sulfate precipitation

The supernatant was fractionated by $(NH_4)_2SO_4$ precipitation according to standard methods with the 0-20%, 20-40% and 40-60%-saturated fraction precipitates being collected by centrifugation. The 40-60% fraction with most of the activity (7.5 g of protein with 442 U of activity) was resuspended in 50 mM MOPS, 1.0 M $(NH_4)_2SO_4$, 5.0 mM $MgCl_2$, pH 8.0.

6.2.3.3 Phenyl-Sepharose hydrophobic interaction chromatography

The dialysed protein was applied to the phenyl-Sepharose column (Section 4.2.4.2), which had been equilibrated in 1.0 M $(NH_4)_2SO_4$, 50 mM MOPS, pH 8.0, 100 mM KCl, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 5.0 mM $MgCl_2$. The fractions were eluted in a linear gradient (total of 3.0 liter) starting with column-equilibration buffer and ending in 50 mM MOPS, 100 mM KCl, 5.0 mM $MgCl_2$, 0.1 mM PMSF, pH 8.0. Active fractions were pooled and dialysed at 4°C against 50 mM Tris-HCl, 5.0 mM $MgCl_2$, 100 mM KCl, 10% glycerol, 0.1 mM PMSF, pH 8.0.

6.2.3.4 Q-Sepharose anion exchange chromatography

The dialysed enzyme (1,546 mg, 382 U) was applied to a 55 ml Q-Sepharose 26/10 column (Section 4.2.3.3) which was equilibrated with the same buffer used

above (50 mM Tris-HCl, 5.0 mM $MgCl_2$, 100 mM KCl, 10% glycerol, 0.1 mM PMSF, pH 8.0) for dialysis. The activity was eluted with a gradient (400 ml) of 50 mM Tris-HCl, 5.0 mM $MgCl_2$, 100 mM KCl, 10% glycerol, 1.0 M NaCl, pH 8.0 at a flow rate of 4.0 ml/min. Active fractions were pooled, concentrated by ultrafiltration with a YM30 membrane (Amicon) and dialysed at 4°C overnight against the following buffer: 25 mM 2-[N-morpholino] ethanesulfonic acid (MES), 5.0 mM $MgCl_2$, 50 mM KCl, 10% glycerol, 0.1 mM PMSF, pH 6.5.

6.2.3.5 Orange dye-ligand chromatography

The recovered fractions (110 mg, 86 U) from the Q-Sepharose step were loaded onto a mimetic orange dye 3 A6xL column, which was equilibrated with buffer containing 25 mM MES, 5.0 mM $MgCl_2$, 50 mM KCl, 10% glycerol, 0.1 mM PMSF, pH 6.5. Proteins were eluted with the above MES dialysis buffer containing 1.0 M NaCl.

6.2.3.6 Hydroxyapatite (HPHT)

Fractions with activity were pooled and dialysed (1.12 mg, 12 U) against starting buffer for HPHT chromatography (Bio-Rad), which was 10 mM Na_2HPO_4, 1.0 mM $MgCl_2$, 1.0 μM $CaCl_2$, pH 7.2. Protein was eluted with high ionic strength buffer containing 350 mM sodium phosphate buffer (175 mM Na_2HPO_4 and 175 mM NaH_2PO_4), 1.0 mM $MgCl_2$, 1.0 μM $CaCl_2$, pH 7.2 at 0.75 ml/min. Purified enzyme (0.3 mg, 8.0 U) was dialysed against 25 mM Tris-HCl buffer and stored at -70°C in 50% glycerol and 7 mM β-mercaptoethanol, 0.1 mM PMSF, 0.1 mM EDTA and 1.0 mM $MgCl_2$, pH 7.2.

6.2.4 Gel electrophoresis, gel filtration chromatography and protein determinations

The protein subunit molecular mass was determined using 10-15% SDS Phast Gels (Pharmacia Biotech) and the *p*I with IEF gels using a pH 3.0-10.0 isoelectric focusing standard (Pharmacia Biotech) which are described in Sections 2.9.2 and 2.9.3. The SDS-PAGE and IEF separations and subsequent silver staining were performed according to the manufacturer instructions. Estimation of the molecular mass of the native protein was obtained using BIOSEP-SEC3000 (Phenomenex, USA). Determination of protein concentrations is described in Section 2.8.

6.2.5 Determination of the thermostability of the *Desulfurococcus* ATP-PFK

The thermostability of the purified ATP-PFK was investigated by incubating the enzyme (50 μg/ml) in following buffer: 50 mM phosphate buffer, 5.0 mM $MgCl_2$, 50 mM KCl, 0.5 mM DTT, 0.02% Triton X-100, pH 7.8 at either 90°C or 100°C (Section 2.10.3).

6.2.6 pH optimum for activity

The pH optimum was determinated under standard conditions described in Section 2.10.1. The assay reactions of PFK activity contained 5.0 mM F-6-P, 1.0 mM ATP, 5.0 mM $MgCl_2$, 0.2 mM NADH, 50 mM KCl.

6.2.7 Substrate specificity and kinetic parameters

The possible phosphoryl donors were investigated as described in Section 4.3.3. Potential allosteric effects were investigated by adding 0.1 mM of the potential modulator, e.g. ADP, AMP, cAMP, F-2,6-P_2, succinate, citrate and PEP to

standard assays using ATP as the phosphoryl donor. Kinetic parameters were determinated at 50°C by varying the concentration of ATP (0.05-2.0 mM) or F-6-P (0.1-5.0 mM) in the assay mixture in the presence of 5.0 mM F-6-P or 1.0 mM of ATP, respectively.

6.2.8 Determination of the N-terminal amino acid sequence

N-terminal sequencing was carried out using Edman degradation chemistry on an Applied Biosystem Procise 492 protein sequencer, at the Australia Proteome Facility, MacQuarie University, New South Wales, Australia.

6.3 RESULTS

6.3.1 Purification and properties of the *D. amylolyticus* ATP-PFK

The *Desulfurococcus* ATP-PFK was purified to homogeneity (shown in Fig. 6.6) in 4 chromatographic steps after the initial 40-60% ammonium sulfate precipitation. The elution profiles are shown in Figures 6.2 to 6.5.

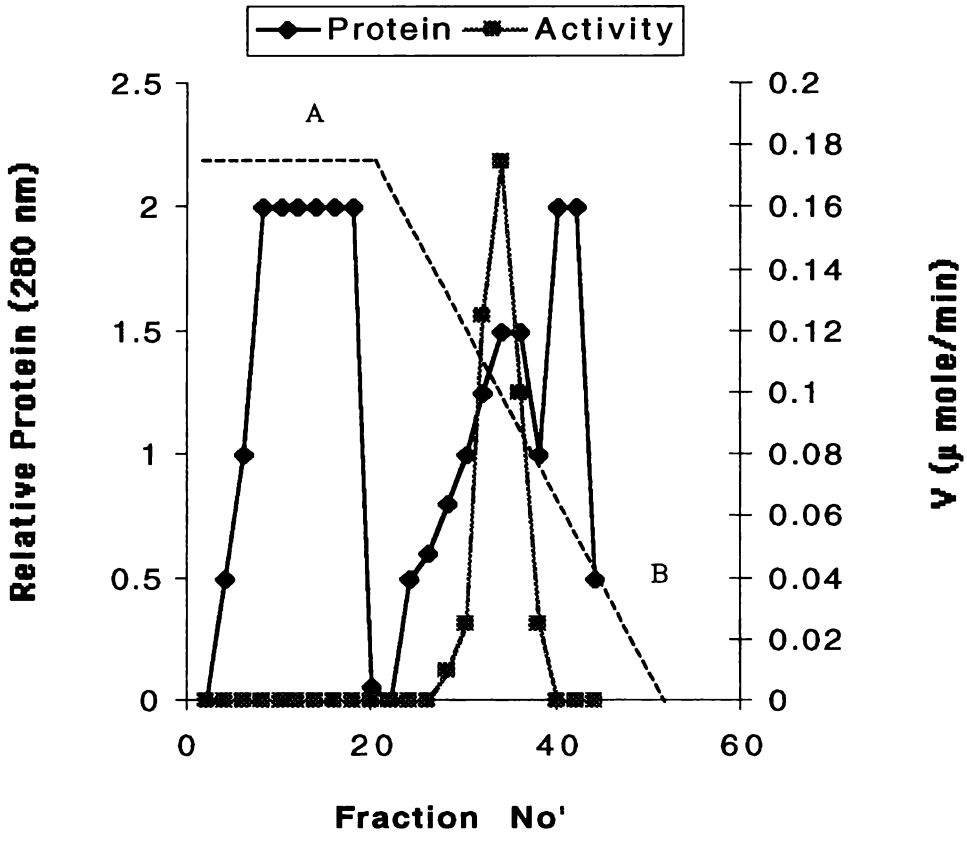


Figure 6.2 The elution profile of the *Desulfurococcus* ATP-PFK from a phenyl-Sepharose column. Unbound protein was eluted from fractions 1 to 20. A linear gradient from 1.0 M to 0.0 M of ammonium sulfate was applied to the column from fractions 11 to 50. A: 1.0 M $(NH_4)_2SO_4$ in 50 mM MOPS buffer (pH 8.0) containing 5.0 mM $MgCl_2$, 100 mM KCl, 0.1 mM PMSF; B: a decreasing linear gradient of 1.0 M $(NH_4)_2SO_4$ in the MOPS buffer. The PFK eluted from the column between 0.25 and 0.6 M of $(NH_4)_2SO_4$ (fractions 30-40). The 280 nm absorbance (A_{280}) plot indicates the relative amount of protein eluting from the column. The reaction velocity (μmole/min) was monitored and calculated based on the 340 nm absorbance.

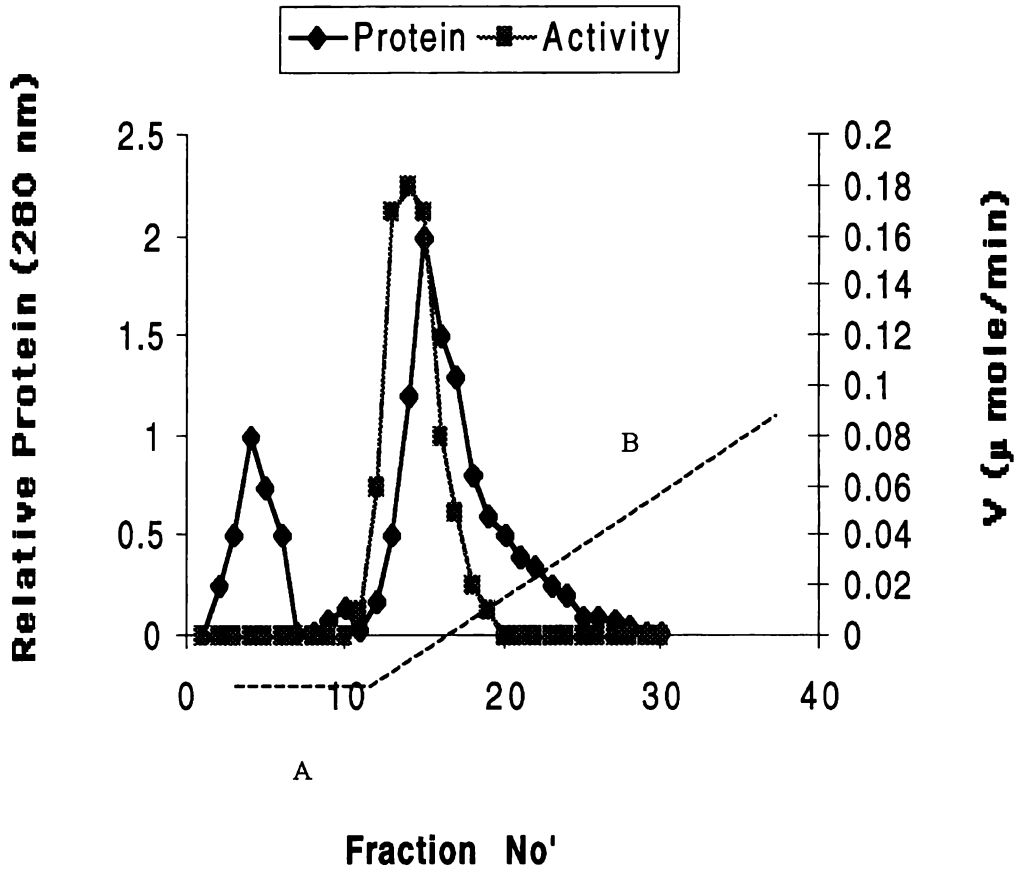


Figure 6.3 The elution profile of the *Desulfurococcus* ATP-PFK from a Q-Sepharose column. Unbound protein was eluted between fractions 1 and 7. The elution profile indicated that most of proteins were bound onto a Q-Sepharose column. The PFK was eluted between 100 and 300 mM NaCl (fractions 12 to 18). A: 50 mM Tris-HCl buffer (pH 8.0) containing 5.0 mM $MgCl_2$, 100 mM KCl, 10% glycerol, 0.1 mM PMSF; B: a linear gradient of NaCl from 0.0 M to 1.0 M in the Tris-HCl buffer. The 280 nm absorbance (A_{280}) plot indicates the relative amount of protein eluting from the column. The reaction velocity (μmole/min) was monitored and calculated based on the 340 nm absorbance.

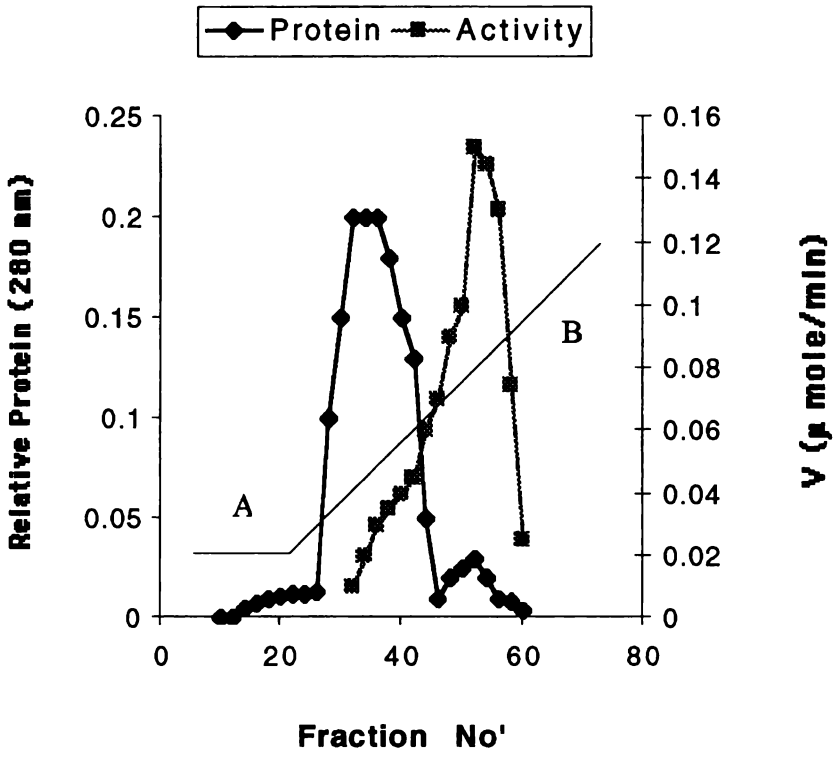


Figure 6.4 Elution profile of the *Desulfurococcus* ATP-PFK from the mimetic orange dye-ligand column. The PFK was eluted between 200 and 400 mM of NaCl (fractions 46 to 60). A: a linear gradient of 0.5 M NaCl in 25 mM MES buffer (pH 8.0) containing 5.0 mM $MgCl_2$, 50 mM KCl, 10% glycerol, 0.1 mM PMSF. The 280 nm absorbance (A_{280}) plot indicates the relative amount of protein eluting from the column. The reaction velocity (μmole/min) was monitored and calculated based on the 340 nm absorbance.

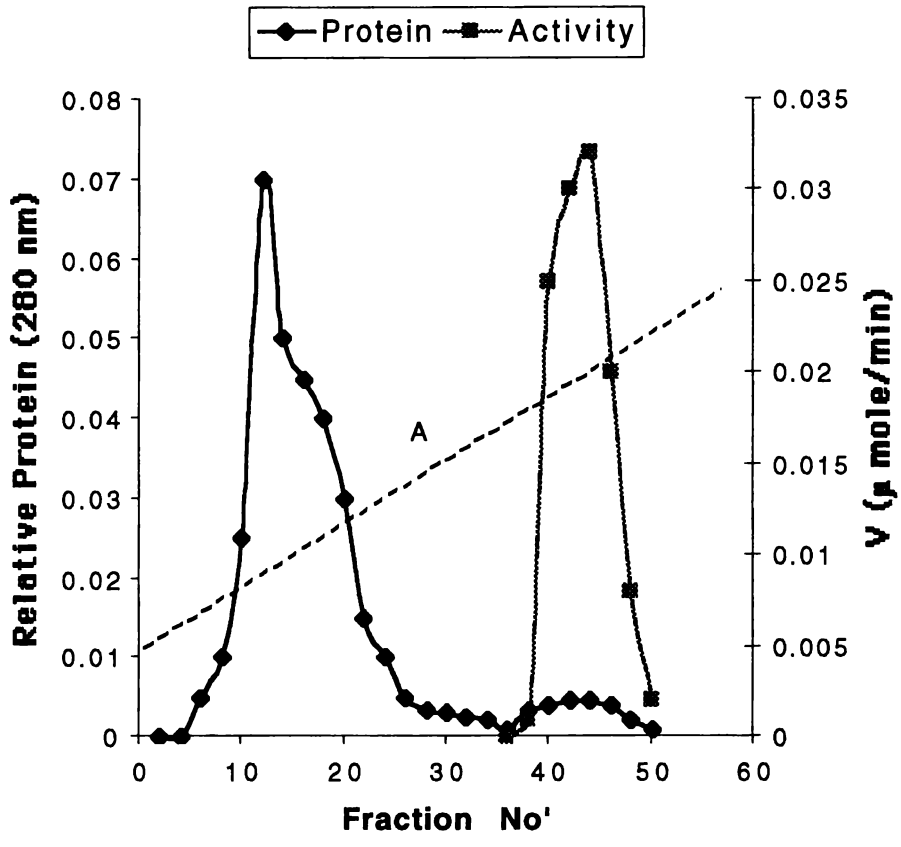


Figure 6.5 The elution profile of the *Desulfurococcus* ATP-PFK from a HPHT column. An increasing gradient of sodium phosphate buffer from 10 to 350 mM. The enzyme eluted between fractions 40 to 50 with a very low protein concentration. The 280 nm absorbance (A_{280}) plot indicates the relative amount of protein eluting from the column. The reaction velocity (μmole/min) was monitored and calculated based on the 340 nm absorbance.

The enzyme was purified 628-fold to homogeneity with a final yield of 1.5% of the activity in the cell-free extract and had a specific activity of 27 U/mg (Table 6.1; Fig. 6.6). Other matrices were trialed during the purification and including phosphocellulose, dye-ligand columns (Cibacron blue dye F3G-A and reactive red 120) and specific elution of the enzyme with ATP, ADP, F-6-P or phosphate. The enzyme did not bind to the phosphocellulose and reactive red 120 columns. Interestingly, the enzyme bound to the F3G-A blue-dye column but the activity was completely lost and could not be eluted with either 2.0 M NaCl or 50% ethanediol, probably due to denaturation on the column or binding that was very

strong preventing elution.

Table 6.1 Purification table for the ATP-PFK from *D. amylolyticus*

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Cell-free extract	520	12,000	0.043	1	100
40-60% $(NH_4)_2SO_4$	442	7512	0.06	1.4	85
Phenyl-Sepharose	382	1546	0.25	5.8	73
Q-Sepharose	86	110	0.78	18.1	17
Orange dye-ligand	12	1.12	10.7	249	2.3
Hydroxyapatite	8	0.3	27	628	1.5

The size of the subunit of the ATP-PFK was estimated to be 37.3 kDa by denaturing SDS-PAGE (Figure 6.6). Gel filtration chromatography with the BIOSEP-SEC 3000 column indicated showed that the native enzyme had a molecular mass of approximately 97 kDa (Fig. 6.6), which suggested that the enzyme might be a dimer or trimer. The protein also migrated as a single band after isoelectric focusing and a *p*I of 5.3 was obtained after comparison with the standards (Table 6.3). The half-life for the enzyme was determined to be about 90 min at 100°C based on amount of activity retained after incubation (Table 6.3). The pH optimum for activity was between 7.6 and 8.0 (Fig. 6.7).

6.3.2 Characterisation of the *Desulfurococcus* ATP-PFK

The apparent K_m values for either F-6-P (0.37 mM) or ATP (0.036 mM) were much lower than those from cell-free extracts reported by Selig et al. (1997). The potential phosphoryl donors of *Desulfurococcus* ATP-PFK were screened (Table 6.3). The purified enzyme was confirmed to have a strong preference for ATP but GTP (19%), CTP (10%) and UTP (6%) could partially be used as an energy sources. The enzyme could not use either PP_i, polyP or ADP as the phosphoryl donor (Table 6.3).

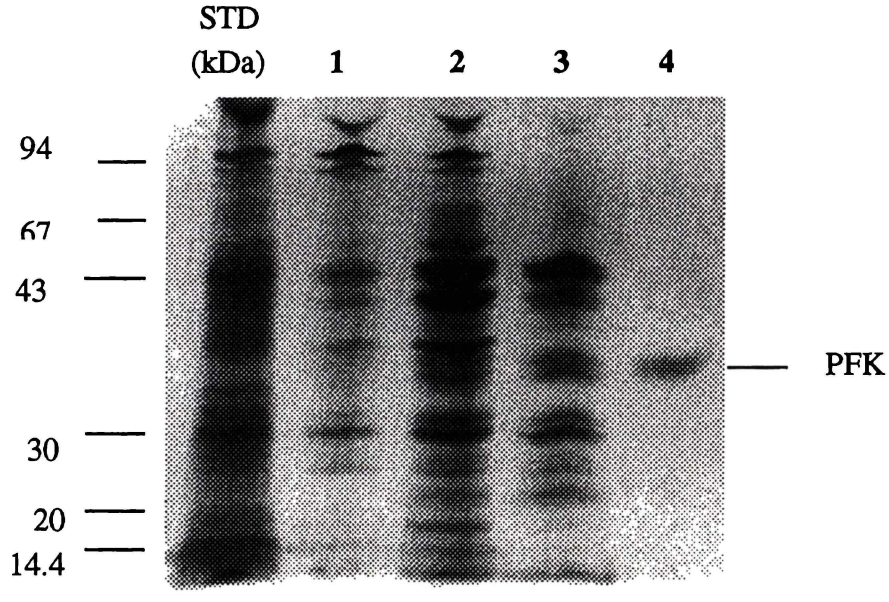


Figure 6.6 SDS-PAGE for the *Desulfurococcus* ATP-PFK. SDS-PAGE gel (silver-stained) of fractions obtained during purification of the ATP-PFK. Lanes 1-4 contained the fractions obtained after purification steps from phenyl-Sepharose to HPHT listed in Table 6.1, respectively; lanes 1 through 4 contained 4.0, 6.0, 5.0, and 0.2 μg of protein, respectively. Lane STD contained the lower molecular weight markers listed in Section 2.9.2.

The pH optimum was determined as described in Section 2.10.1. Like most ATP-PFKs from eukaryotes and bacteria, the *Desulfurococcus* ATP-PFK also possesses a optimum pH between 8.0 and 8.5 (Fig. 6.7). A summary of some properties (apparent K_m values, thermostability, *p*I, optimum Mg concentration) of the archaeal ATP-PFK are listed in Table 6.2.

The effects of a number of potential activators and inhibitors were also investigated. The traditional allosteric effectors, i.e. PEP, ADP, F-2,6-P_2, citrate and succinate had no significant effect on enzyme activity. Strikingly, the enzyme activity was stimulated by K^+ and Na^+ and Mn^{2+} (Table 6.3). The divalent cation Mg^{2+} was necessary for activity; other divalent cations as chloride salts, e.g. Ca^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, could not replace Mg^{2+} (not shown).

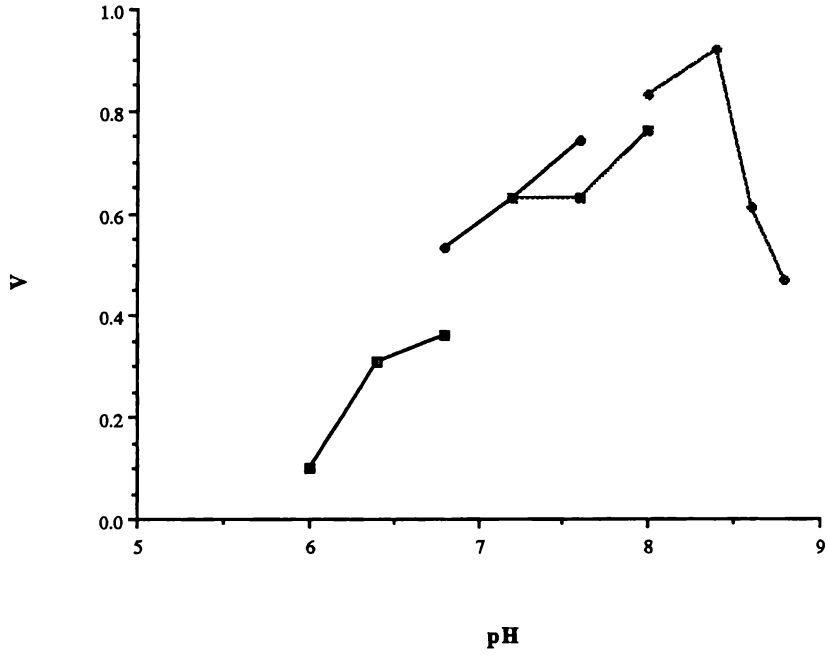


Figure 6.7 Determination of pH optimum from the *Desulfurococcus* ATP-PFK. The buffers used were described in Section 2.10.1. V: reaction velocity (μmole/min) was obtained and calculated based on the 340 nm absorbance.

Table 6.2 Summary of the properties of the *Desulfurococcus* ATP-PFK

Molecular mass (SDS-PAGE)	37.3 kDa
Molecular mass (Gel filtration)	97.0 kDa
pH optimum	8.0-8.5 mM
*p*I (IEF)	5.3
Optimum Mg^{2+}	3.0-7.0 mM
K_m for F-6-P	0.37 mM
K_m for ATP	0.036 mM
Thermostability (Half-life)	90 min at 100°C

Table 6.3 Phosphoryl donors and activators/inhibitors tests for the ATP-PFK from *D. amylolyticus*

Substrate	Phosphoryl Donors[a] % Relative activity	Activators/Inhibitors[b] % Relative activity
ATP	100	100
ADP	ND[c]	108
TTP	ND	90
TDP	ND	85
GTP	19	108
GDP	ND	93
CTP	10	75
CDP	ND	79
UTP	6	92
UDP	ND	96
PP_i	ND	90
F-2,6-P_2	-	105
PEP	-	77
AMP	-	91
Succinate	-	88
Citrate	-	70
Acetyl-CoA	-	70
L-Arginine	-	97
CoA	-	107
Glucose-6-phosphate	-	99
Iso-citrate	-	83
Na_3citrate	-	96
Malic acid	-	79
Glutamine	-	102
Oxalo-acetate	-	93
Phosphoglycerate	-	98
3-Phosphoglycerate	-	87
KCl (10 mM)	-	181
NaCl (10 mM)	-	161
$MnCl_2$ (0.1 mM)	-	158
$CuCl_2$ (1.0 mM)	-	0
$CuCl_2$ (0.01 mM)	-	43

[a]30 mM Tris-HCl (pH 8.0 at 50°C) containing 5.0 mM F-6-P, 5.0 mM $MgCl_2$, 0.1 mM of phosphoryl donors, 0.01 mM DTT, 100 mM KCl.

[b]30 mM Tris-HCl (pH 8.0 at 50°C) containing 0.5 mM F-6-P, 5.0 mM $MgCl_2$, 0.05 mM ATP, 1.0 mM of activators or inhibitors

Unfortunately, no N-terminal sequence was obtained after 2 attempts, suggesting that the N-terminus was blocked.

6.4 DISCUSSION

The first archaeal ATP-PFK has been purified and characterised from the hyperthermophilic crenarchaeon *D. amylolyticus*. The enzyme was found to be specific for ATP and the enzyme activity was not significantly affected by either traditional modulators or numerous other activators or inhibitors. During small-scale purification trials, it was found that the ATP-PFK activity steadily decreased unless glycerol (10%) and PMSF were added after each step. As most ATP-PFKs are activated by KCl, KCl (50-100 mM) was also added in various buffers to help maintain activity. Both ATP- and ADP-PFK activities were initially found in the cell-free extract, however, the ADP-PFK activity (likely due to contaminating adenylate kinase activity) was removed to an undetectable level after the final purification step.

Bacterial ATP-PFKs usually have a tetrameric structure of identical subunits each with a molecular mass of approximately 35 kDa and are often allosterically regulated by PEP and ADP (Uyeda, 1979). ATP-PFKs in mammals also have a tetrameric structure but the subunits are larger (approximately 85 kDa) and described in Chapter 1. The yeast ATP-PFK is an exception and has an octameric structure composed of two non-identical subunits with molecular masses of 120 and 113 kDa, which have arisen as a result of gene duplication and fusion events (Poorman et al., 1984). The enzyme from *D. amylolyticus* possesses a 37.3 kDa subunit and a 97 kDa native molecular mass. The results indicated a similar structure to that described for the PP_i-PFK from *T. tenax*, with a molecular mass of 37 kDa on SDS gel, a native molecular mass of 100 kDa and nonallosteric properties (Siebers et al., 1998). The subunit molecular mass for the *D.*

amylolyticus enzyme is similar to that of bacterial PFKs and significantly smaller than the ADP-PFK of archaea which is approximately 52 kDa (Ronimus et al., 1999a).

The apparent K_m values (Table 6.2) for ATP and F-6-P for the purified enzyme in this study were much lower than those reported from the cell-free extract assays (Table 1.1; Selig et al., 1997). A higher apparent K_m value might be caused by an impurity or different ionic strength in the crude cell-free extracts as apparent K_m values with the *T. maritima* enzyme have been found to be highly related to different concentrations of KCl (Table 7.1). Surprisingly, the *Desulfurococcus* ATP-PFK was not only nonallosteric but also possessed a low K_m for F-6-P. Similar results have also been reported for the ATP-PFKs from *L. delbrueckii* (Le Bras et al. 1991), *D. discoideum* (Martínez-Costa et al. 1994), *T. brucei* (Michels et al., 1997), *D. amylolyticus* (Hansen et al., 2000) and for the PFK-2 of *E. coli* (Uyeda, 1979). In contrast to these findings, most ATP-PFKs from eukarya and bacteria have a high K_m value for their substrate F-6-P. The optimum pH for the *D. amylolyticus* ATP-PFK was similar to most ATP-PFKs, which is approximately 8.0-8.5. The divalent cation Mg^{2+} was essential for the *D. amylolyticus* ATP-PFK enzyme activity and other divalent cations were not able to substitute for it under experimental conditions. The ATP-PFK from *D. amylolyticus* possessed a high thermal stability (90 min at 100°C) which could be due to the protein amino acid composition, a high degree of hydrophobicity, overall chain rigidity and/or the overall tightness of intramolecular packing (Jaenicke et al., 1996).

The purification and characterisation of the ATP-PFK from *D. amylolyticus* was undertaken in order to examine whether its biochemical properties would provide support for the notion that the enzyme belongs to either the major allosteric PFKs from eukarya or bacteria or whether it perhaps represented a more ancestral and

nonallosteric type of ATP-PFK. The phylogeny of archaea as described from 16S rRNA sequence comparisons suggests that *T. tenax* (with a PP_i-PFK) is from a more ancient lineage than *D. amylolyticus* (with a ATP-PFK; Brock et al., 1994). In addition, an alignment of PFK amino acid sequences containing the *T. tenax* PP_i-PFK, *Dictyoglomus* PP_i-PFK, *A. methanolica* PP_i-PFK, *S. coelicolor* ATP-PFK and *T. brucei* ATP-PFK supports the idea that PP_i-PFKs are more ancient than ATP-PFKs (Alves et al., 1997; Michels et al., 1997; Siebers et al., 1998; Ding et al., 2000a).

CHAPTER SEVEN

CLONING, EXPRESSION AND CHARACTERISATION OF A PP_i-PFK AND AN ATP-PFK FROM THE HYPERTHERMOPHILIC BACTERIUM *THERMOTOGA MARITIMA*

7.1 INTRODUCTION

Small subunit ribosomal RNA (SSU rRNA) phylogenies have shown that *T. maritima* is one of the deepest and most slowly evolving lineages in the bacterial domain (Huber et al., 1986; Woese, 1990). In addition, Schröder et al. (1994) had demonstrated that *T. maritima* possessed an ATP-PFK in a cell-free extract. Interestingly, the complete genome of *T. maritima* has been sequenced, revealing that this organism possesses two genes encoding for both a PP_i- and an ATP-PFK (Nelson et al., 1999). If both genes are expressed as functional proteins, it suggests that there might be interesting mechanisms of regulation of glycolysis in this organism. In this chapter, the cloning of the two genes and the expression and characterisation of both PP_i and ATP-PFKs genes from *T. maritima* were investigated.

7.2 MATERIALS AND METHODS

7.2.1 Bacterial strains and plasmids

T. maritima was obtained from the TRUCC which is equivalent to DSM 3109. Cells were grown at 80°C in the *T. maritima* medium described by Huber et al. (1986). *E. coli* strain DH5α and expression plasmid pPROEX HTb were used for gene cloning

and expression. *E. coli* was grown at 30°C with vigorous aeration (200 rpm) in LB broth supplemented with 100 μg/ml ampicillin.

7.2.2 DNA techniques

The chemicals, reagents and enzyme assays used in this chapter were described in Sections 2.2 and 2.3. Genomic DNA from *T. maritima* was prepared as described in Section 2.11.3. Large-scale plasmid DNA was purified from *E. coli* by using the alkaline lysis method combined with caesium chloride gradient purification (Section 2.11.4). DNA fragments produced by PCR, and restriction digests subsequently used for sequencing and cloning experiments were separated on 1% TAE preparative gels using the "freeze and squeeze" technique and phenol and chloroform extractions according to the methods by Ronimus (1993) and Sambrook et al. (1989).

7.2.3 Sequence and restriction map analysis for both genes

The sequences of both PP_i- and ATP-PFK genes were obtained from *Thermotoga* genomic sequence (Nelson et al., 1999). The restriction mapping of both genes was carried out using DNA Strider program (version 1.2).

7.2.4 Construction of the *Thermotoga* PP_i- and ATP-PFKs expression clones

The open reading frames (ORFs) representing the full-length sequences of both PP_i- and ATP-PFK genes (Appendix C) were amplified by PCR using genomic DNA from *T. maritima.* The design of the primers was based on the gene sequences of the 5' and

3' ends of the putative PFK genes identified in the full genome sequence (Nelson et al., 1999). After the restriction map studies, the forward primer for the PP_i-PFK gene, corresponded to the N-terminus, contained an upstream *Sfo* I site (in bold) and 5' end-spacer (5'-GGAA **GGC GCC** ATG GCT GAA AGA TTG GGG ATA CTC G-3'); and the reverse primer, corresponded to the C-terminus, containing a flanking *Hin*d III site (in bold) and a 5' end-spacer (5'-GCTA **AAG CTT** TAT GGA AGC TCT GTC GTA TGC CAG-3').

The primers for the ATP-PFK gene also containing *Sfo* I and *Hin*d III sites were (5'-GGCT **GGC GCC** ATG AAG AAG ATA GCA GTA TAC -3') and (5'-CCA **TAA GCT** TTA TGA AAG CAT ATG TGC TAT TTC-3') for forward and reverse primers, respectively. Ampli *Taq* Gold DNA polymerase was used for PCR with an initial "hot start" of 9 min at 94°C which was described in Section 5.2.3.1. The remainder of the profile (45 sec at 94°C, 45 sec at 50°C, and 2 min at 72°C) was repeated for 35 cycles, and the final 72°C extension step was for 6 min. After electrophoresis, both PCR products were sliced from the gels and followed by "freeze and squeeze", phenol:chloroform purification, ethanol precipitation, then quantification for ligation and transformation. The amplified PCR products were sequenced from both ends and used to search the database (BLAST) and aligned with the *Thermotoga* genome sequence (Nelson et al., 1999).

The PCR-generated DNA fragments were cloned into the pPROEX HTb expression vector after restriction digestion (100 ng DNA of each gene) of the ends with *Sfo* I (5.0 U) and *Hin*d III (5.0 U) at 37°C overnight, followed by ligation with T_4 DNA ligase (0.5 U/100-300 ng DNA) using the protocols of Sambrook et al. (1989). The ligation mixture of restricted plasmid pPROEX HTb and PCR products was used to

transform *E. coli* strain DH5α by electroporation as described in Section 2.12.11. Screening of the clones for those with inserts was carried out through alkaline lysis miniprep plasmid isolation (Sambrook et al., 1989) followed by restriction enzyme analysis.

7.2.5 Gene expression and enzyme purification

For small-scale (10 ml) experiments of gene expression of either the PP_i-PFK or the ATP-PFK in *E. coli* DH5α, the protocols used were essentially the same as those described in Section 5.2.6. For large-scale gene expression, the protocol used was as follows: a single colony of *E. coli* DH5α with either *pfp* or *pfk* gene was inoculated onto 10 ml LB media containing 100 μg/ml ampicillin and grown overnight at 30°C with agitation (200 rpm). The 10 ml of overnight culture was then inoculated onto 600 ml LB media with ampicillin and grown at 30°C for 3-4 h with agitation (200 rpm). IPTG was added to the culture to a final concentration 1.0 mM when the culture reached an A_{600} of 0.5-1.0. The induced cells were harvested by centrifugation (11,000 x *g*, 20 min at 4°C) after 5 h IPTG induction. Cell pellets were resuspended in Ni-NTA buffer A [20 mM Tris-HCl (pH 8.5 at 4°C), 100 mM KCl, 5 mM β-mercaptoethanol, 10% glycerol, 20 mM imidazole]. After sonication and centrifugation (11,000 x *g*, 20 min at 4°C), the supernatant was transferred to a new centrifuge tube and heat-treated in a waterbath at 80°C for 40 min. The denatured *E. coli* proteins were removed by centrifugation (11,000 x *g*, 20 min at 4°C). The supernatant was dialysed at 4°C overnight against the Ni-NTA buffer A. The purification protocols used followed the manufacturer's instructions (Life Technology). In addition, the Ni-NTA resin was regenerated with $NiSO_4$ after 5-8 batches of purification of a single PFK type. The method for regeneration of this

resin is described in Appendix D. The clones with the appropriate genes were resuspended in 50% glycerol and stored at –70°C.

7.2.6 Determination of molecular sizes and enzyme thermostabilities

The native molecular mass and subunit molecular sizes were measured as described in Sections 2.6 and 2.9.2. The thermostabilities of the recombinant *Thermotoga* PFK enzymes were investigated by incubating the enzymes (each at 100 μg/ml final concentration) at 90°C in buffer containing either 50 mM phosphate, MOPS or Bis-Tris buffer, 3.0 mM $MgCl_2$, 0.02% Triton X-100, pH 7.0 at 90°C, under a mineral oil overlay for between 0 and 400 min, then using the conditions described in Section 2.10.3.

7.2.7 pH dependence, cation specificities and molecular properties of recombinant enzymes

The pH optima of both enzymes were determined as described in Section 2.10.1. The determination of cation specificities was carried out by replacing Mg^{2+} with alternative divalent cations, all at 1.0 mM, and comparison of the activity with that of the control with 1.0 mM $MgCl_2$. The effects of cations on the activity of both enzymes were also examined in the presence of Mg^{2+} using the standard assay with addition of the different cations. The optimal concentrations of KCl for activity of both enzymes were investigated using KCl with a range of final concentrations (0-500 mM).

7.2.8 Determination of kinetic parameters, activators and inhibitors

The apparent K_m values for both enzymes were measured using the methods described in Section 2.10.5. All assays were carried out in the presence of an optimal KCl concentration of 175 mM.

The traditional metabolic regulators for both enzymes were tested using the concentrations of cosubstrates (PP_i, PPP_i, polyP, ATP or F-6-P) near their apparent K_m values. The regulators tested and the concentration used were PEP (0.01, 0.05, 0.1, 1.0, 5.0, 10.0 mM), ADP (0.05, 0.1, 1.0 mM), GDP (0.05, 0.1, 1.0 mM), succinate (0.05, 0.1, 1.0 mM), AMP (0.05, 0.1, 1.0 mM), cAMP (0.05, 0.1, 1.0 mM), F-2,6-P_2 (0.5, 1.0, 5.0 mM) and citrate (0.1, 1.0 mM).

7.3 RESULTS

7.3.1 Sequence analysis of the *Thermotoga* PP_i- and ATP-PFKs

After an analysis of the restriction map for the gene sequences, the design of the primers for PCR was based on the sequences of the 5' and 3' ends of the putative PFK genes identified in the full genome sequence (Nelson et al., 1999). For both the PP_i- and ATP-PFK genes (Fig. 7.1a and 7.1b), the forward primer corresponding to the N-terminus contained an upstream *Sfo* I site; and the reverse primer corresponding to the C-terminus contained a flanking *Hin*d III site, since these restriction sites were not found within the genes.

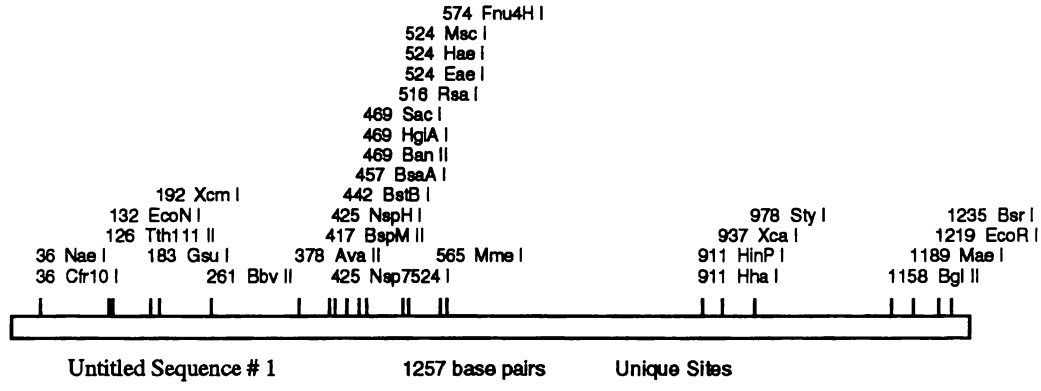


Figure 7.1a Restriction map of the *Thermotoga* PP_i-PFK

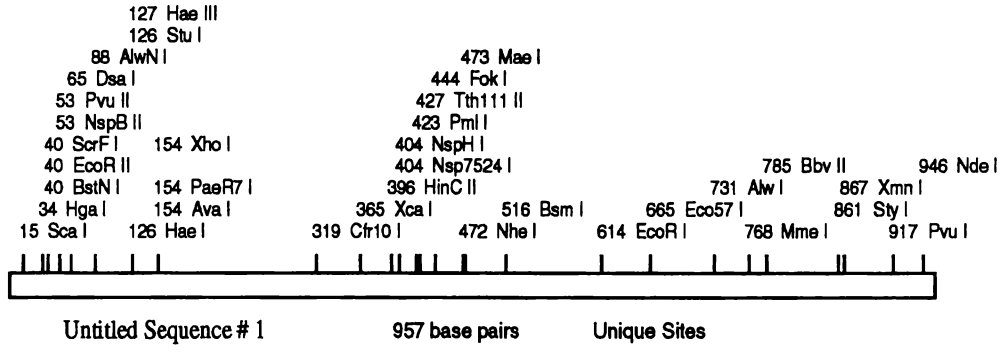


Figure 7.1b Restriction map of the *Thermotoga* ATP-PFK

7.3.2 Amplification of the two genes by PCR

The PCR products corresponding to the two genes (*pfp* and *pfk*) were of the sizes predicted from their gene sequences, approximately 1,200 bp and 950 bp, respectively (Fig. 7.2).

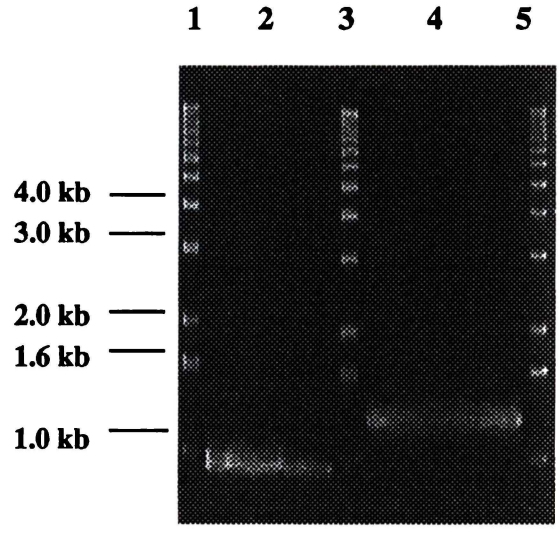


Figure 7.2 PCR amplification of both *pfp* and *pfk* genes from *T. maritima*. Lanes 1, 3, and 5: 1.0 kb ladder marker; lane 2: *pfk* gene (approximately 950 bp); lane 4: *pfp* gene (approximately 1.2 kb).

The PCR products were sequenced and aligned with *Thermotoga* genome sequence (Appendix C). A single band for either the PP_i-PFK or ATP-PFK was obtained after PCR amplifications. The PCR products were run on 1% TAE preparative gels for subsequent sequencing and cloning procedures.

The pPROEX plasmid had a linear molecular size of 4,7 kb and an approximate size of 2,9 kb for the supercoiled form (Fig. 7.3, lane 10). A linear size about 6.2 kb and a supercoiled size about 3.2 kb were found from lanes 2 to 7 with *Thermotoga pfk* gene (950 bp). The clones with the *Thermotoga pfp* gene (1.3 kb) possessing sizes of 3.4 and 6.5 kb are found in either lane 8 or lane 9.

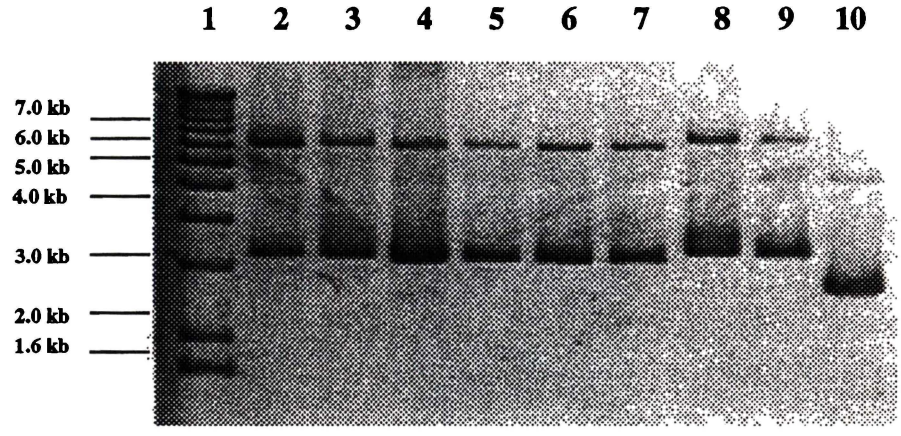


Figure 7.3 Miniprep plasmid analysis of both the *Thermotoga pfp* and *pfk* genes from transformed *E. coli* clones. Lane 1: 1 kb standard marker; lanes 2 to 7: clones with plasmid for the *Thermotoga pfk* gene cut with *Hin*d III; lanes 8 to 9: clones with plasmid for the *Thermotoga pfp* gene cut with *Hin*d III; lane 10: pPROEX plasmid without inserts.

7.3.3 Molecular sizes and thermostabilities of both enzymes

The enzymes were purified with the Ni-NTA resin according to the manufacturer's instructions. The molecular weights of the *Thermotoga* PP_i- and ATP-PFK were approximately 48,000 and 38,000, respectively (Fig. 7.4).

Both the PP_i- and ATP-PFK from *Thermotoga* were extremely thermostable. The half-lives of both were greater than 5 h at 90°C when phosphate buffer was used (not shown). The thermostabilities of the enzyme were greatly affected by which buffer was present during the incubations. For example, the half-life at 90°C of the ATP-PFK was only 15 min in 50 mM MOPS buffer. Similar results were obtained with the PP_i-PFK in these buffers (not shown).

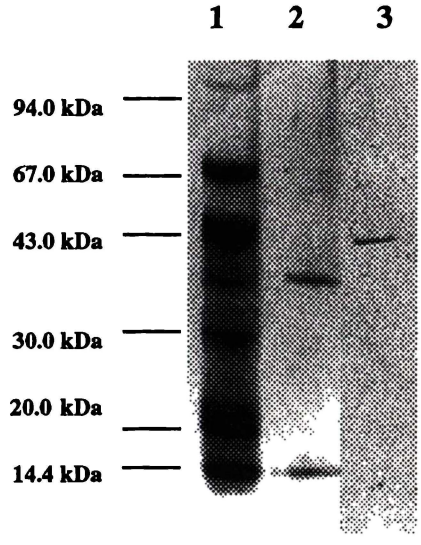


Figure 7.4 SDS-PAGE for both purified PP_i- and ATP-PFK from *T. maritima*. Lane 1 contained lower molecular markers; lane 2: purified *Thermotoga* ATP-PFK (1.0 μg protein); lane 3: purified *Thermotoga* PP_i-PFK (0.75 μg protein).

The recombinant PP_i-PFK had a native molecular mass of 96 kDa as determined by its elution during gel filtration chromatography, which suggests the active molecule exists as a homodimer. In contrast, the molecular mass of the ATP-PFK was 200 kDa (not shown), and thus a homotetramer is the most probable quaternary structure.

7.3.4 pH optima, cation specificities and molecular properties

The initial attempts of screening the optimal pH for both enzymes were carried out using different buffers with a wide range from pH 4.6 to 9.6 as described in Section 2.10.1. Bis-Tris and Tris-HCl buffers were used for the final determination of the pH optima within a narrow pH range for the *Thermotoga* PP_i- and ATP-PFK,

respectively. The results showed that the *Thermotoga* PP_i- and ATP-PFK had pH optima of 5.6-6.0 and 7.8-8.0 (pH values at 50°C), respectively.

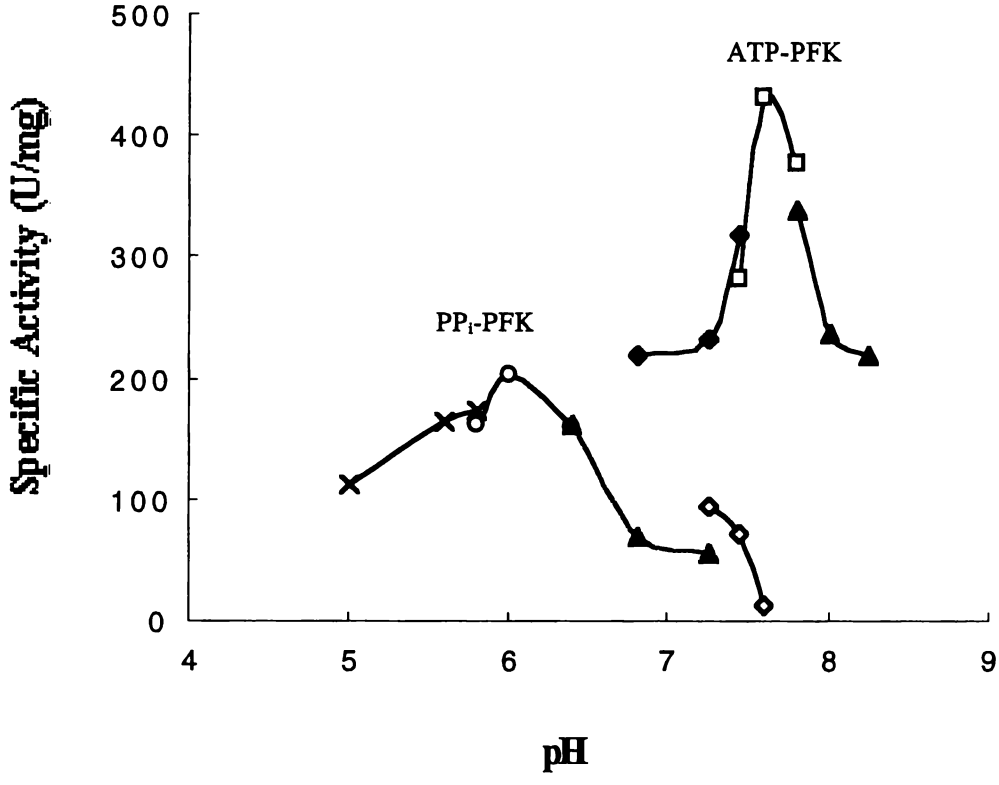


Figure 7.5 Determination of the pH optima for activity of the *Thermotoga* PP_i- and ATP-PFKs

Both enzymes required KCl for optimum activity. The results showed (Fig. 7.6) that the enzyme activities were activated 2.3-fold and 1.6-fold at 175 mM for the PP_i- and ATP-PFK, respectively. Both enzymes were inhibited when the concentration of KCl exceeded 200 mM (Fig. 7.6).

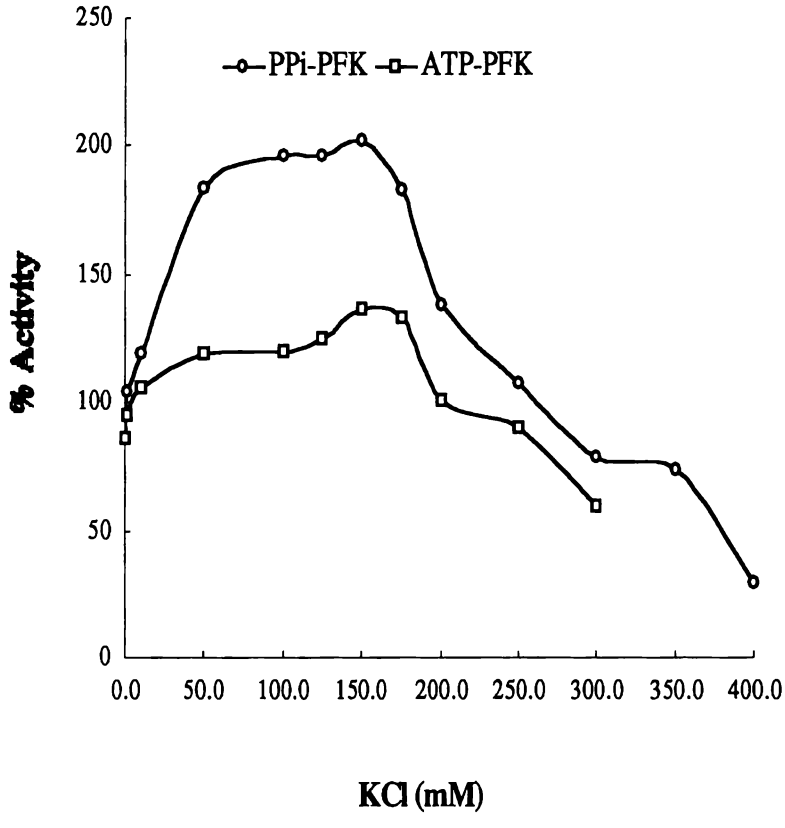


Figure 7.6 Effect of KCl on enzyme activity from the *Thermotoga* PP_i- and ATP-PFK

As can be seen in Table 7.1, the apparent K_m values of PP_i and F-6-P for the *Thermotoga* PP_i-PFK varied with different concentrations of KCl. The optimum KCl concentration for the PP_i-PFK is about 175 mM which was associated with the lowest apparent K_m values.

Table 7.1 Effects of KCl on the apparent K_m values of PP_i and F-6-P from the *Thermotoga* PP_i-PFK

	KCl (mM)	50	175	250	500
K_m-PP_i [a]	(mM)	0.044	0.025	0.043	0.057
K_m-F-6-P [b]	(mM)	0.957	0.504	0.980	2.680

The assays were carried out in 30 mM Bis-Tris, pH 5.8 (at 50°C) containing

[a] 3.0 mM F-6-P, 5.0 mM $MgCl_2$

[b] 0.3 mM PP_i, 5.0 mM $MgCl_2$

Both of the *Thermotoga* PP_i- and ATP-PFKs were highly activated by KCl and extremely sensitive to Cu^{2+} and Zn^{2+} (Table 7.2). In addition, the activities from both enzymes were partially inhibited by Co^{2+} and Mn^{2+} at 0.1 mM of concentration. Interestingly, Fe^{2+} and Fe^{3+} had the similar effects on both enzymes. The function of Mg^{2+} for both enzymes could not be replaced by any other tested divalent cations (not shown).

Table 7.2 Cation effects on the activity of the *T. maritima* PP_i- and ATP-PFKs

		Relative Activity (%)	
Cation	mM	PP_i-PFK[a]	ATP-PFK[b]
$MgCl_2$	3.0	100	100
KCl	10	133	130
KCl	50	140	135
NaCl	50	114	105
$MnCl_2$	0.1	49	76
$CaCl_2$	0.1	84	100
$CoCl_2$	0.1	53	43
$SeCl_4$	0.1	67	76

Table 7.2 (continued)

LiCl	0.1	80	90
$NiCl_2$	0.1	59	70
$FeCl_2$	0.1	36	104
$FeCl_3$	0.1	36	104
$ZnCl_2$	0.1	0	0
$ZnCl_2$	0.01	28	10
$ZnCl_2$	0.001	56	72
$CuCl_2$	0.1	0	10
$CuCl_2$	0.01	46	53
$CuCl_2$	0.001	57	72

[a] 30 mM Bis-Tris (pH 5.8 at 50°C), 3.0 mM F-6-P, 5.0 mM $MgCl_2$, 0.75 mM PP_i

[b] 30 mM Tris-HCl (pH 8.0 at 50°C), 3.0 mM F-6-P, 5.0 mM $MgCl_2$, 0.05 mM ATP

7.3.5 Determination of phosphoryl donors, kinetics parameters, activators and inhibitors

Different phosphoryl donors, apparent K_m values and some activators and inhibitors for both *T. maritima* PP_i- and ATP-PFKs were investigated and showed in Table 7.3. The experimental conditions were essentially the same as those in Chapters 4, 5 and 6.

Table 7.3 Kinetic parameters from the *Thermotoga* PP_i-PFK

Substrate	K_m (mM)	V_{max} (U/mg)	V_{max}/K_m
PP_i as phosphoryl donor			
PP_i	0.067	203	3×10^3
F-6-P	0.980	358	3.6×10^2
F-1,6-P_2	0.305	46	1.5×10^2
P_i	2.220	31	0.14×10^2

Table 7.3 (continued)

PPP_i as phosphoryl donor			
PPP_i	0.010	249	2.7×10^4
F-6-P	0.482	220	4.6×10^2
PolyP (n=15±3) as phosphoryl donor			
polyP	0.0038	319	8.0×10^4
F-6-P	0.374	280	7.5×10^2

Table 7.4 Kinetic parameters from the *Thermotoga* ATP-PFK

Substrate	K_m (mM)	V_{max} (U/mg)	V_{max}/K_m
Apparent K_m values for ATP, F-6-P and GTP			
ATP	0.148	432	5.0×10^3
F-6-P	0.432	464	1.1×10^3
GTP	1.220	294	2.4×10^2
Effects of PEP on the apparent K_m values for F-6-P			
0.1	0.324	250	7.7×10^2
0.5	0.305	250	8.2×10^2
1.0	0.271	252	9.3×10^2
5.0	0.669	260	4.0×10^2
Effects of PP_i, PPP_i, polyP (n=15±3) and citrate on the apparent K_m values for F-6-P			
0.01 mM PP_i	0.436	148	3.4×10^2
0.025 mM PP_i	0.691	136	1.97×10^2
0.05 mM PPP_i	0.983	256	2.6×10^2
0.05 mM polyP	1.369	278	2.0×10^2
1.0 mM citrate	0.811	224	2.76×10^2

Various phosphoryl donors were tested as substrates for the *Thermotoga* enzymes. All the graphical data relating to the determination of apparent K_m values for both

Thermotoga PP_i- and ATP-PFK are shown in Appendix E.1-20. Compared to the apparent K_m values with different phosphoryl donors, the *Thermotoga* PP_i-PFK is more likely to be a polyP dependent PFK as the apparent K_m values are decreased from 0.067 (PP_i), 0.01 (PPP_i) to 0.0038 (polyP) mM, respectively. In addition, the V_{max} values for PP_i, PPP_i and polyP increased steadily (Table 7.3). The traditional metabolic regulators did not have a significantly effect on the *Thermotoga* PP_i-PFK activity.

The putative *Thermotoga* ATP-PFK displayed the highest activity with ATP as the phosphoryl donor but had significant activity when this was replaced by GTP (42%), UTP (14%), CTP (13%) and TTP (10%). No activity was detected with, either PP_i, PPP_i, polyP or ADP as the phosphoryl donor. It was also found that *Thermotoga* ATP-PFK was strongly inhibited by higher concentrations (2.0 mM) of ATP (Appendix E.9).

Interestingly, the apparent K_m and V_{max} values for F-6-P from the *Thermotoga* ATP-PFK did not change significantly when different concentrations of PEP (0.1-5.0 mM) were added (Table 7.3), which suggested that PEP did not have an significant effect on *Thermotoga* ATP-PFK activity. A range of concentrations of PEP (0.01-5.0 mM) was investigated but the normal hyperbolic kinetic curve for F-6-P did not noticeably change into a sigmoidal type of kinetic curve even at the higher concentrations (5.0 mM) of PEP (Appendix E.12-15).

The *Thermotoga* PP_i-PFK activity was not influenced significantly by the presence of either ATP, ADP, AMP, TTP, TDP, GTP, GDP, CTP, CDP, UTP, UDP, cAMP or P_i, (each at 0.1 mM; not shown). Activity was also unaffected by either F-2,6-P_2,

succinate or citrate (each at 1.0 mM; not shown). The *Thermotoga* ATP-PFK activity was only slightly inhibited by citrate at 1.0 mM concentration. The apparent K_m for F-6-P increased from 0.43 μM to 0.81 μM when citrate (1.0 mM) was present in the reaction mix (Table 7.1).

The *Thermotoga* ATP-PFK activity was extremely inhibited by PP_i, PPP_i and polyP at very low concentration (30-50% left at 0.1 mM; Fig. 7.7). The enzyme activity was completely inhibited by 1.0 mM of either PP_i, PPP_i or polyP (not shown), however, PEP had no significantly effect at higher concentration (53% remaining activity at 5.0 mM concentration, not shown).

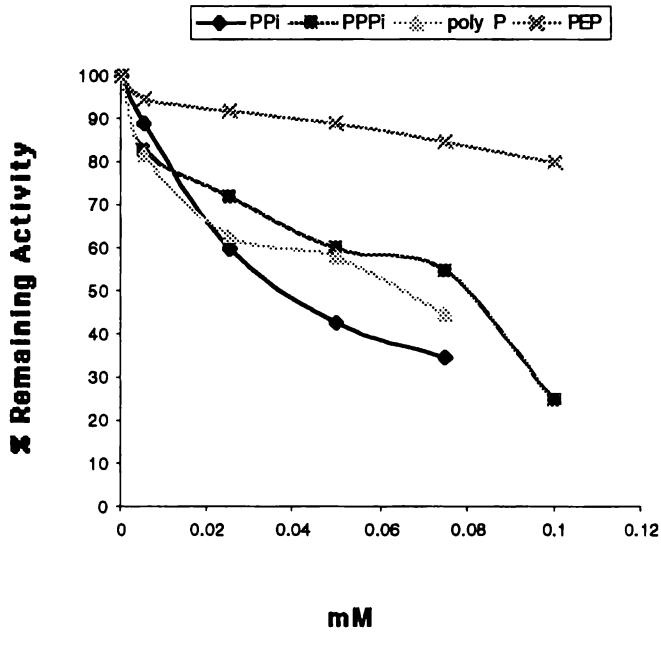


Figure 7.7 Effects of PP_i, PPP_i, polyP and PEP on the *Thermotoga* ATP-PFK activity

ADP slightly inhibited the ATP-PFK (70% of control at 1.0 mM; not shown) at high concentration and the activity increased slightly (129% of control; not shown) at the

lower concentration of 0.05 mM. The inhibition of ATP-PFK activity by PP_i could be partially alleviated by the presence of nucleotide diphosphates, i.e. ADP, GDP, UDP or TDP (Table 7.4).

Table 7.5 Effects of some compounds on PP_i inhibition of the *Thermotoga* ATP-PFK

Effectors	% Relative activity
None	100
0.1 mM PP_i	20
0.1 mM PP_i, 0.1 mM ADP	57
0.1 mM PP_i, 0.1 mM AMP	22
0.1 mM PP_i, 0.1 mM ADP, 0.1 mM AMP	57
0.1 mM PP_i, 0.1 mM TDP	69
0.1 mM PP_i, 0.1 mM GDP	68
0.1 mM PP_i, 0.1 mM CDP	27
0.1 mM PP_i, 0.1 mM UDP	47

30 mM Tris-HCl, pH 7.8 (at 50°C), 0.5 mM F-6-P, 0.25 mM ATP, 5 mM $MgCl_2$, 175 mM KCl.

7.3.6 Sequence alignment and phylogenetic analysis of for both the *Thermotoga* enzymes

Eleven representative amino acid sequences of PFKs from eukaryotes, bacteria and the crenarchaeon *T. tenax* were retrieved from sequence data bases and aligned with both of PP_i- and ATP-PFK sequences from *T. maritima* which was shown in Figure 5.13. An unrooted phylogenetic tree incorporating 22 amino acid sequences of PFKs is presented in Figure 5.14 and shows the *Thermotoga* PP_i-PFK to have greatest homology with eukaryotic PP_i-PFKs from *T. vaginalis* and *N. fowleri* (Mertens et al., 1998; Wessberg et al., 1995). The *Thermotoga* PP_i-PFK and those from the two primitive eukaryotic organisms form a branch within group II enzymes based on the classification scheme proposed by Siebers et al. (1998). The *Thermotoga* ATP-PFK

is most closely related to the ATP-PFK from *T. thermophilus* and *A. aeolicus* and belongs to group I PFKs.

7.4 DISCUSSION

It has been confirmed from this work that both putative PP_i- and ATP-PFK genes identified in the genome sequence of *T. maritima* (Nelson et al., 1999) encode for distinct and functional PFKs. *Thermotoga* is thus the only prokaryote reported which possesses two different phosphoryl donor subtypes of PFK. We had previously detected both PP_i- and ATP-PFK activity from cell-free extracts of this organism with the PP_i-PFK activity representing approximately 25% of ATP-PFK activity at pH 7.0 (Table 3.1). When extracts were assayed at the pH optima for the respective enzymes, the PP_i-based activity (pH 5.8 at 50°C) was slightly higher than the activity with ATP (at pH 7.8 at 50°C; not shown). This suggests that both enzymes might be expressed *in vivo*.

Both the *Thermotoga* PFKs possess unusual biochemical properties when compared to the more typical enzyme for each phosphoryl subtype. PFKs in other organisms generally have a requirement for a low concentration (10-50 mM) of either sodium or potassium ions for optimal activity (Ding et al., 1999, 2000a; Ronimus et al., 1999). With respect to the *Thermotoga* enzymes, the requirement for potassium ions and the stimulation of activity by their presence is more pronounced, e.g. the PP_i-PFK activity increased approximately 230% at KCl concentrations ranging from 0 to 170 mM, which suggested that KCl might play a role in enzyme tertiary structure. The higher concentration of this monovalent cation for activity possibly reflects the marine environment from which the organism was isolated (Huber et al., 1986).

Surprisingly, both *Thermotoga* enzymes are extremely sensitive to Cu^{2+} and Zn^{2+}. Our previous work also demonstrated that the PFK activity from either *D. thermophilum* and *D. amylolyticus* was strongly inhibited by Cu^{2+} (Ding et al., 1999, 2000a). The investigation on the pyruvate ferredoxin oxidoreductases (POR) from the archaeon *P. furiosus* and bacterium *T. maritima* by Smith et al. (1994). They found Cu^{2+} and Zn^{2+} are integral parts of the *P. furiosus* POR and participated directly in catalysing acetyl-CoA production, but not in the *T. maritima* POR.

As the PP_i-PFK has a preference for longer chain phosphoester substrates (Table 7.3), it could be correctly described as a polyP-PFK. This is the first report of a PFK with such a characteristic. PolyP is a component of volcanic condensates and deep-oceanic hydrothermal vents, is ubiquitously distributed in all living organisms (Kornberg, 1995) and possibly played a role in the prebiotic evolution of metabolism (Baltscheffsky, 1996; Yamagata, 1991). Significantly, polyP has been used as an alternate phosphoryl/energy source to ATP for other enzymes involved with glucose metabolism. For example, polyP-dependent glucokinase activity has been reported in *M. tuberculosis* (Hsieh et al., 1993) and *P. freudenreichii* (Uryson and Kulaev, 1968; Wood and Clark, 1988) and a polyP-fructokinase in *M. phlei* (Wood and Clark, 1988). The polyP-glucokinase from *P. freudenreichii* was particularly responsive to phosphoester chain length with the apparent K_m values declining from 4.3 μM to 0.2 nM for polymer lengths of 30 and 724 residues, respectively (Wood and Clark, 1988). The PP_i-PFK from *Thermotoga* demonstrated a similar, though less pronounced, effect with a decline in the apparent K_m values from 67 to 3.8 μM as phosphoester chain length increased from 2 to 18. In contrast, the PP_i-PFKs from *D. thermophilum* and *S. thermophila* favor the PP_i substrate (Ding et al., 1999, 2000a; Ronimus et al., 1999).

The *Thermotoga* ATP-PFK also possesses unusual characteristics. In particular, activity was strongly inhibited by both PP_i and polyP at concentrations reported to be common in bacteria (10-100 μM) (Kulaev, 1979, 1994). Although PP_i and polyP are effective chelators of Mg^{2+} (Yoshida, 1972), the low concentrations (0.1 mM) of phosphoryl donor causing inhibition rules out the free Mg^{2+} concentration (30 mM) as a contributing factor in the decrease observed. This is the first report of allosteric control of ATP-PFK activity by such effectors. In contrast, the ATP-PFK was not appreciably affected by common allosteric effectors such as GDP, PEP, F-2,6-P_2, citrate and succinate. ADP also had opposing effects on *Thermotoga* ATP-PFK activity as the enzyme was slightly activated at a low concentration of ADP and partially inhibited at higher concentrations, but the magnitude of these effects does not reflect allosteric control. Similar results concerning ADP-related effects have been reported for the ATP-PFK from the extremely thermophilic bacterium *Flavorbacter thermophilum* which has a valid name of *Thermus thermophilus* (Yoshida, 1972).

PEP is often a strong inhibitor of ATP-PFKs (Uyeda, 1979), but the *Thermotoga* enzyme displayed virtually no significant response even at very high concentrations (10 mM, not shown). The allosteric responses of the ATP-PFKs from *E. coli* and *B. stearothermophilus* are mediated by a glutamic acid residue at position 187 via the binding of PEP (Auzat et al., 1994b; Evans and Hudson, 1979). The sequence alignment shows that the *Thermotoga* ATP-PFK also possesses an identical glutamic acid residue (Glu^{-187}) but the biochemical properties from this work suggests that PEP is not vital for regulating the enzyme, and thus probably not glycolysis in this organism (Fig. 5.13). The strong inhibition by PP_i and polyP suggests that these effectors might replace, either partially or fully, the function of PEP and other

potential modulators within this organism. Although nonallosteric ATP-PFKs have also been reported from the archaeon *D. amylolyticus* (Hansen and Schönheit, 2000), the primitive eukaryote *T. brucei* (Michels et al., 1997), and the bacterium *L. bulgaricus* (Branny et al., 1993), the response of these enzymes to polyP has not been investigated.

The results presented here indicate that the control of the EM pathway in *Thermotoga* may be mediated by a quite different mechanism than that conventionally found, where the activity of ATP-PFK is allosterically controlled by either PEP, ADP AMP, F-2,6-P_2, citrate, succinate or a combination of these. The pH optimum for the ATP-PFK of *Thermotoga* is between 7.8-8.0 (which presumably reflects the intracellular pH of the organism). For ATP-PFK dependent glycolysis, the PP_i and polyP concentrations would have to remain low (<100 μM). If polyP accumulated and/or the pH fell, then the ATP-PFK would be inhibited and the PP_i-PFK activity would predominate. The PP_i-PFK catalyses a typically reversible reaction, but the pH optima for the forward and reverse reactions are unusually close with the *Thermotoga* enzyme in comparison to other PP_i-PFKs which actually have an acidic forward optimum and an slightly alkaline reverse optimum pH. Since no allosteric effector has been detected for this enzyme from bacteria and most primitive eukaryotes, the reaction mode will be dictated simply by the concentrations of intracellular metabolites.

As mentioned previously, polyP is regarded as being ubiquitously present in all organisms (Kornberg, 1994, 1995, 2000), and Kulaev (1979) has reports concentrations of between 10-100 μM/g dry weight in fourteen species of bacteria. PolyP concentrations at this level would affect glycolysis in *Thermotoga*.

Interestingly, no gene has been identified in the genome of *Thermotoga* encoding a polyP kinase, though in other organisms other enzymes have also been implicated in the synthesis of polyP, e.g. adenylate kinase in *Acinetobacter johnsonii* (Resnick and Zahnder, 2000). Possibly, the PP_i-PFK could produce polyP by means of the reverse reaction at intracellular pH values between pH 6.0 and 7.0 since FBPase gene has not been identified in *Thermotoga* genome sequence. Although the implications of possessing these enzymes are uncertain, it is likely that additional investigation of glycolysis and its control in this organism would be rewarding.

A phylogenetic tree constructed from published sequences of PFK genes (Fig. 5.13) indicates that the *Thermotoga* PP_i-PFK shares the highest degree of homology to the sequences from an amitochondriate flagellated protist, *T. vaginalis* and the mitochondriate, *N. fowleri,* and this is supported by high bootstrap values (Fig. 5.13). The origin of the *Thermotoga pfp* gene is of interest as some isolates of *Naegleria* have been obtained from hot pools. Although the PP_i-PFK from *Thermotoga* has some biochemical properties which are similar to the PP_i-PFK enzymes from these eukaryotes, e.g. an acidic pH optimum for the F-1,6-P_2 forming reaction and a homodimeric structure with individual subunit sizes of 45-48 kDa, the *N. fowleri* PP_i-PFK was sensitive to AMP (Mertens et al., 1993) whereas the *Thermotoga* enzyme is AMP insensitive. The homology of the *Thermotoga* PP_i-PFK to non-thermophilic eukaryotes is somewhat surprising since gene analysis of the *Thermotoga* genome indicates that the vast majority of homologies are with other thermophilic bacteria and archaea (Nelson et al., 1999). For example, of the 174 genes analysed which are involved with energy metabolism in *Thermotoga*, only one matched more closely to a gene from a eukaryotic source (Nelson et al., 1999). In contrast to the phylogeny of

the PP_i-PFK, the phylogeny of the ATP-PFK is more conventional and it groups with group I PFKs from other phylogenetically related thermophilic bacteria (Fig. 5.13).

In summary, *Thermotoga* appears to be unique in that it contains the genes for two distinct PFKs and both genes can express functional enzymes. Both enzymes have unique properties, in particular, their responses to PP_i and polyP. It is possible that PP_i and polyP may play a central role in the control of glucose metabolism in this organism.

CHAPTER EIGHT

GENERAL DISCUSSION AND CONCLUSION

It is generally believed that the last universal common ancestor of all extant life on this planet was a hyperthermophile (Woese et al., 1990; Doolittle, 1999). It follows, therefore, that an understanding of the biochemical characteristics of enzymes, and of their respective phylogenies, especially of the key enzymes in central metabolic pathways from hyperthermophilic microorganisms, is particularly important. The original hypothesis that guided this research was based on two concepts supporting the presence of a PP_i-PFK in the early development of glycolysis/gluconeogenesis metabolic pathways. The first concept was that in the glycolytic direction a PP_i-PFK would be able to utilise an energy source that was being constantly replenished in the environment (PP_i). Whereas ATP can be produced under simulated pre-biotic conditions, its relative instability at high temperature would have led to its supply being erratic and discontinuous. This would hardly have been conducive to the evolution of archaeal metabolic pathways. PP_i, on the other hand, is significantly more thermostable and would have been continuously present at low concentration (Yamagata et al., 1991; Russell and Hall, 1997). The second concept of the hypothesis guiding this thesis was that in the gluconeogenic direction, the use of a PP_i-PFK would have an enormous advantage because the reaction is more reversible under physiological conditions. In contrast, the ATP-PFK reaction is essentially irreversible. If an autotrophic origin of life is upheld, as advocated by Wächterhäuser (1990), then using a PP_i-PFK makes more metabolic sense.

PFKs with either PP_i or ATP as phosphoryl donors from three hyperthermophilic microorganisms have been studied in this thesis. The data from this study support the hypothesis that glycolysis was present in all current domains of life indicating that the origin of the pathway occurred prior to the last universal common ancestor (Fothergill-Gilmore and Michels, 1993).

Overall, the results of the biochemical study of PFKs from extremely thermophilic bacteria and archaea in this study showed that there were some significant similarities in the biochemical properties examined counterparts these extremely thermophilic and other mesophilic PFKs. For example, the requirement of Mg^{2+} for optimal PFK activity is common in all PFKs studied in this thesis and to PFKs in general (Uyeda, 1979; Johnson and Reinhart, 1992). It has also been found that KCl is an activator to all the enzymes studied in this thesis although the enzymes from different organisms had varying optimal concentrations of KCl. For example, the enzymes from *D. thermophilum* and *D. amylolyticus* required 10-50 mM of KCl but both the PP_i- and ATP-PFK from *T. maritima* required 150-175 mM. Strikingly, the apparent K_m values for the *T. maritima* PP_i-PFK activity were highly related to the concentration of KCl. In addition, the *Dictyoglomus* enzyme was found to be a dimer and to be nonallosteric, similar to most other PP_i-PFKs from bacteria and primitive eukaryotes (Morgan and Ronimus, 1998; Mertens, 1991, 1993).

Surprisingly, Cu^{2+} was found to be a potential toxin to these extremely thermophilic enzymes in this study. If this inhibition was common to other mesophilic and thermophilic PFKs, it might need further confirmation. Interestingly, the inhibition by Cu^{2+} from other enzyme activities has also been investigated in nitri-oxide synthase (Perry et al., 2000), cytochrome b6f complex (Rao et al., 2000) and the

glycolytic enzymes- triosephosphatase, hexokinase and muscle PFK (Jannasch et al., 1999; Lai and Blass, 1984).

The sequence alignments (Fig. 5.13) suggested that most PFKs, except *E. coli* PFK-2 (probably representing a novel or inducible group of PFKs) and ADP-PFK, have evolved from a common ancestral enzyme as they all possess the conserved binding sites for F-6-P. Three groups of PFKs were delineated from the 22 different amino acid phylogenetic tree (Fig. 5.14). Although a direct comparison cannot be made between the 16S rRNA and the PFK phylogenetic trees. Nonetheless, the PFK tree itself has some advantages. The lengths and relative positions of the lineages in the PFK phylogenetic tree could partially reflect both the enzyme functions and their origin. For example, while most ATP-PFKs, either thermophilic or mesophilic, are clustered in group I, PP_i-PFKs are located in both groups II and III. Group III PFKs including PP_i-PFKs from *T. tenax* and *D. thermophilum* have been suggested to be represent the most ancient lineage of PFK (Siebers et al., 1998). Strikingly, two ATP-PFKs, those from *T. brucei* and *S. coelicolor*, have been shown to have evolved from PP_i-PFKs in group II and III (Alves et al., 1997; Michels et al., 1997). In addition, the likelihood of a lateral gene transfer events, exemplified by the *T. maritima* PP_i-PFK, was also identified from the PFK tree and confirms the original identification proposed from the genome sequence data (Nelson et al., 1999). Lateral gene transfer is widely believed to have existed in bacteria and archaea and raises the interesting possibility that the *T. tenax* PP_i-PFK was obtained in this manner (Ralph and McClelland, 1994; Nelson et al., 1999; Ochman et al., 2000).

A. aeolicus is one of the earliest bacterial lineages to diverge, and is one of the most thermophilic bacteria known. It is a strictly autolithotrophic bacterium and grows on

hydrogen, oxygen, CO_2 and mineral salts. *A. aeolicus* represents the most deeply branching organism within the bacterial domain on the basis of phylogenetic analysis of 16S rRNA sequence, although analyses of individual protein sequences vary in their placement of *Aquifex* relative to other groups. The genome sequence indicates that an ATP-PFK gene is present in this organism. The phylogenetic position of the *A. aeolicus* ATP-PFK is highly related to other thermophilic ATP-PFKs from *T. maritima* and *T. thermophilus*, which are clustered in PFK group I (Fig. 5.14). It is presently unknown whether this gene encodes a functional enzyme or not as this organism does not grow on a number of organic substrates including sugars, amino acids, yeast extract or meat extract (Decker et al., 1998).

Utilisation of PP_i, PPP_i and polyP as an energy source for the growth of microorganisms has also been investigated since the 1960s (Harold, 1966; Liu et al., 1982; Cruden et al., 1983; Peck et al., 1983; Patterson, 1989). The results from those studies suggest that microoganisms capable of utilising PP_i as a source of energy for growth are widely distributed in nature and are not limited to the sulfate reducing bacteria. The ability of the *D. thermophilum* and *T. maritima* PP_i-PFKs to utilise PP_i, PPP_i and polyP is potentially relevant with regard to the origins of PFK activity. From energetic aspects, there is no reason to exclude PP_i or polyP as a source of energy and it has been proposed that PP_i or polyP is an evolutionary precursory of ATP (Lipmann, 1965). The first indirect evidence that PP_i could serve as a source of energy was the observation of Baltscheffsky (1996) that PP_i synthesis is coupled to electron transfer in photosynthetic bacteria. In addition, the using of PP_i as energy substrate enables the organism to have a higher energy efficiency, assuming that the PP_i utilised is a by-product of biosynthetic reactions and is otherwise wastefully hydrolysed by pyrophosphatase. From this standpoint, the organism could have a

significant energetic advantage in the production of ATP when the ATP is produced by glycolysis only.

Significantly, polyP has been used as an alternate phosphoryl/energy source to ATP for other enzymes involved with glucose metabolism (Hsieh et al., 1993; Uryso and Kulaev, 1968; Wood and Clark, 1988). Compared to the *Dictyoglomus* and *Spirochaeta* enzymes (Ding et al., 1999; Ronimus et al., 1999), the *Thermotoga* PP_i-PFK has a striking preference for longer chain phosphoester substrates (the decline in apparent K_m values with the increasing chain length of polyP), and the enzyme could be more correctly described as a polyP-PFK.

FUTURE RESEARCH

On a personal level, the discovery of the unusual regulation of the *T. maritima* PP_i-PFK by PP_i and polyP has driven myself to investigate further the global functions of gene expression suing DNA microarray technique and effects of these inorganic phosphates on other enzyme activities from another thermophilic archaeon *Methanosarcina thermophila.* This is to be undertaken in a postdoctoral position over the next three years in Professor J. G. Ferry's laboratory in the Department of Biochemistry and Molecular Biology, Pennsylvania State University, U.S.A.

Appendix A
MEDIA FOR MICROORGANISMS

The composition of each medium was originally from either ATCC, DSM or TRUCC. The preparations of all media used in this study followed exactly the protocols described by these institutions.

a. Media for nonthermophilic bacteria

1. Medium for *S. alkalica*

Na_2CO_3	10.0	g
$NaHCO_3$	15.0	g
NaCl	10.0	g
K_2HPO_4	0.2	g
NH_4Cl	1.0	g
KCl	0.2	g
$Na_2S \cdot 9H_2O$	1.0	g
Yeast extract	0.5	g
Sucrose	5.0	g
Vitamin solution (Wolin's vitamins)*	10.0	ml
Trace element solution (DSM 141)**	1.0	ml
Distilled water (Final volume)	1000.0	ml

***Vitamin solution:**

Biotin	2.0	mg
Folic acid	2.0	mg
Pyridoxine-HCl	10.0	mg
Thiamine-HCl	5.0	mg
Riboflavin	5.0	mg
Nicotinic acid	5.0	mg
DL-calcium pantothenate	5.0	mg
Vitamin B_{12}	0.1	mg
p-Aminobenzoic acid	5.0	mg
Lipoic acid	5.0	mg
Distilled water	1000.0	ml

**** Trace element solution (DSM medium 141):**

Nitrilotriacetic acid	1.50	g
$MgSO_4 \cdot 7H_2O$	3.00	g

$MnSO_4 \cdot 2H_2O$	0.50 g
NaCl	1.00 g
$FeSO_4 \cdot 7H_2O$	100.0 mg
$CoSO_4 \cdot 7H_2O$	180.0 mg
$CaCl_2 \cdot 2H_2O$	100.0 mg
$ZnSO_4 \cdot 7\ H_2O$	180.0 mg
$CuSO_4 \cdot 5H_2O$	10.0 mg
$KAl(SO_4)_2 \cdot 12H_2O$	20.0 mg
H_3BO_3	10.0 mg
$Na_2MoO_4 \cdot 2H_2O$	10.0 mg
$NiCl_2 \cdot 6H_2O$	30.0 mg
$Na_2SeO_3 \cdot 5H_2O$	0.3.0 mg
Distilled water (Final volume)	1000.0 ml

The nitrilotriacetic acid was dissolved, the pH adjusted to 6.5 with KOH, then minerals added. The final pH of the medium was adjusted at 7.0 with either 1.0 N HCl or 1.0 N NaOH.

2. Medium for *S. halophila*

Peptone	2.0 g
Yeast extract	4.0 g
Inorganic salts solution*	980.0 ml

***Inorganic salts solution:**

$CaCl_2 \cdot 2H_2O$	1.3 g
NaCl	43.8 g
$MgSO_4 \cdot 7\ H_2O$	49.4 g
Distilled water (Final volume)	1000.0 ml

The pH was adjusted to 7.5 with KOH and sterilised by autoclaving at 121°C for 15 min. After sterilisation, the filter-sterilised (0.2 μm) 25% maltose was added to a final concentration of 0.5% (20 ml of a 25% maltose solution to 980 ml of medium). The salts were added in the order listed to avoid the formation of a precipitate.

3. Medium for *S. isovalerica*

Glucose	2.0	g
Trypticase	1.0	g
Yeast extract	1.0	g
L-cysteine-HCl	0.5	g
0.2% Resazurin	1.0	ml
Tris-HCl buffer (5.0 mM, pH 7.5)	250.0	ml
Seawater	750.0	ml
Distilled water	200.0	ml

The final pH was adjusted to 7.5. The tubes and serum bottles were capped with rubber stoppers under 97% N_2 and 3% H_2.

4. Medium for (DSM 173) *S. litoralis*

Glucose	2.0	g
Trypticase	1.0	g
Yeast extract	1.0	g
L-cysteine HCl	0.5	g
0.2% Resazurin	1.0	ml
I.0 M Tris-HCl buffer (pH 7.5)	50	ml
Seawater	750.0	ml
Distilled water	250.0	ml

The medium was adjusted to final pH 7.2 after autoclaving and dispensed anaerobically under 100% N_2 atmosphere.

5. Medium (DSM 169) for *S. zuelzerae*

Solution 1:

KH_2PO_4	0.75	g
$NaH_2PO_4 \cdot H_2O$	0.25	g
L-cysteine-HCl	0.5	g
Distilled water	480.0	ml
pH 7.2		

Solution 2:

Yeast extract	0.2	g
NH_4Cl	1.0	g

$MgSO_4 \cdot 7H_2O$	0.5	g
$CaCl_2 \cdot 2H_2O$	0.02	g
0.2% Resazurin	1.0	ml
$FeCl_3 \cdot 6H_2O$ (250 mg/L)	10	ml
Trace element solution*	2	ml
Distilled water	480	ml

Solution 3:

$NaHCO_3$	5%

Solution 4:

Glucose	10%

***Trace element solution:**

H_3BO_3	56.0	mg
$ZnSO_4 \cdot 7H_2O$	44.0	mg
$CoCl_2 \cdot 6H_2O$	20.0	mg
$CuSO_4 . 5H_2O$	2.0	mg
$MnCl_2$	2.0	mg
$Na_2MoO_4 \cdot 2H_2O$	75.0	mg
Distilled water	100	ml

Solutions 1 and 2 were autoclaved at 121°C for 15 min, separately. Solutions 3 and 4 were filter-sterilised (0.2 μm). Solution 1 (480.0 ml) was mixed with Solution 2 (480.0 ml) under 80% N_2 and 20% CO_2. While gassing, 20.0 ml of $NaHCO_3$ solution and 20.0 ml of glucose solution were added anaerobically and aseptically. The pH of the medium was adjusted to 7.2 with either 1.0 N HCl or 1.0 N NaOH.

6. Medium for *S. thermophila* Rt118 B.1 and GAB 76

$(NH_4)_2SO_4$	0.5	g
KH_2PO_4	0.28	g
$MgSO_4 \cdot 7H_2O$	0.25	g
$CaCl_2 \cdot 2H_2O$	0.07	g
Yeast extract	0.5	g
Tryptone	0.5	g
Maltose	3.0	g
HEPES buffer	2.4	g

0.2% Resazurin	1.0	ml
Distilled water	100.0	ml
pH	7.2	

7. Medium for *T. bryantii*

Rumen fluid*	30.0	ml
Glucose	0.05	g
Maltose	0.05	g
Soluble starch	0.05	g
Peptone	0.05	g
Yeast extract	0.05	g
$(NH_4)_2SO_4$	0.05	g
0.2% Resazurin	1.0	ml
L-cysteine-HCl	50.0	mg
Salts solution**	50.0	ml
Distilled water	20.0	ml
pH	7.0	

***Rumen fluid:**

The fresh rumen fluid obtained from Dairying Research Company (Hamilton, New Zealand) was filtered through two layers of cheese cloth to remove large particles and centrifuged at 11,000 x *g* at 4°C for 40 min. The clear supernatant was used for medium preparation.

****Salts solution:**

$CaCl_2 \cdot 2H_2O$	0.2	g
$MgSO_4 \cdot 7H_2O$	0.2	g
KH_2PO	1.0	g
$NaHCO_3$	10.0	g
NaCl	2.0	g

$CaCl_2 \cdot 2H_2O$ and $MgSO_4 \cdot 7H_2O$ were dissolved in 300 ml of distilled water, then water (500 ml) and remaining salts were added while swirling slowly. Another aliquot of water (200.0 ml) was added and the solution stored at 4°C. All of the ingredients were mixed in an Erlenmeyer flask. The medium was boiled for 10-20 min until medium changed from pink to yellowish. The L-cysteine was added when the medium was cold. The pH of medium was adjusted to 7.0 with either 1.0 N NaOH or 1.0 N HCl.

8. Medium for *T. denticola*

Brain heart infusion broth	12.5	g
Trypticase	10.0	g
Yeast extract	2.5	g
Sodium thioglycollate	0.5	g
L-cysteine-HCl	1.0	g
L-asparagine	0.3	g
Glucose	2.0	g
Distilled water (Final volume)	1000.0	ml

Supplements:

0.2% (wt/vol) Thiamine pyrophosphate	3.0	ml
Volatile fatty acids*	2.0	ml
10% Sodium bicarbonate	20.0	ml
Rabbit serum (filter-sterilised)	20.0	ml

Volatile fatty acids:

Isobutyric acid	0.5	ml
D,L-2-methylbutyric acid	0.5	ml
Isovaleric acid	0.5	ml
Valeric acid	0.5	ml
0.1 N KOH	100.0	ml

All the supplements were filter-sterilized (0.2 μm). The medium was prepared and dispensed anaerobically under an atmosphere of 80% N_2, 10% H_2 and 10% CO_2. The pH of medium was adjusted to 7.0 with 1.0 N NaOH or 1.0 N HCl.

9. Medium for *T. pectinovorum*

Pectin	0.8	g
KH_2PO_4	2.0	g
Polypeptone	5.0	g
Heart infusion broth	5.0	g
Yeast extract	5.0	g
$(NH_4)_2SO_4$	2.0	g
L-cysteine-HCl	0.7	g
Distilled water	500.0	ml

Rumen fluid	500.0	ml
0.2% Resazurin	1.0	ml
pH	7.0-7.2	

The medium was prepared anaerobically and inoculated under an atmosphere of 90% N_2 and 10% CO_2. The pH of medium was adjusted to 7.0-7.2 with either 1.0 N NaOH or 1.0 N HCl.

10. Medium for *L. biflexa*

Peptone	0.3	g
Beef extract	0.2	g
NaCl	0.5	g
Sterile rabbit serum	100.0	ml
0.05% Hemin*	2.5	ml
Distilled water	900.0	ml
pH	7.3	

*The hemin was dissolved in 0.5-1.0 ml NaOH and then 100.0 ml of distilled water was added, then sterilised at 121°C for 15 min. The pH of medium was adjusted to 7.3 with either 1.0 N NaOH or 1.0 N HCl.

11. Luria Bertani (LB) broth for *E. coli*

NaCl	10.0	g
Tryptone	10.0	g
Yeast extract	5.0	g
pH	7.2	

The ingredients were mixed in 1,000.0 ml of distilled water. Sterilised by autoclaving at 121°C for 20 min. The pH of medium was adjusted to 7.0 with either 1.0 N NaOH or 1.0 N HCl.

b. Media for thermophilic bacteria

12. Medium for either *C. fervidus* or *F. nodosum*

Solution 1:

NH_4Cl	0.9	g
$MgCl_2 \cdot 6H_2O$	0.2	g

KH_2PO_4	0.75	g
K_2HPO_4	1.5	g
0.2% Resazurin	1.0	ml
Trace element solution*	9.0	ml
10% $FeSO_4 \cdot 7H_2O$	0.03	ml
Vitamin solution (same as Medium 1)	5.0	ml
Trypticase peptone	10.0	ml
Distilled water	850.0	ml

Solution 2:

Yeast extract	3.0	g
Distilled water	100	ml

Solution 3:

Glucose	5.0	g
Distilled water	50.0	ml

Solutions 1 and 2 were autoclaved separately at 121°C for 45 min. Solution 3 and the vitamin solution were filter-sterilised. The pH of medium was adjusted to 7.0 with 1.0 N NaOH or 1.0 N HCl.

***Trace element solution:**

H_3BO_3	10.0	mg
$ZnCl_2$	100.0	mg
$CoCl_2 \cdot 6H_2O$	17.0	mg
$CuCl_2$	20.0	mg
$MnCl_2 \cdot 4H_2O$	100.0	mg
$Na_2MoO_4 \cdot 2H_2O$	10.0	mg
$FeCl_3$	20.0	mg
$CaCl_2 \cdot 2H_2O$	100.0	mg
NaCl	1000.0	mg
$Na_2SeO_3 \cdot 5H_2O$	20.0	mg
Distilled water	1000.0	ml

13. Medium for *C. saccharolyticus*

NaCl	0.9	g
NH_4Cl	0.9	g
$MgCl_2 \cdot 6H_2O$	0.2	g
KH_2PO_4	0.8	g

K_2HPO_4	1.5	g
Yeast extract	1.0	g
L-cysteine·HCl	0.75	g
Cellobiose	1-2.0	g
0.2% Resazurin	1.0	ml
SL 10 Trace element solution*	9.0	ml
0.28% $FeCl_2 \cdot 4H_2O$	1.0	ml
Wolin's vitamin solution	5.0	ml
Trypticase peptone	2.0	ml
Selenite/tungstate**	1.0	ml
pH	7.0	

***SL 10 Trace elements solution:**

H_3BO_3	6.0	mg
$ZnCl_2$	70.0	mg
$CoCl_2 \cdot 6H_2O$	190.0	mg
$Cu\ Cl_2$	2.0	mg
$Na_2MoO_4 \cdot 2H_2O$	36.0	mg
$FeCl_3 \cdot 6H_2O$	1.5	mg
$CaCl_2 \cdot 2H_2O$	100.0	mg
$MnCl_2 \cdot 4H_2O$	100.0	mg
$NiCl_2 \cdot 6H_2O$	24.0	mg
HCl (25%, 7.7 M)	10.0	ml
Distilled water	990.0	ml

$FeCl_2 \cdot 4H_2O$ was dissolved in the HCl then diluted in water.

****Selenite/tungstate:**

NaOH	0.5	g
$Na_2SeO_3 \cdot 5H_2O$	3.0	mg
$Na_2WO_4 \cdot 2H_2O$	4.0	mg

14. Medium for *D. thermophilum* and *Thermococcus* strain Wai 21 S1

KH_2PO_4	1.5	g
$Na_2HPO_4 \cdot 12H_2O$	4.2	g
NH_4Cl	0.5	g
$MgCl_2 \cdot 6H_2O$	0.4	g
Na_2CO_3	1.0	g

Yeast extract	2.0	g
Trypticase peptone	2.0	g
Soluble starch	5.0	g
L-cysteine-HCl	1.0	g
$Fe(NH_4)(SO_4)_2 \cdot 6H_2O$	40	mg
$CaCl_2 \cdot 2H_2O$	50	mg
Dictyoglomus trace element solution*	1.0	ml
Wolin's vitamin solution	1.0	ml
0.2% Resazurin	1.0	ml
Distilled water	1000.0	ml
pH	7.2	

***_Dictyoglomus_ trace element solution:**

$CoCl_2 . 6H_2O$	2.9	mg
$Na_2MoO_4 . 2H_2O$	2.4	mg
$Na_2SeO_3 . 5H_2O$	0.2	mg
$MnCl_2 . 4H_2O$	2.0	mg
$ZnSO_4$	2.8	mg

15. Medium for *T. maritima*

Soluble starch	5.0	g
KH_2PO_4	0.5	g
NaCl	20.0	g
Yeast extract	0.5	g
$Na_2S \cdot 9H_2O$	0.5	g
$NiCl_2 \cdot 6H_2O$	2.0	mg
0.2% Resazurin	1.0	ml
*Trace elements solution (DSM 141)	15.0	ml
Sea water	250.0	ml
Distilled water	750.0	ml
pH	6.5	

The gas atmosphere was 100% N_2

c. Media for archaea

15 Medium for *T. zilligii*

K_2HPO_4	1.5	g
$MgCl_2 \cdot 6H_2O$	0.3	g

NaCl	2.5	g
Trypticase	8.0	g
Sodium thioglycollate	0.5	g
Sulphur, powdered	2.0	g
Wolin's vitamins	1.0	ml
Zeikus trace elements*	5.0	ml
0.2% Resazurin	1.0	ml
Distilled water (Final volume)	1000.0	ml
pH	7.4	

***Zeikus trace element solution:**

$FeCl_3$	200.0	mg
$MnCl_2 \cdot 4H_2O$	100.0	mg
$CoCl_2 \cdot 6H_2O$	17.0	mg
$CaCl_2 \cdot 2H_2O$	100.0	mg
$ZnCl_2$	100.0	mg
$CuCl_2$	20.0	mg
H_3BO_3	10.0	mg
$Na_2MoO_4 \cdot 2H_2O$	10.0	mg
$Na_2SeO_3 \cdot 5H_2O$	20.0	mg
NaCl	1000.0	mg
Distilled water	990.0	ml

16. Medium for *D. amylolyticus*

NH_4Cl	0.33	g
KH_2PO_4	0.33	g
KCl	0.33	g
$CaCl_2 \cdot 2H_2O$	0.44	g
$MgCl_2 \cdot 6H_2O$	0.44	g
NaCl	0.5	g
$NaHCO_3$	0.8	g
$Na_2S \cdot 9H_2O$	0.5	g
SL-10 trace element solution	1.0	ml
Wolin's vitamin solution	1.0	ml
0.2% Resazurin	1.0	ml
Starch or trypticase peptone	5.0	g
Yeast extract	0.2	g
Sulfur, powdered	10.0	g

pH	6.2-6.4

The medium was prepared anaerobically under 80% N_2 + 20% CO_2 gas phase

Appendix B.1

Programme #2 for SDS-PAGE with PhastGel Gradient 10-15

Sample	Application	Down	at 1.1	1 Vh	
Sample	Application	Up	at 1.1	10 Vh	
Sep 1.1	250 V	10.0 mA	3.0 W	15°C	60 Vh
Sep 1.2	50 V	0.1 mA	0.5W	15°C	0 Vh

Appendix B.2

Programme #4 for IEF with PhastGel Gradient IEF 3-9

Sample	Application	Down at	1.2	0 Vh	
Sample	Application	Up at	1.3	0 Vh	
Extra alarm to sound at 1.1		73 Vh			
Sep 1.1	2000 V	2.5 mA	3.5 mA	15°C	75 Vh
Sep 1.2	200 V	2.5 mA	3.5 mA	15°C	15 Vh
Sep 1.3	2000 V	2.5 mA	3.5 mA	15°C	410 Vh

Appendix B.3

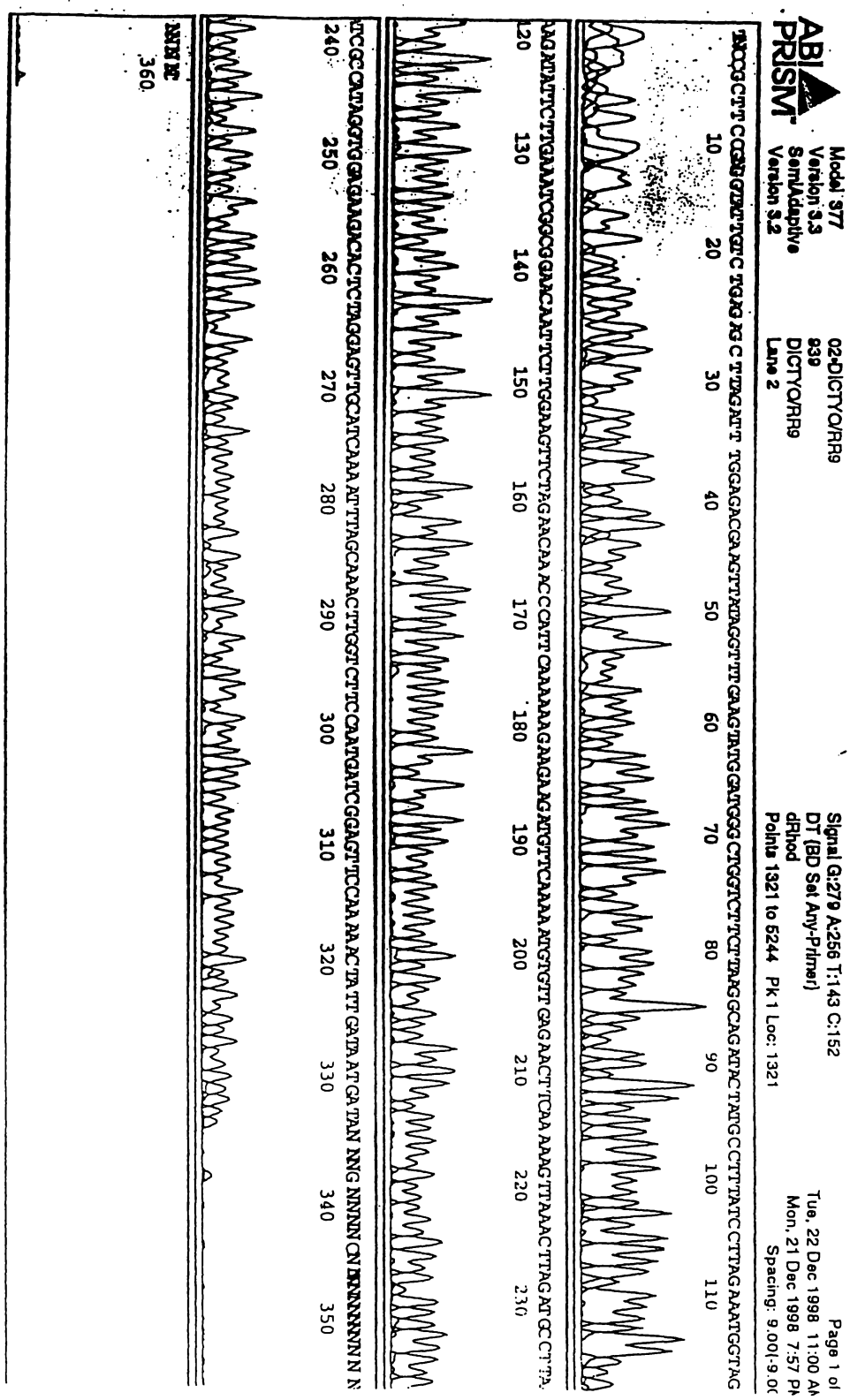
Protocol of miniprep plasmid purification from Wizard *Plus* SV Minipreps DNA purification Systems

1. Harvest 1-5 ml of high copy number plasmid or 10 ml of low copy number plasmid of bacterial culture by centrifugation for 5 min at 14,000 x *g* in a table-top centrifuge. Pour off the supernatant and blot the inverted tube on a paper towel to remove excess media.
0. Add 250 µl of Cell Resuspension Solution and completely re-suspend the cell pellet by vortexing well or pipetting.
3. Add 250 µl of Cell Lysis Solution and mix by inverting the tube 4 times (do not vortex). Incubate until the cell suspension clears, approximately 1-5 min.
4. Add 10 µl of Alkaline Protease Solution and mix by inverting the tube 4 times. Incubate for 5 min at RT.
5. Add 350 µl of Wizard Plus SV Neutralisation Solution and immediately mix by inverting the tube 4 times (do not vortex).
6. Centrifuge the bacterial lysate at 14,000 x *g* in a microcentrifuge for 10 min at RT.
7. Transfer the cleared lysate (approximately 850 µl) to the prepared Spin Column by decanting. Avoid disturbing or transferring any of the white precipitate with the supernatant.

8. Centrifuge the supernatant at 14,000 x *g* in a microcentrifuge for 1 min at RT. Remove the Spin Column from the tube and discard the flowthrough from the Collection Tube. Reinsert the Spin Column into the Collection Tube.
9. Add 750 ml of Column Wash Solution, previously diluted with 95% ethanol, to the Spin Column.
10. Centrifuge at 14,000 x *g* in a microcentrifuge for 1 min at RT. Remove the spin Coumn from tube and discard the flowthrough. Reinsert the Spin Column into the Collection Tube.
11. Repeat the wash procedure using 250 µl of Column Wash solution.
12. Centrifuge at 14,000 x *g* in a micrcentrifuge for 2 min at RT.
13. Transfer the Spin Column to a new sterile 1.5 ml microcentrifuge tube being careful not to transfer any of the Column Wash Solution with the Spin Column.
14. Elute the plasmid DNA by adding 100 µl of Nuclease-Free Water to the Spin Column. Centrifuge at 14,000 x *g* for 1 min at RT in microcentrifuge.
15. DNA is store at 4°C in TE buffer.
16. Cap the microcentrifuge tube and store the purified plasmid DNA at -20°C

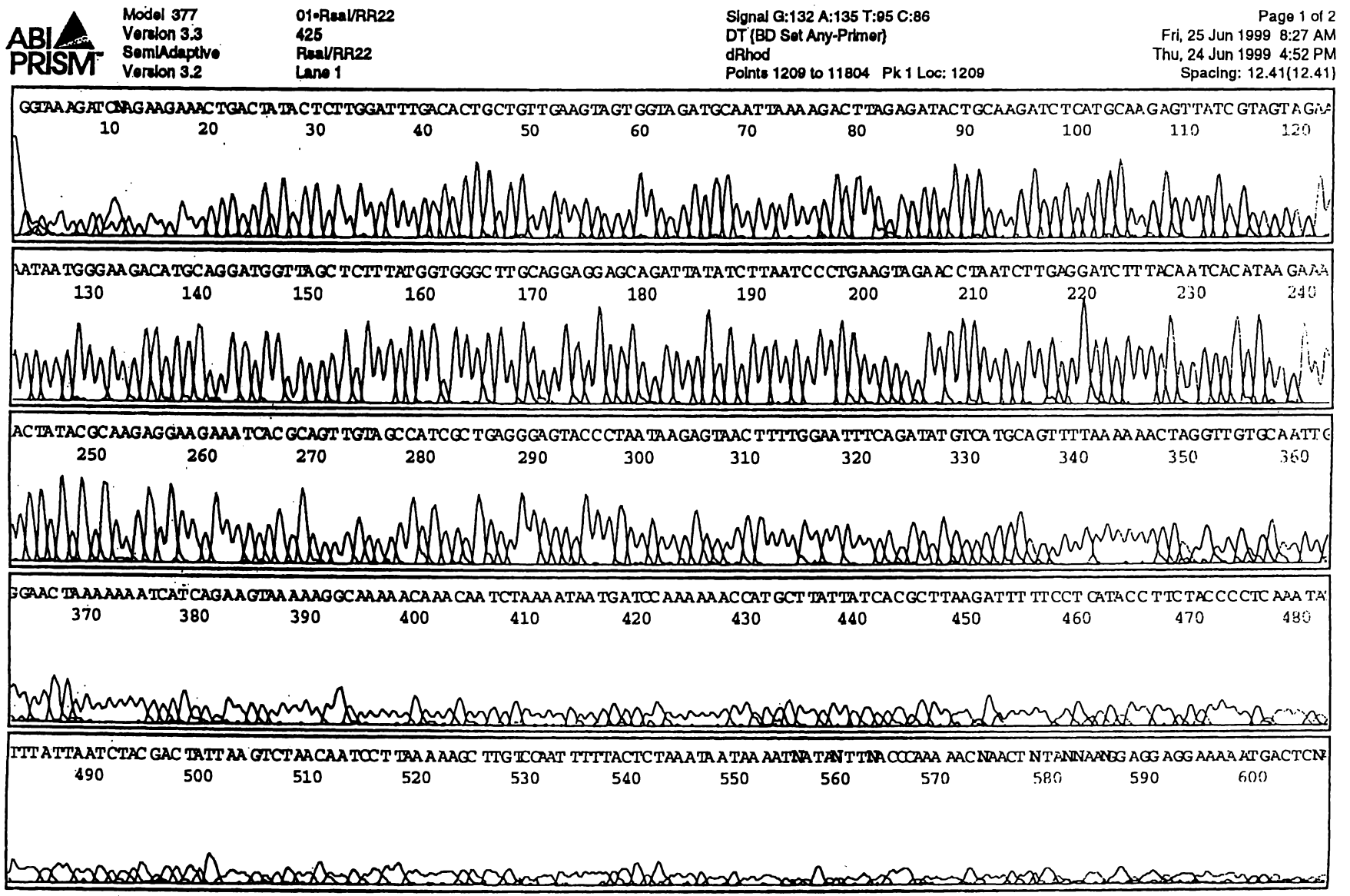
Appendix B.4

Sequences of PCR and inverse-PCR for *Dictyoglomus pfp*



Sequence from a 350 bp PCR fragment to be used for probe

Sequence from a 360 bp inverse-PCR fragment after *Sau* 3A I digestion



Sequence from a 750 bp inverse-PCR fragment after *Rsa* I digestion

Sequence from a 2,100 bp inverse-PCR fragment after *Sau* 96 I digestion

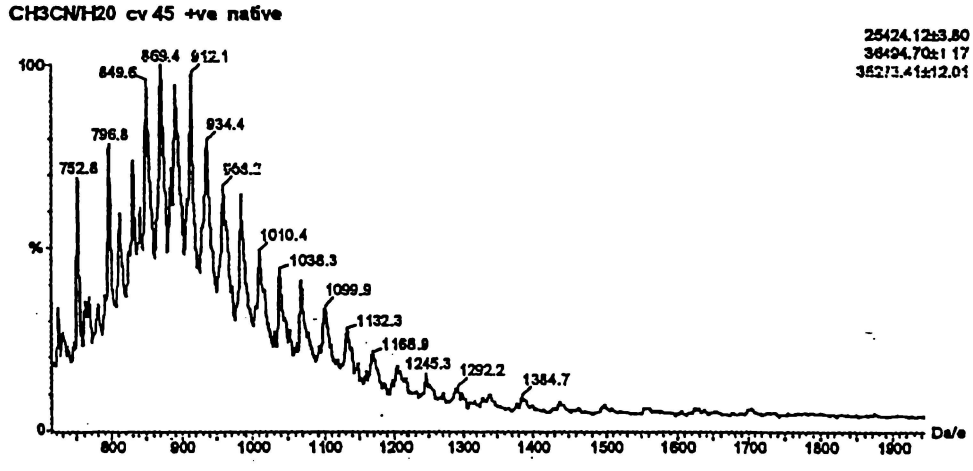
Sequence from N-terminal amplification of the *Dictyoglomus pfp* gene

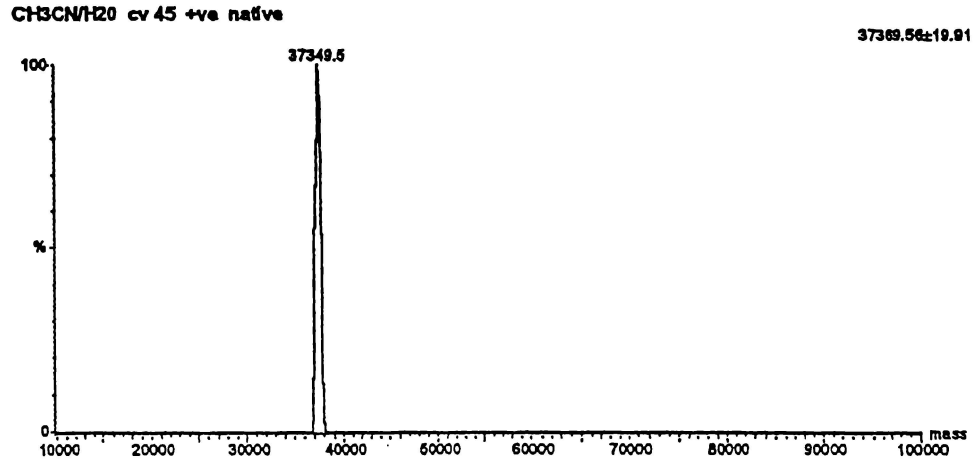
Sequence from C-terminal amplification of the *Dictyoglomus pfp* gene

Appendix B.5

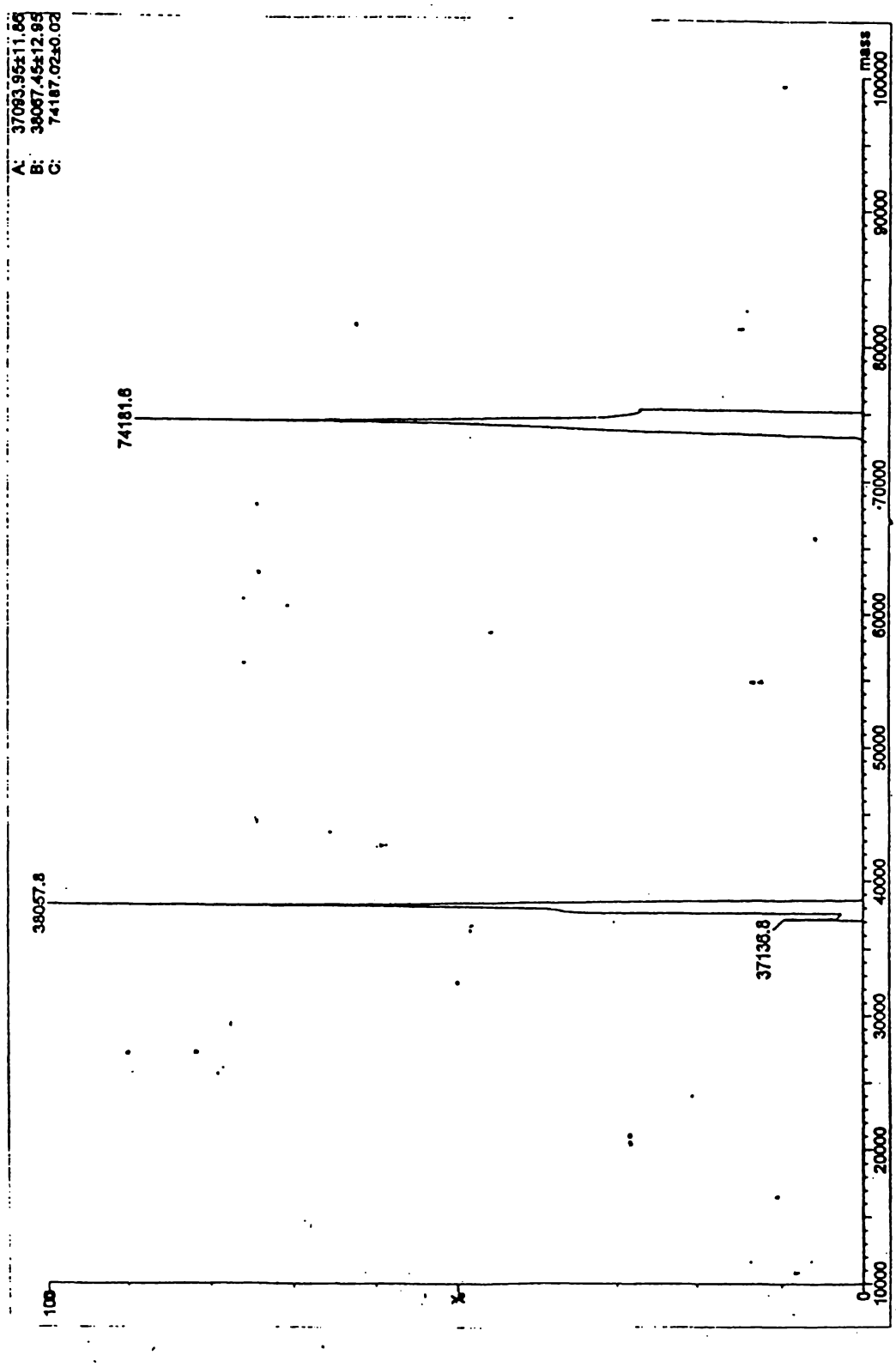
Mass spectrometry for both the native and the recombinant PP_i-PFKs of *D. thermophilum*

The native molecular mass from the *D. thermophilum* PP_i-PFK





The recombinant molecular mass from the *D. thermophilum* PP_i-PFK



Appendix C
GENE SEQUENCES OF *THERMOTOGA pfp* AND *pfk*

C.1 The nucleotide and peptide sequences from *Thermotoga* PP_i-PFK

Nucleotide sequence:

GTGGCTGAAAGATTGGGGATACTCGTTGGTGGAGGGCCGGCACCTGGAATCAACAGTGTG

ATAAGTTCTGTTACCATCGAAGCTATAAACAACGGACTCGAAGTGATCGGAATATACGAT

GGATTCAAACACCTCGTAGAGGGAAAAACGAATATGGTGAAAAAACTTTCCATCGAAGAT

GTCTCCAGAATCCACATCGAAGGTGGATCCATTCTCAGAACCTCGAGGGTGAATCCTGCC

AAATCCGAAGAAACCCTCGAGAAGACAGTTCAGACTCTCAAAAAGCTCGGGATAAAGTAT

CTTGTTACTATCGGCGGGGACGACACCGCGTTCTCCGCGAGCAAAGTGTGTGAAAGATCG

AAGGGCGAAATAAAGGTGGTCCACGTTCCAAAGACTATAGACAACGATCTCCCTCTTCCG

GAGAACATGCCCACGTTCGGTTTCGAAACAGCTCGTCACGTAGCCACAGAGCTCGTCTAC

AATCTCATGCAAGACTCAAGAACAACGAACAGGTGGTACTTTGTGGCCATGATGGGAAGA

GAAGCCGGGCACCTCGCTCTCGGCGTTGGAAAAGCGGCGAGTGCAACGATCACGATCATT

CCTGAAGAGTTCAAAGAGGGAGTGACGCTCGAAGAAGTGTGCGACGTTCTTGATGGAGCG

ATTCTCAAGAGAAAACTCATGGGAAGAGACGATGGAGTGGCGGTCATAGGTGAAGGTATC

GCAGAAAAGATGGATCCTGAAGAACTCGCCAACATTCCCGGTGTGATAGTCGAAAAAGAC

CCACACGGACACCTGAGACTTGCAGAGATCCCGCTCGCCACCATTCTGAAACGGGCGATC

GAGAGGAGATACGCTGAAAGAGGTGAAAGAATCCACATCGTCGATGTCACAATAGGATAC

GAACTCAGAAGCGCACGCCCTATACCTTTCGACATAGTATACACCAGAACACTCGGTTAC

GGTGCAGTGAGATTTCTCCTTGGAGACTATTCCGATCTCCCTGGCGGGATGGTGTGCGTG

GTTGGTGGCAGAATCAAGATTCTTCCCTTCGACGCTTTCATGGATCCAAAGACCGGAAGG

ACAAAGGTGAGAGTGGTGGATGTTCGATCGGAAGATTACAGAGTAGCAAGAAAATACATG

ATAAGGCTGGAAAAGAAAGATCTCGAAGATCCCGAAACTCTCGAAAAACTAGCAAAGCTC

GCAAAGATGGAACCTGAAGAATTCAAAAAGAAATACTGGCATACGACAGAGCTTCCA

Amino acid sequence:

MAERLGILVGGGPAPGINSVISSVTIEAINNGLEVIGIYDGFKHLVEGKTNMVKKLSIED

VSRIHIEGGSILRTSRVNPAKSEETLEKTVQTLKKLGIKYLVTIGGDDTAFSASKVCERS

KGEIKVVHVPKTIDNDLPLPENMPTFGFETARHVATELVYNLMQDSRTTNRWYFVAMMGR

EAGHLALGVGKAASATITIIPEEFKEGVTLEEVCDVLDGAILKRKLMGRDDGVAVIGEGI

AEKMDPEELANIPGVIVEKDPHGHLRLAEIPLATILKRAIERRYAERGERIHIVDVTIGY

ELRSARPIPFDIVYTRTLGYGAVRFLLGDYSDLPGGMVCVVGGRIKILPFDAFMDPKTGR

TKVRVVDVRSEDYRVARKYMIRLEKKDLEDPETLEKLAKLAKMEPEEFKKKYWHTTELP

C.2 The nucleotide and peptide sequences from *Thermotoga* ATP-PFK

Nucleotide sequence:

TTGAAGAAGATAGCAGTACTTACAAGCGGCGGAGACGCACCTGGAATGAACGCAGCTGTG
AGAGCCGTGGTCAGGTACGGTGTCAGGCAGGGACTGGAAGTGATCGGAGTGAGAAGAGGT
TACTCAGGCCTCATCGACGGCGATTTTGTAAAACTCGAGTACAAAGATGTGGCAGGAATC
ACAGAAAAGGGAGGAACAATTCTGAGAACTTCCAGATGTGAGGAGTTCAAGACAGAAGAG
GGCAGGGAACTCGCTGCGAAACAGATAAAAAAACATGGTATAGAAGGACTCGTCGTCATA
GGTGGTGAAGGGAGTCTCACCGGCGCTCATCTTCTTTACGAAGAACACAAAATACCCGTT
GTCGGTATACCAGCAACCATAGACAACGACATTGGGTTGACTGACATGTGCATAGGGGTG
GACACGTGTTTGAACACGGTGATGGATGCTGTTCAAAAGCTCAAAGACACCGCTAGCTCG
CATGAGAGAGCTTTCATTGTGGAAGTCATGGGGAGGCATTCCGGCTACATCGCTCTCATG
GCGGGACTGGTGACTGGTGCAGAAGCCATCATCGTACCAGAGATTCCGGTGGATTATTCA
CAGCTCGCCGATAGAATTCTCGAAGAAAGGAGAAGAGGAAAGATCAACAGCATAATCATA
GTCGCTGAAGGGGCAGCGAGTGCCTATACCGTCGCAAGACACCTCGAATACAGGATAGGC
TACGAAACGAGGATCACCATACTCGGACACGTACAGAGAGGTGGTTCTCCAACGGCTTTC
GACAGAAGACTGGCACTGAGTATGGGAGTTGAAGCGGTCGATGCTCTTCTGGACGGAGAG
GTAGACGTGATGATAGCGCTCCAAGGGAACAAGTTCGTGAGAGTTCCTATAATGGAAGCG
CTCTCCACAAAGAAAACGATCGACAAAAAACTTTACGAAATAGCACATATGCTTTCA

Amino acid sequence:

MKKIAVLTSGGDAPGMNAAVRAVVRYGVRQGLEVIGVRRGYSGLIDGDFVKLEYKDVAGI
TEKGGTILRTSRCEEFKTEEGRELAAKQIKKHGIEGLVVIGGEGSLTGAHLLYEEHKIPV
VGIPATIDNDIGLTDMCIGVDTCLNTVMDAVQKLKDTASSHERAFIVEVMGRHSGYIALM
AGLVTGAEAIIVPEIPVDYSQLADRILEERRRGKINSIIIVAEGAASAYTVARHLEYRIG
YETRITILGHVQRGGSPTAFDRRLALSMGVEAVDALLDGEVDVMIALQGNKFVRVPIMEA
LSTKKTIDKKLYEIAHMLS

Appendix D

PROTOCOL OF NI-NTA RESIN REGENERATION

The Ni-NTA resin is regenerated for either different recombinant proteins or after 5 purifications for a particular protein. The protocol for regeneration of this resin is described as follows:

2 volumes of 6 M guanidine hydrochloride/0.2 M acetic acid

2 volumes of MilliQ water

3 volumes of 2% ethanol

1 volume of 25% ethanol

1 volume of 50% ethanol

1 volume of 75% ethanol

5 volumes of 100% ethanol

1 volume of 75% ethanol

1 volume of 50% ethanol

1 volume of 25% ethanol

1 volume of MilliQ water

5 volumes of 100 mM EDTA, pH 8.0

2 volumes of MilliQ water

2 volumes of 100 mM $NiSo_4$

2 volumes of MilliQ water

2 volumes of 6 M guanidine hydrochloride/0.2 M acetic acid

The resin is equilibrated with starting buffer as described in the manufacturer's instructions.

Appendix E.1-20
Determinations of kinetic parameters from both the *Thermotoga* PP_i- and ATP-PFKs

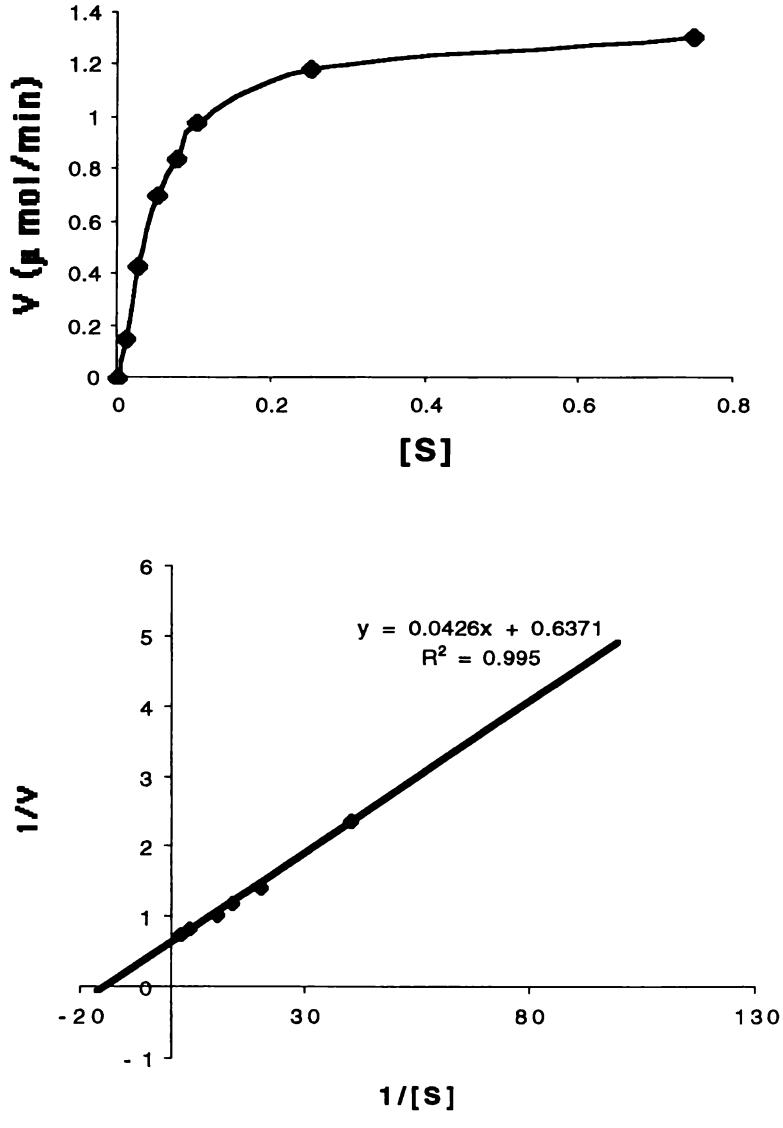


Figure E.1 Double reciprocal (1/*v* versus 1/[S]) Lineweaver-Burk plots for determination of the apparent K_m value of PP_i from the *Thermotoga* PP_i-PFK. The *Thermotoga* PP_i-PFK was assayed in 25 mM Bis-Tris (pH 5.8 at 50°C) containing 0.2 mM NADH, 5 mM $MgCl_2$, 5 mM F-6-P, 175 mM KCl. The results were obtained from an average value of three assays. The apparent K_m value for PP_i was 0.067 mM.

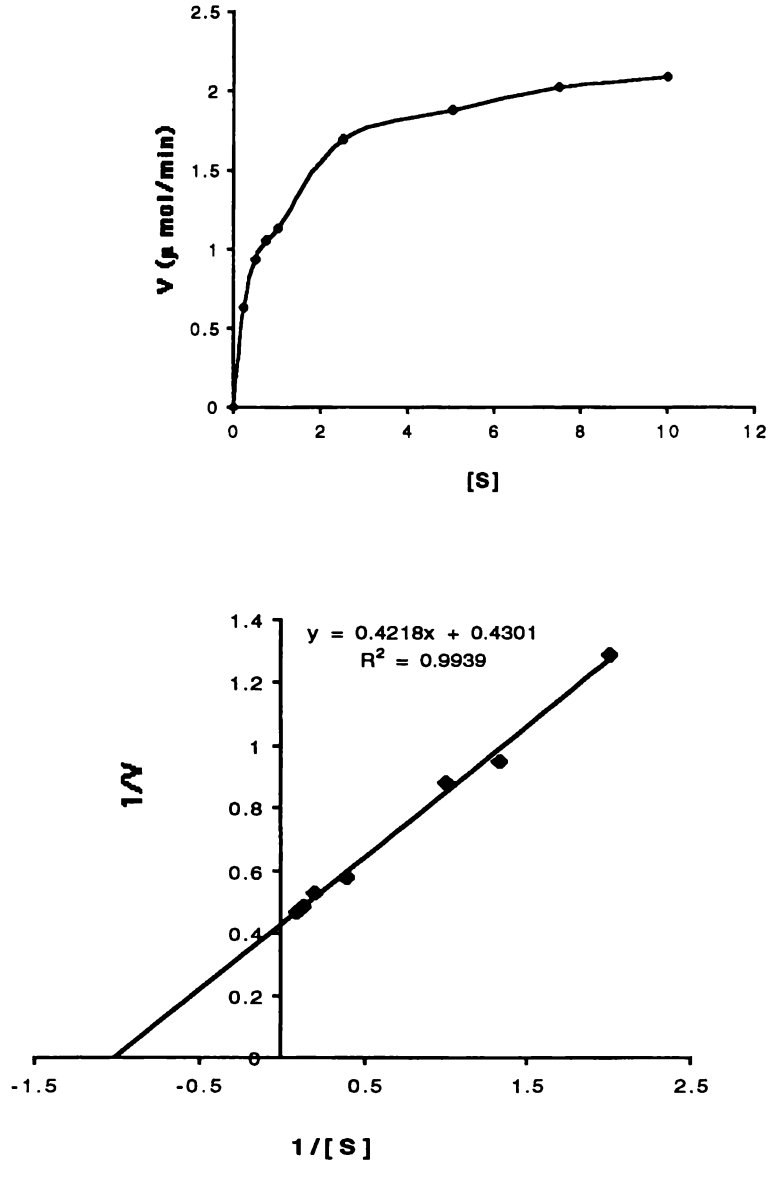


Figure E.2 Double reciprocal (1/*v* versus 1/[S]) Lineweaver-Burk plots for determination of the apparent K_m value of F-6-P from the *Thermotoga* PP_i-PFK. The *Thermotoga* PP_i-PFK was assayed in 25 mM Bis-Tris (pH 5.8 at 50°C) containing 0.2 mM NADH, 5 mM $MgCl_2$, 1.0 mM PP_i and 175 mM KCl. The results were obtained from an average value of three assays. The apparent K_m value for F-6-P was 0.98 mM.

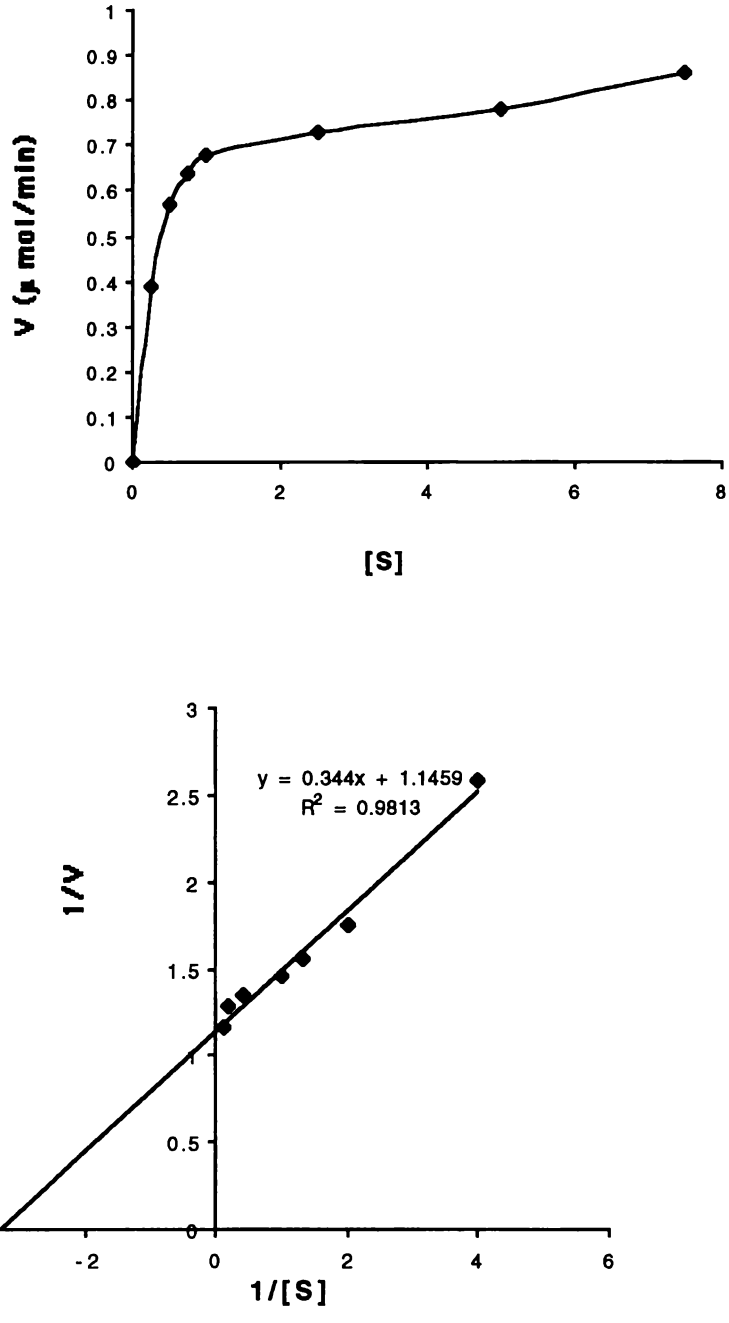
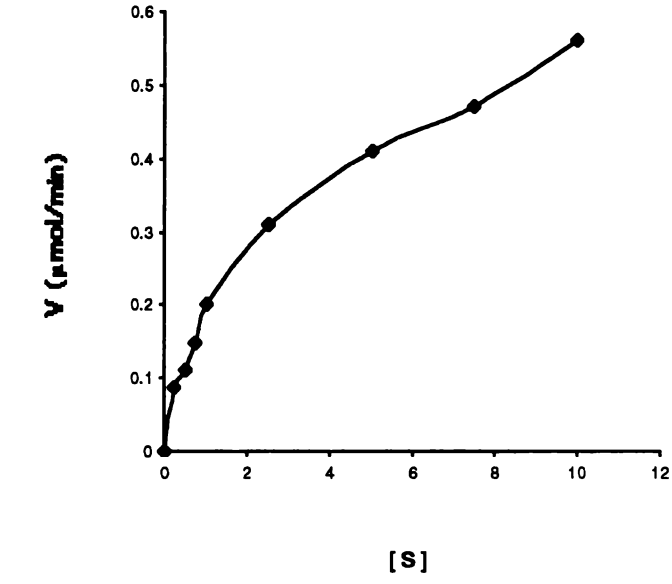


Figure E.3 Double reciprocal (1/*v* versus 1/[S]) Lineweaver-Burk plots for determination of the apparent K_m value of F-1,6-P_2 from the *Thermotoga* PP_i-PFK. The *Thermotoga* PP_i-PFK was assayed in 25 mM Bis-Tris (pH 6.0 at 50°C) containing 5.0 mM NADP, 5 mM $MgCl_2$, 20.0 mM P_i and 175 mM KCl. The results were obtained from an average value of three assays. The apparent K_m value for F-1,6-P_2 was 0.3 mM.



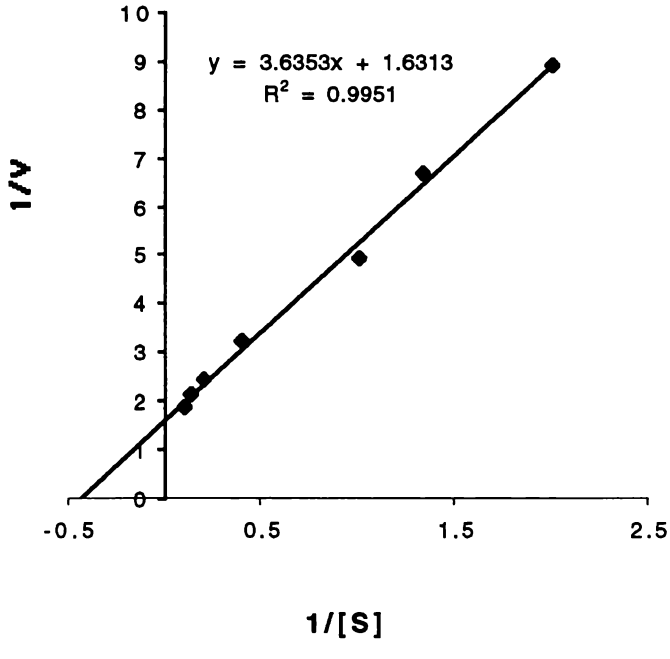


Figure E.4 Double reciprocal (1/*v* versus 1/[S]) Lineweaver-Burk plots for determination of the apparent K_m value of P_i from the *Thermotoga* PP_i-PFK. The *Thermotoga* PP_i-PFK was assayed in 25 mM Bis-Tris (pH 6.0 at 50°C) containing 5.0 mM $NADP^+$, 5 mM $MgCl_2$, 10.0 mM F-1,6-P_2 and 175 mM KCl. The results were obtained from an average value of three assays. The apparent K_m value for F-1,6-P_2 was 2.22 mM.

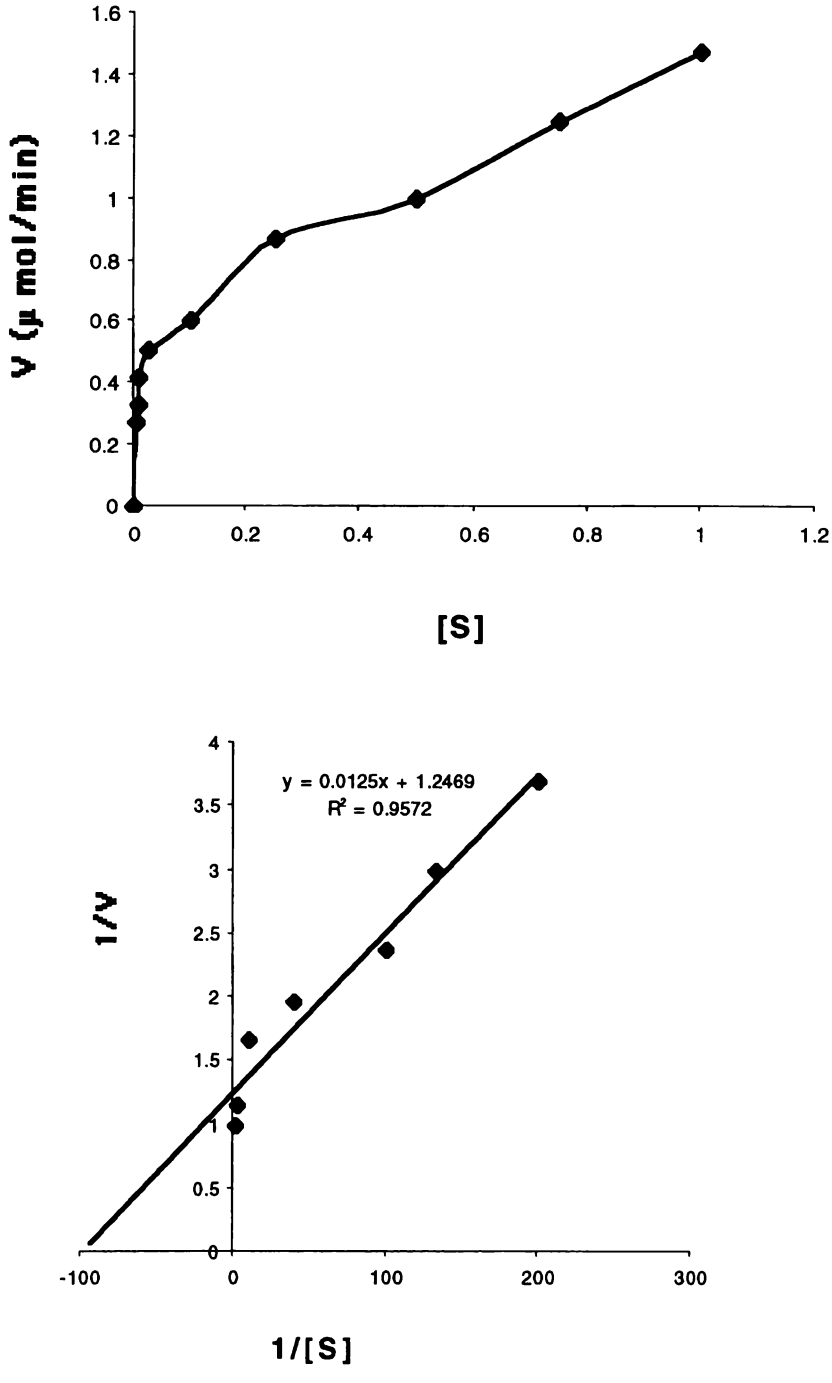


Figure E.5 Double reciprocal (1/*v* versus 1/[S]) Lineweaver-Burk plots for determination of the apparent K_m value of PPP_i from the *Thermotoga* PP_i-PFK. The *Thermotoga* PP_i-PFK was assayed in 25 mM Bis-Tris (pH 5.8 at 50°C) containing 0.2 mM NADH, 5.0 mM $MgCl_2$, 3.0 mM F-6-P, 175 mM KCl. The results were obtained from an average value of three assays. The apparent K_m value for PPP_i was 10.0 μM.

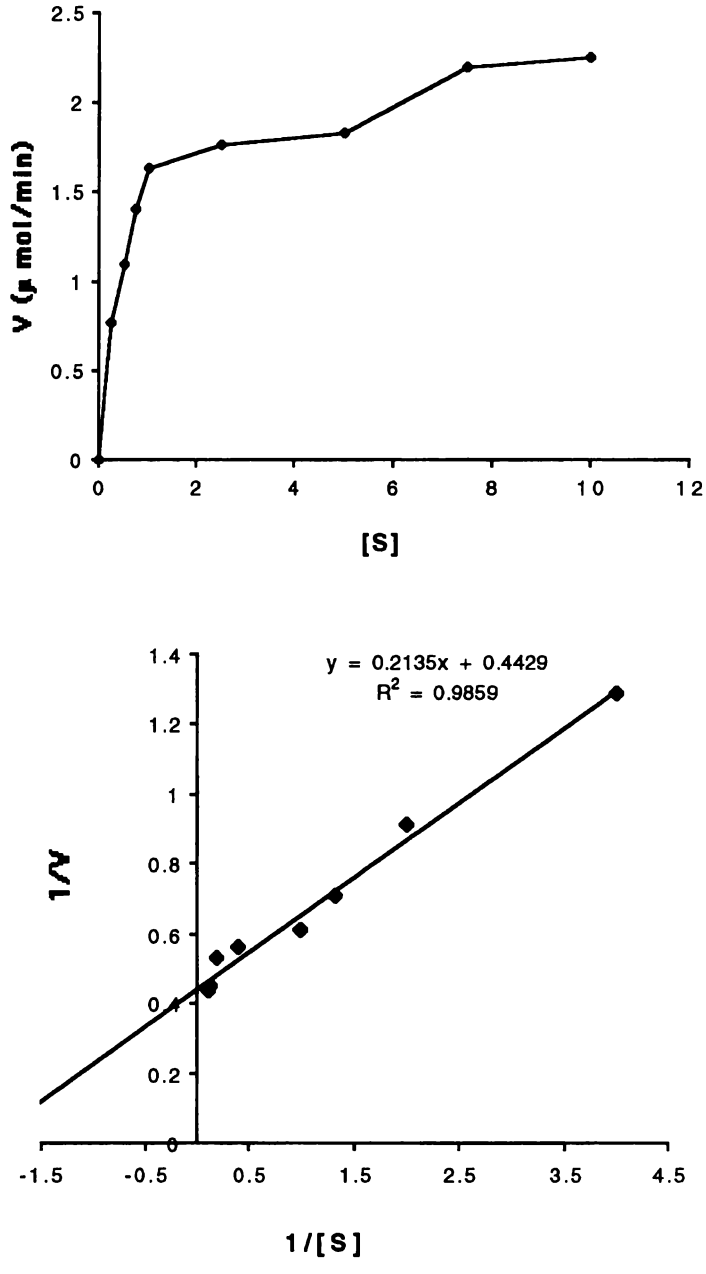


Figure E.6 Double reciprocal (1/*v* versus 1/[S]) Lineweaver-Burk plots for determination of the apparent K_m value of F-6-P using PPP_i as phosphoryl donor from the *Thermotoga* PP_i-PFK. The *Thermotoga* PP_i-PFK was assayed in 25 mM Bis-Tris (pH 5.8 at 50°C) containing 0.2 mM NADH, 5 mM $MgCl_2$, 1.0 mM PPP_i, 175 mM KCl. The results were obtained from an average value of three assays. The apparent K_m value for PP_i was 0.482 mM.

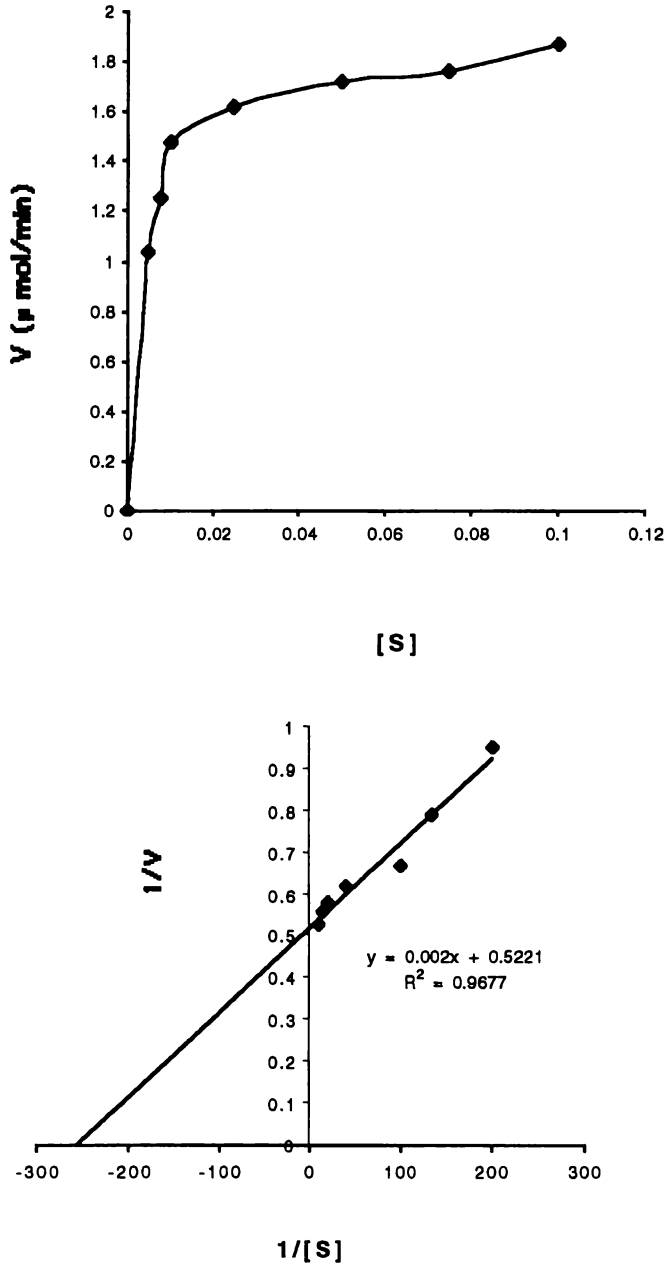


Figure E.7 Double reciprocal (1/*v* versus 1/[S]) Lineweaver-Burk plots for determination of the apparent K_m value of polyP (n=15±3) from the *Thermotoga* PP_i-PFK. The *Thermotoga* PP_i-PFK was assayed in 25 mM Bis-Tris (pH 5.8 at 50°C) containing 0.2 mM NADH, 35 mM $MgCl_2$, 5 mM F-6-P and 175 mM KCl at 50°C. The results were obtained from an average value of three assays. The apparent K_m value for polyP (n=15±3) was 3.8 μM.

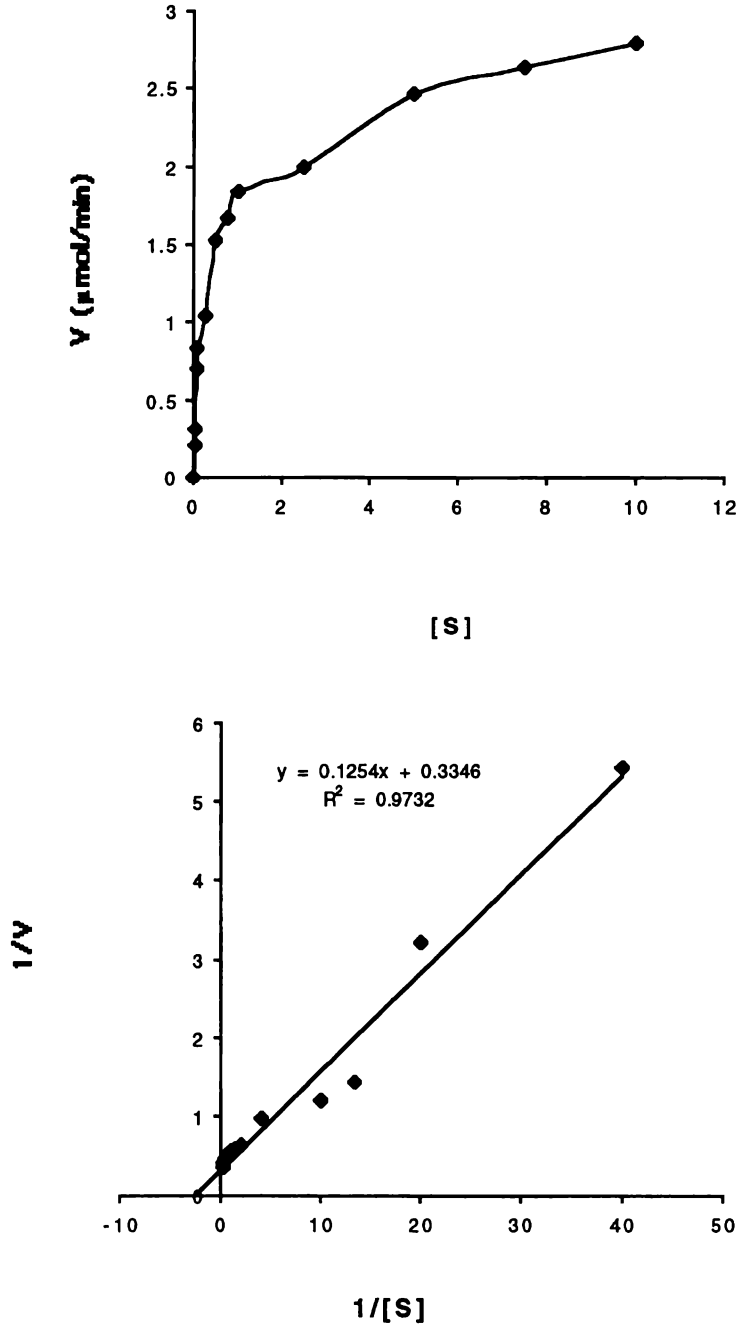


Figure E.8 Double reciprocal (1/*v* versus 1/[S]) Lineweaver-Burk plots for determination of the apparent K_m value of F-6-P using polyP (n=15±3) as phosphoryl donor from the *Thermotoga* PP_i-PFK. The *Thermotoga* PP_i-PFK was assayed in 25 mM Bis-Tris (pH 5.8 at 50°C) containing 0.2 mM NADH, 35 mM $MgCl_2$, 0.5 mM polyP (n=15±3), 175 mM KCl. The results were obtained from an average value of three assays. The apparent K_m value for F-6-P was 0.374 mM.

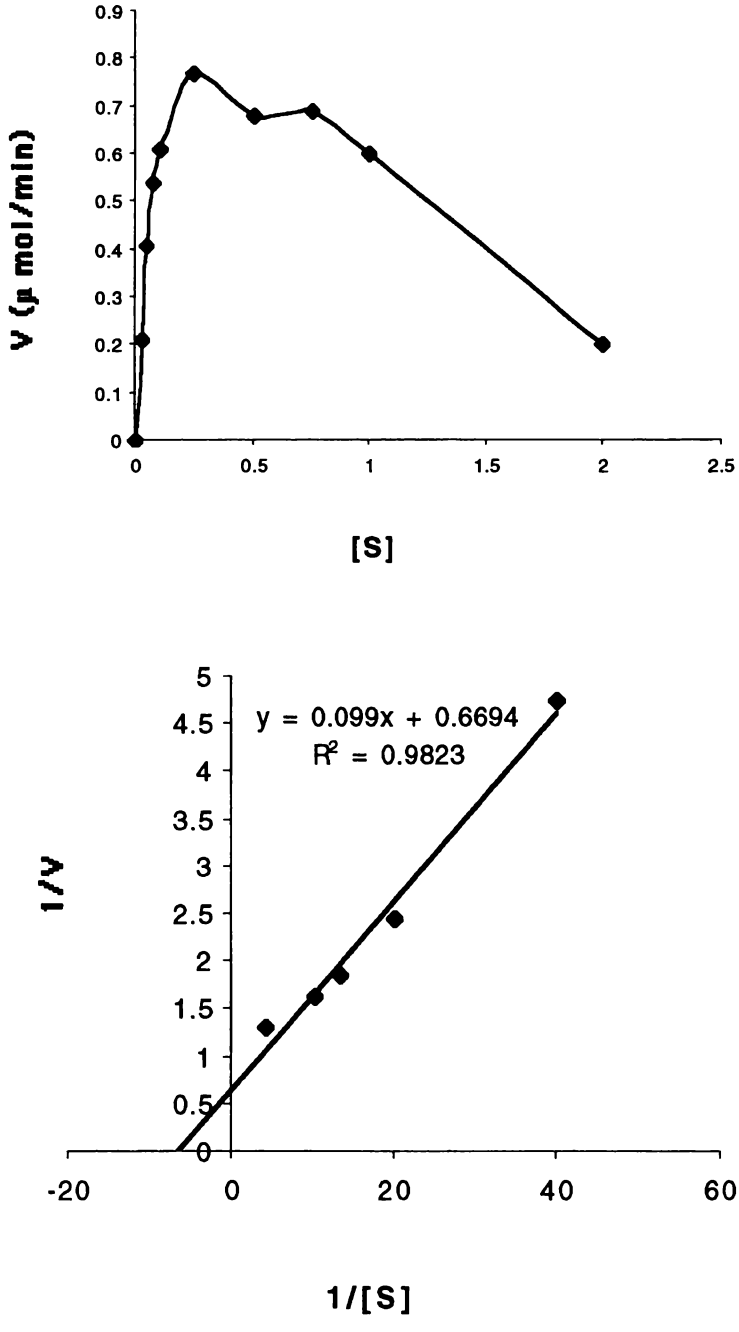


Figure E.9 Double reciprocal (1/*v* versus 1/[S]) Lineweaver-Burk plots for determination of the apparent K_m value of ATP from the *Thermotoga* ATP-PFK. The *Thermotoga* ATP-PFK was assayed in 30 mM Tris-HCl (pH 8.0 at 50°C) containing 0.2 mM NADH, 5 mM $MgCl_2$, 3 mM F-6-P and 150 mM KCl. The results were obtained from an average value of three assays. The apparent K_m value for ATP was 0.148 mM.

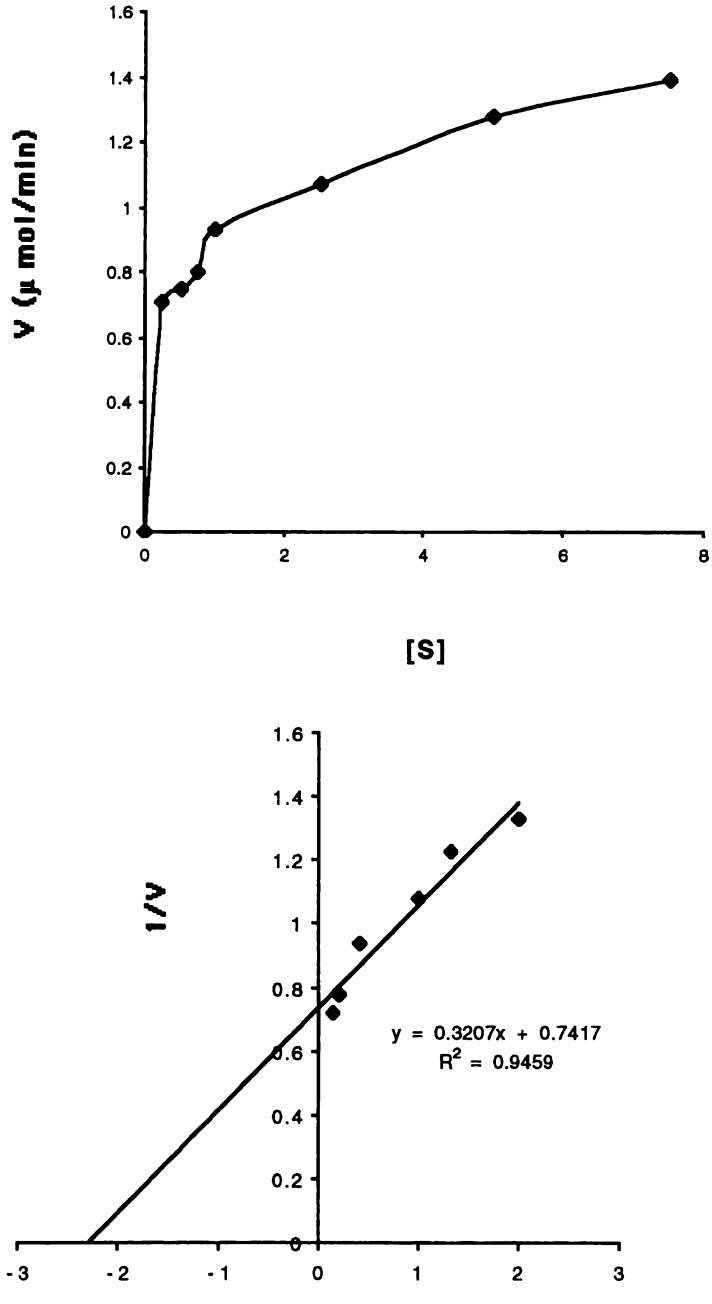


Figure E.10 Double reciprocal (1/*v* versus 1/[S]) Lineweaver-Burk plots for determination of the apparent K_m value of F-6-P from the *Thermotoga* ATP-PFK. The *Thermotoga* ATP-PFK was assayed in 30 mM Tris-HCl (pH 8.0 at 50°C) containing 0.2 mM NADH, 5 mM $MgCl_2$, 0.25 mM ATP and 150 mM KCl. The results were obtained from an average value of three assays. The apparent K_m value for F-6-P was 0.432 mM.

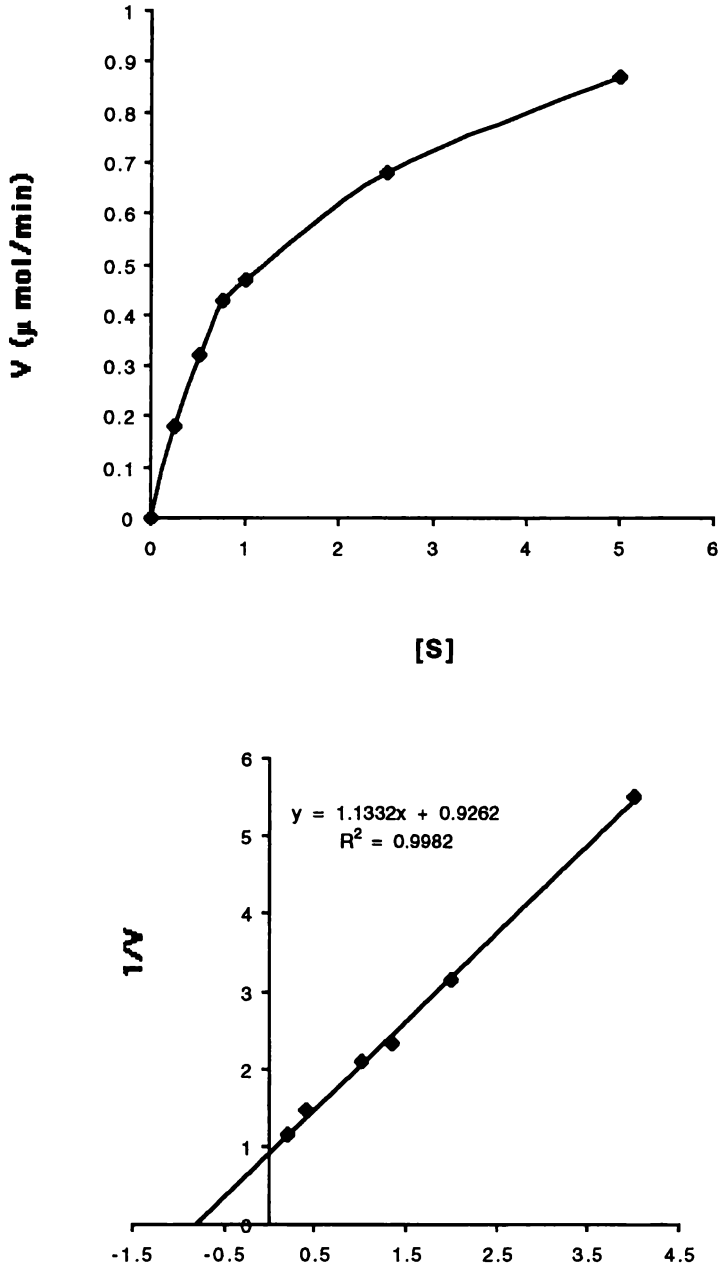


Figure E.11 Double reciprocal (1/*v* versus 1/[S]) Lineweaver-Burk plots for determination of the apparent K_m value of GTP from the *Thermotoga* ATP-PFK. The *Thermotoga* ATP-PFK was assayed in 30 mM Tris-HCl (pH 8.0 at 50°C) containing 0.2 mM NADH, 5 mM $MgCl_2$, 3 mM F-6-P and 150 mM KCl. The results were obtained from an average value of three assays. The apparent K_m value for GTP was 1.22 mM.

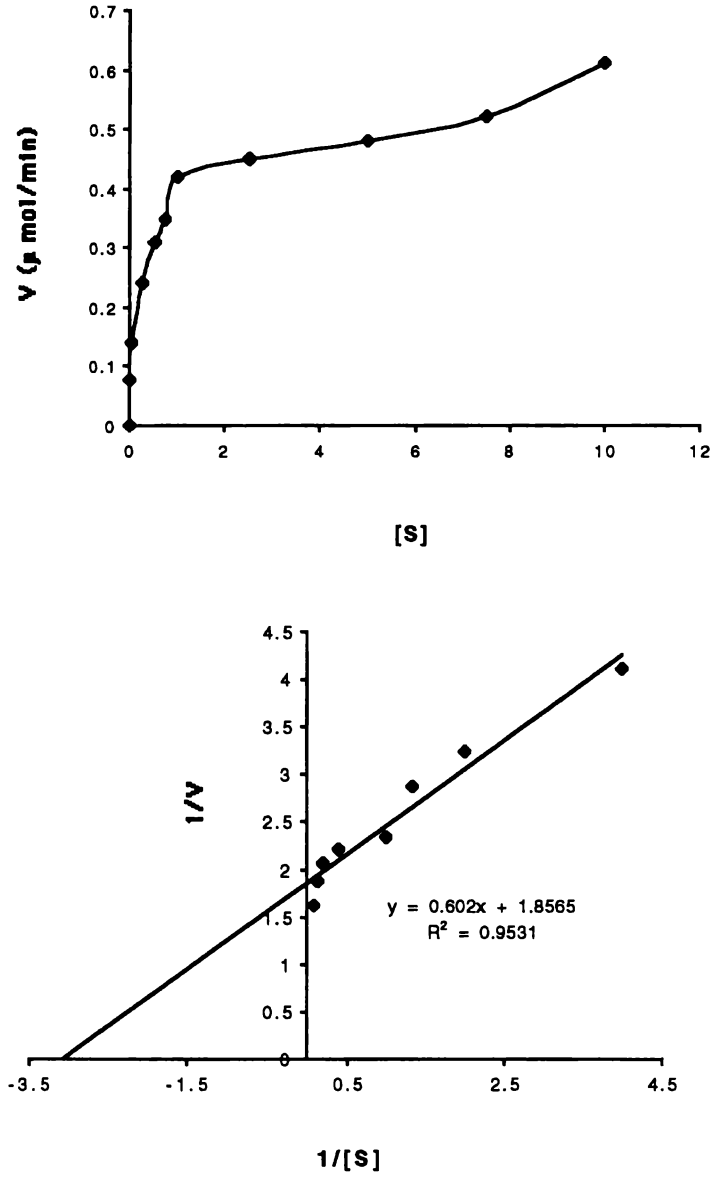


Figure E.12 Double reciprocal (1/*v* versus 1/[S]) Lineweaver-Burk plots for determination of the apparent K_m value of F-6-P with 0.1 mM PEP from the *Thermotoga* ATP-PFK. The *Thermotoga* ATP-PFK was assayed in 30 mM Tris-HCl (pH 8.0 at 50°C) containing 0.1 mM PEP, 0.2 mM NADH, 5 mM $MgCl_2$, 0.25 mM ATP and 150 mM KCl. The results were obtained from an average value of three assays. The apparent K_m value for F-6-P was 0.324 mM.

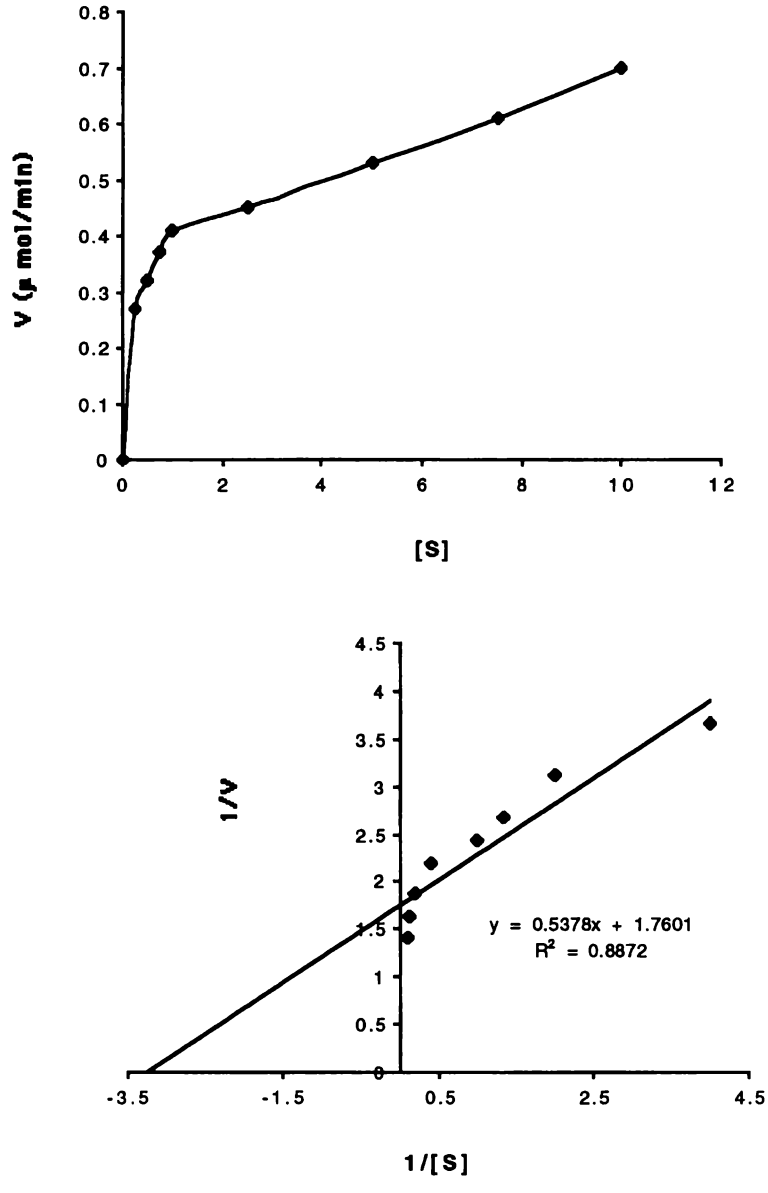


Figure E.13 Double reciprocal (1/*v* versus 1/[S]) Lineweaver-Burk plots for determination of the apparent K_m value of F-6-P with 0.5 mM PEP from the *Thermotoga* ATP-PFK. The *Thermotoga* ATP-PFK was assayed in 30 mM Tris-HCl (pH 8.0 at 50°C) containing 0.5 mM PEP, 0.2 mM NADH, 5 mM $MgCl_2$, 0.25 mM ATP and 150 mM KCl. The results were obtained from an average value of three assays. The apparent K_m value for F-6-P was 0.305 mM.

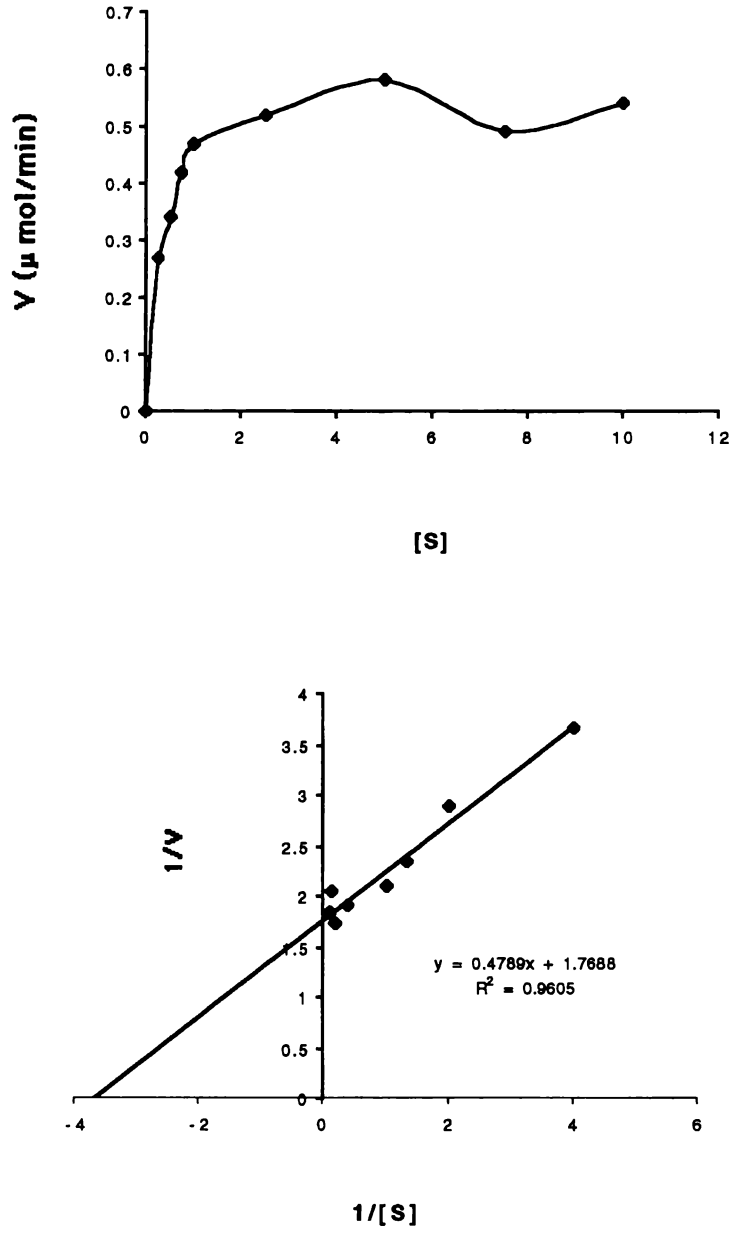


Figure E.14 Double reciprocal (1/*v* versus 1/[S]) Lineweaver-Burk plots for determination of the apparent K_m value of F-6-P with 1.0 mM PEP from the *Thermotoga* ATP-PFK. The *Thermotoga* ATP-PFK was assayed in 30 mM Tris-HCl (pH 8.0 at 50°C) containing 1.0 mM PEP, 0.2 mM NADH, 5 mM $MgCl_2$, 0.25 mM ATP and 150 mM KCl. The results were obtained from an average value of three assays. The apparent K_m value for F-6-P was 0.271 mM.

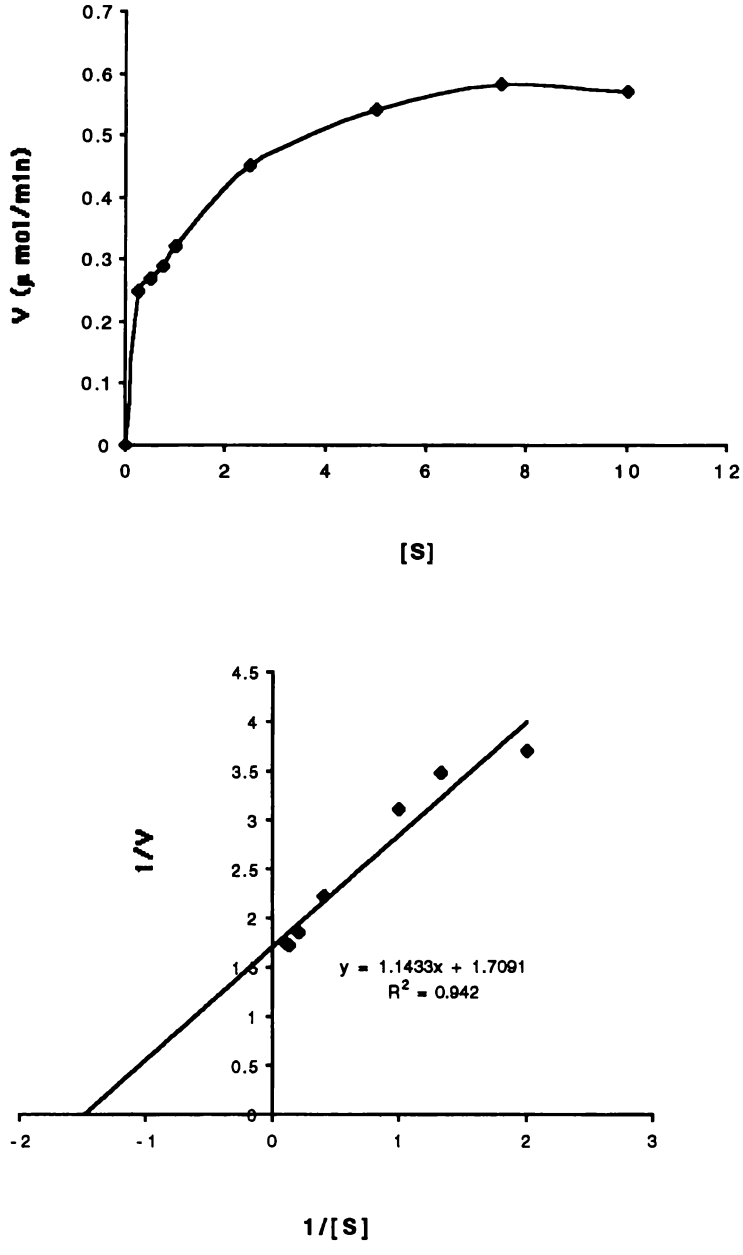


Figure E.15 Double reciprocal (1/*v* versus 1/[S]) Lineweaver-Burk plots for determination of the apparent K_m value of F-6-P with 5.0 mM PEP from the *Thermotoga* ATP-PFK. The *Thermotoga* ATP-PFK was assayed in 30 mM Tris-HCl (pH 8.0 at 50°C) containing 5.0 mM PEP, 0.2 mM NADH, 5 mM $MgCl_2$, 0.25 mM ATP and 150 mM KCl. The results were obtained from an average value of three assays. The apparent K_m value for F-6-P was 0.669 mM.

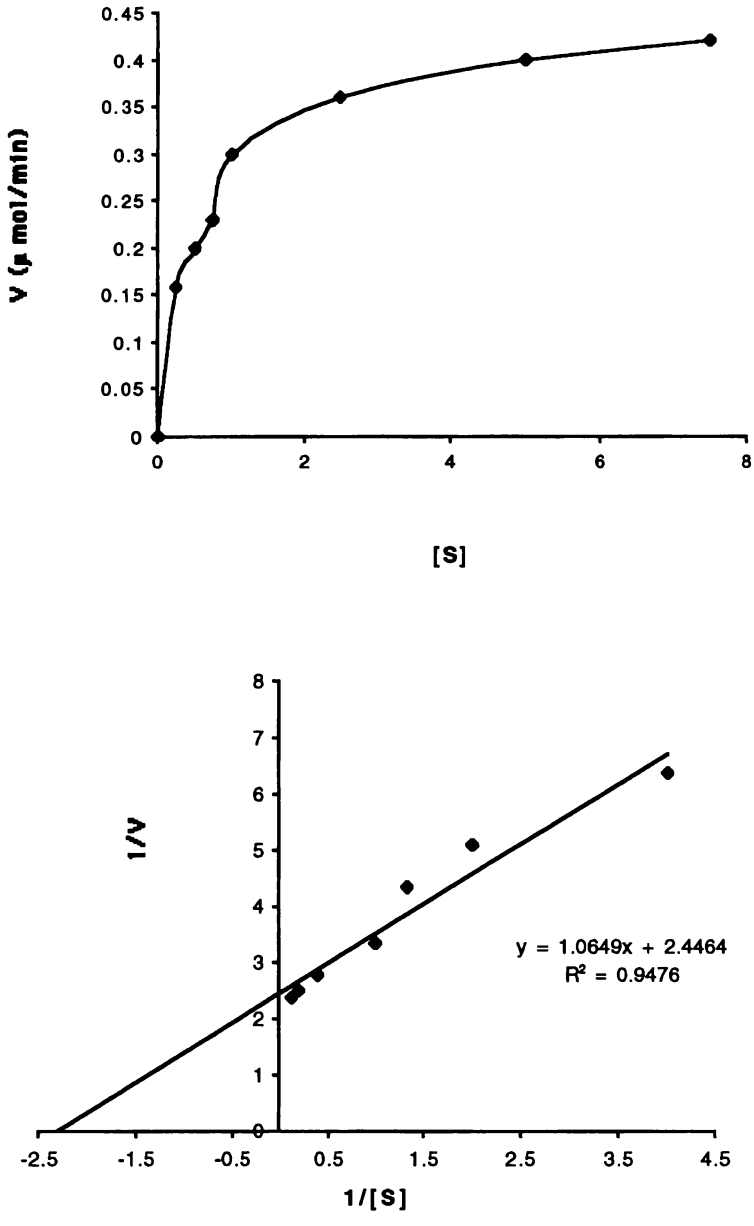


Figure E.16 Double reciprocal (1/*v* versus 1/[S]) Lineweaver-Burk plots for determination of the apparent K_m value of F-6-P with 0.01 mM PP_i from the *Thermotoga* ATP-PFK. The *Thermotoga* ATP-PFK was assayed in 30 mM Tris-HCl (pH 8.0 at 50°C) containing 0.01 mM PP_i, 0.2 mM NADH, 5 mM $MgCl_2$, 0.25 mM ATP and 150 mM KCl. The results were obtained from an average value of three assays. The apparent K_m value for F-6-P was 0.435 mM.

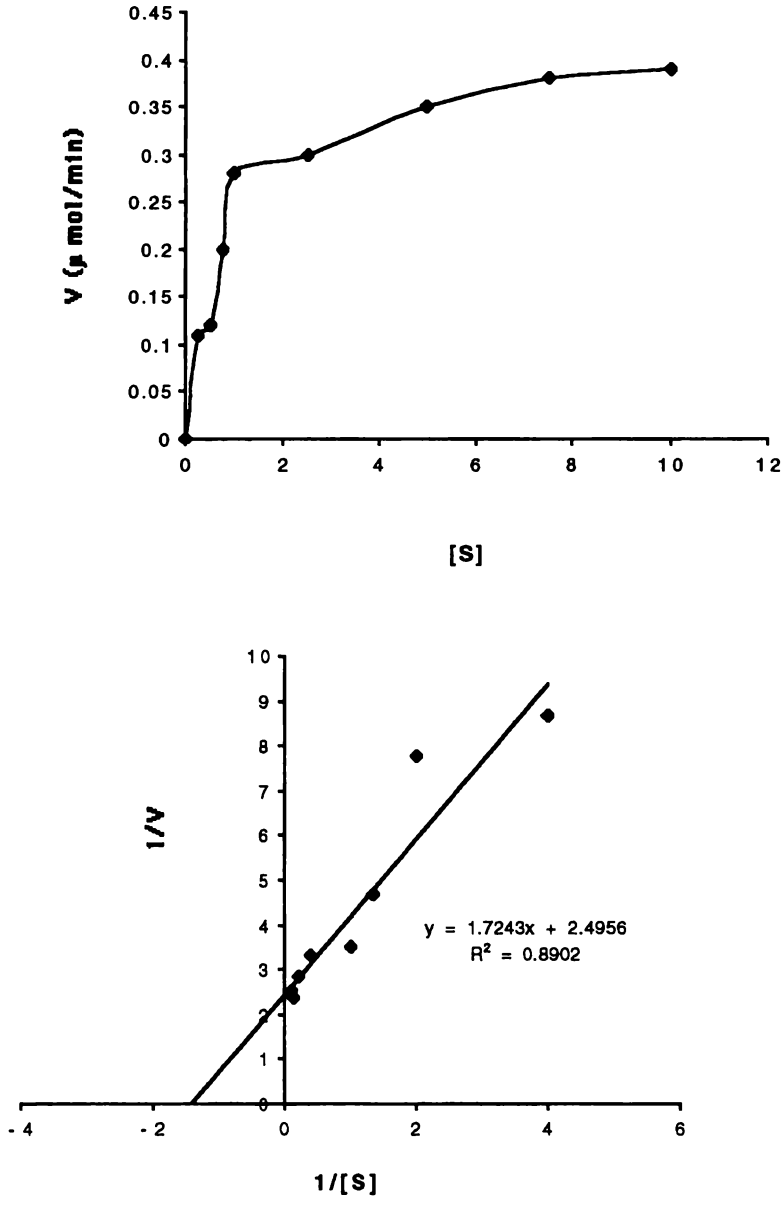


Figure E.17 Double reciprocal (1/*v* versus 1/[S]) Lineweaver-Burk plots for determination of the apparent K_m value of F-6-P with 0.025 mM PP_i from the *Thermotoga* ATP-PFK. The *Thermotoga* ATP-PFK was assayed in 30 mM Tris-HCl (pH 8.0 at 50°C) containing 0.025 mM PP_i, 0.2 mM NADH, 5 mM $MgCl_2$, 0.25 mM ATP and 150 mM KCl. The results were obtained from an average value of three assays. The apparent K_m value for F-6-P was 0.691 mM.

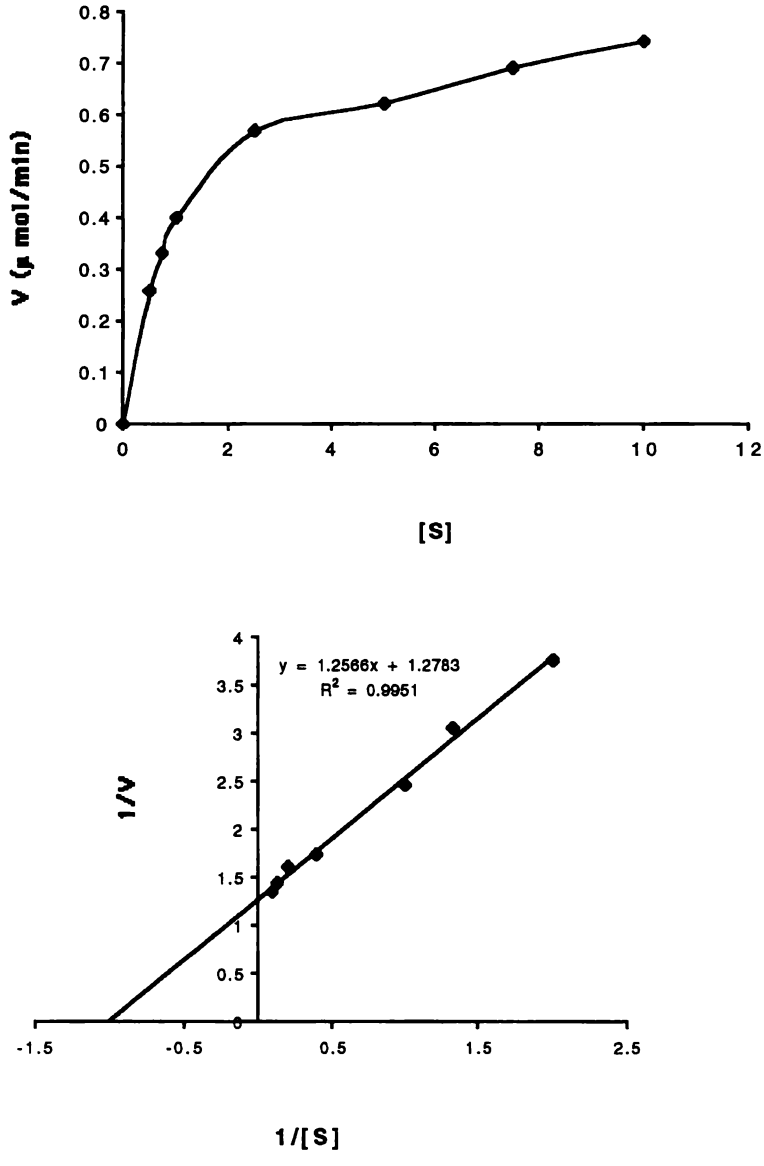


Figure E.18 Double reciprocal (1/*v* versus 1/[S]) Lineweaver-Burk plots for determination of the apparent K_m value of F-6-P with 0.05 mM PPP_i from the *Thermotoga* ATP-PFK. The *Thermotoga* ATP-PFK was assayed in 30 mM Tris-HCl (pH 8.0 at 50°C) containing 0.05 mM PPP_i, 0.2 mM NADH, 5 mM $MgCl_2$, 0.25 mM ATP and 150 mM KCl. The results were obtained from an average value of three assays. The apparent K_m value for F-6-P was 0.983 mM.

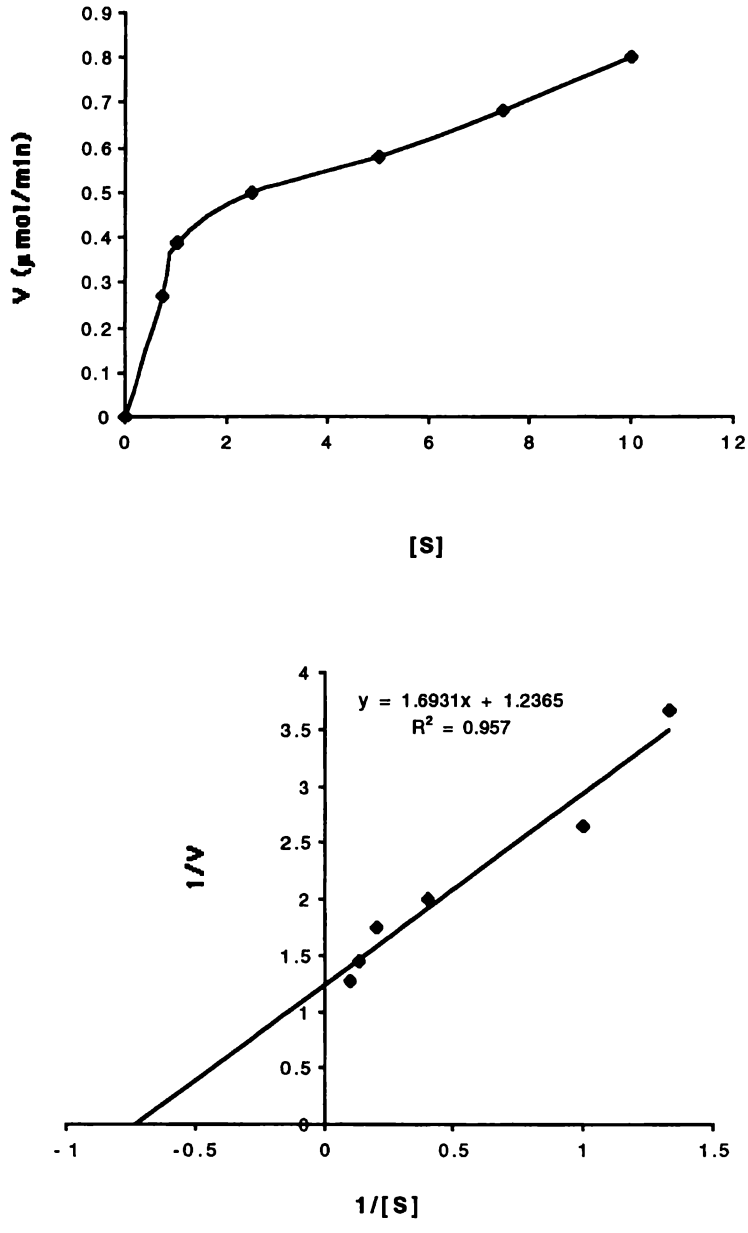
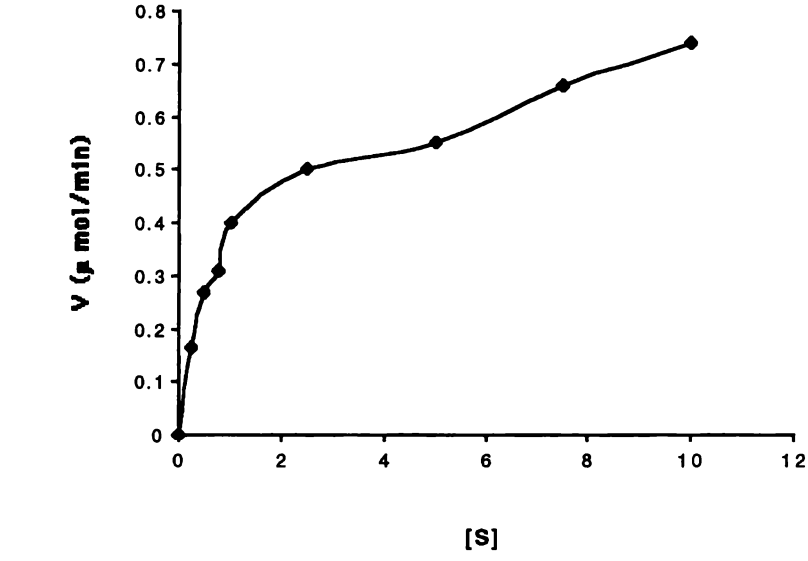


Figure E.19 Double reciprocal (1/*v* versus 1/[S]) Lineweaver-Burk plots for determination of the apparent K_m value of F-6-P with 0.05 mM polyP from the *Thermotoga* ATP-PFK. The *Thermotoga* ATP-PFK was assayed in 30 mM Tris-HCl (pH 8.0 at 50°C) containing 0.05 mM polyP, 0.2 mM NADH, 5 mM $MgCl_2$, 0.25 mM ATP and 150 mM KCl. The results were obtained from an average value of three assays. The apparent K_m value for F-6-P was 1.369 mM.



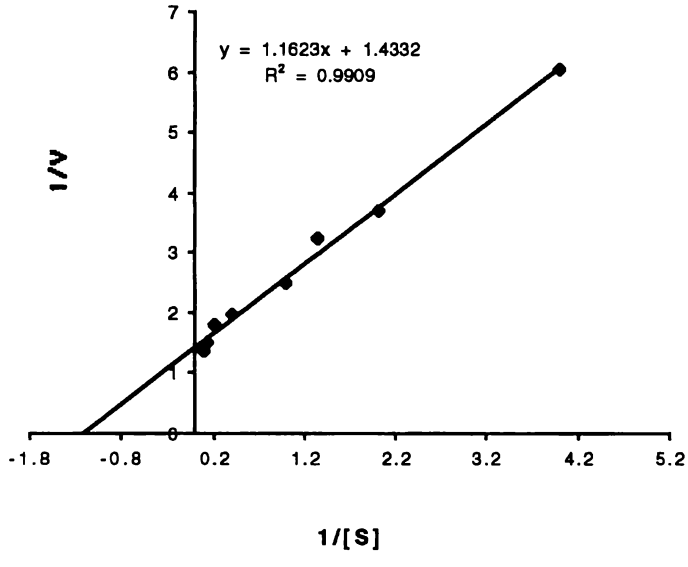


Figure E.20 Double reciprocal (1/*v* versus 1/[S]) Lineweaver-Burk plots for determination of the apparent K_m value of F-6-P with 1.0 mM citrate from the *Thermotoga* ATP-PFK. The *Thermotoga* ATP-PFK was assayed in 30 mM Tris-HCl (pH 8.0 at 50°C) containing 1.0 mM citrate, 0.2 mM NADH, 5 mM $MgCl_2$, 0.25 mM ATP and 150 mM KCl. The results were obtained from an average value of three assays. The apparent K_m value for F-6-P was 0.811 mM.

REFERENCES

Achenbach-Richter, L., Gupta, R., Stetter, K. O. and Woese, C. R. (1987). Were the original eubacteria thermophiles? *Systematic and Applied Microbiology* 9:34-39

Aksenova, H. Y., Rainey, F. A., Janssen, P. H., Zavarzin, G. A. and Morgan, H. W. (1992). *Spirochaeta thermophila* sp. nov., an obligately anaerobic, polysaccharolytic, extremely thermophilic bacterium. *International Systematic Bacteriology* 42:175-177

Altschul, S. F., Gish, W., Miller, W., Myers, W. and Lipman, D. L. (1997). Basic local alignment search tool. *Journal of Molecular Biology* 215:403-410

Altekar, W. and Rangaswamy, V. (1990). Indication of a modified EMP pathway for fructose breakdown in a halophilic archaebacterium. *FEMS Microbiology Letters* 69:139-144

Altekar, W. and Rangaswamy, V. (1991). Ketohexokinase (ATP:D-fructose 1-phosphotransferase) initiates fructose breakdown via the modified EMP pathway in halophilic archaea. *FEMS Microbiology Letters* 83:241-246

Alves, A. M. C. R., Euverink, G. J. W. and Dijkhuizen, L. (1997). Identification of ATP-dependent phosphofructokinase as a regulatory step in the glycolytic pathway of the *Actinomyces* A3(2). *Applied and Environmental Microbiology* 63:956-961

Alves, A. M. C. R., Meijer, W. G., Vrijbloed, J. W. and Dijkhuizen, L. (1996). Characterisation and phylogeny of the *pfp* gene of *Amycolatopsis methanolica* encoding PP_i-dependent phosphofructokinase. *Journal of Bacteriology* 178:149-155

Ashihara, H. and Sato, F. (1993). Pyrophosphate:fructose-6-phosphate 1-phosphotranferase and biosynthetic capacity during differentiation of hypocotyls of *Vigna* seedlings. *Biochimica et Biophysica Acta* 1156:123-127

Auzat, I., Byrnes, W. M., Garel, J-R. and Chang, S. H. (1995). Role of residue 161 in the allosteric transitions of two bacterial phosphofructokinases. *Biochemstry* 34:7062-7068

Auzat, I., Le Bras, G. and Garel, J-R. (1994a). The cooperativity and allosteric inhibition of *Escherichia coli* phosphofructokinase depend on the interaction between threonine-125 and ATP. *Proceedings of the National Academy of Sciences USA* 91:5242-5246

Auzat, I., Bras, G., Le. Branny, P., Torre, F. D. L., Theunissen, B. and Garel, J-R. (1994b). The role of Glu 187 in the regulation of phosphofructokinase by phosphoenolpyruvate. *Journal of Molecular Biology* 235:68-72

Baltscheffsky, H. (1996). Energy conversion leading to the origin and early evolution of life: did inorganic pyrophosphate precede adenosine triphosphate? In: Origin and evolution of biological energy conversion. eds H. Baltscheffsky, VCH Publishers, New York p 1-9

Banks, J. F. and Whitehouse, C. M. (1996). Electrospray ionization mass spectrometry. *Methods in Enzymology* 270:486-519

Barnes, W. M. (1987). Sequencing DNA with dideoxyribonucleotides as chain terminators: hints and strategies for big projects. *Methods in Enzymology* 152:538-556

Belouski, E., Watson, E. E. and Bennett, G. N. (1998). Cloning, sequence and expression of the phosphofructokinase gene of *Clostridium acetobutylicum* ATCC 824 in *Esherichia coli*. *Current Microbiology* 37:17-22

Berger, S. A. and Evans, P. R. (1990). Active-site mutants altering the cooperativity of *E. coli* phosphofructokinase. *Nature* 343:575-576

Berger, S. A. and Evans, P. R. (1992). Site-directed mutagenesis identifies catalytic residues in the active site of *Escherichia coli* phosphofructokinase. *Biochemistry* 31:9237-9242

Bertagnolli, B. L. and Cook, P. F. (1984). Kinetic mechanism of pyrophosphate-dependent phosphofructokinase from *Propionibacterium freudenreichii*. *Biochemistry* 23:4104-4108

Birnboim, H. C. (1983). A rapid alkaline extraction method for the isolation of plasmid DNA. *Methods in Enzymology* 100:243-255

Black, C. C., Mustardy, S. S., Kormanik, P. P., Xu, D-P. and Paz, N. (1986). Regulation and roles for alternative pathways of hexose metabolism in plants. *Physiologia Plantarum* 69:387-394

Blamey, J. M. and Adams, M. W. W. (1994). Characterisation of an ancestral type of pyruvate ferredoxin oxidoreductase from the hyperthermophilic bacterium, *Thermotoga maritima*. *Biochemistry* 33:1000-1007

Botha, A-M. and Botha, F. C. (1993). Induction of pyrophosphate dependent phosphofructokinase in watermelon (*Citrullus lanatus*) cotyledons coincides with insufficient cytosolic D-fructose-1,6-bisphosphate 1-phosphohydrolase to sustain gluconeogenesis. *Plant Physiology* 101:1385-1390

Botha, F. C. and Small, J. G. C. (1987). Comparison of the activities and some properties of pyrophosphate and ATP-dependent fructose-6-phosphate 1-phosphotransferases of *Phaseolus vulgaris* seeds. *Plant Physiology* 83:772-777

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principles of protein-dye binding. *Analytical Biochemstry* 72:248-254

Branny, P., De La Torre, F. and Garel, J-R. (1993). Cloning, sequencing and expression in *Escherichia coli* of the gene coding for phosphofructokinase in *Lactobacillus bulgaricus*. *Journal of Bacterology* 175:5344-5349

Branny, P., De La Torre, F. and Garel, J-R. (1996). The genes for the phosphofructokinase and pyruvate kinase of *Lactobacillus delbrueckii* subsp. *bulgaricus* constitute an operon. *Journal of Bacterology* 178:4727-4730

Brennan, S. O., Davis, P. F. and Midwinter, G. G. (1974). Structural aspects of sheep-heart phosphofructokinase. *European Journal of Biochemistry* 42:489-494

Brock, T. D., Madigan, M. T., Martinko, J. M. and Parker, J. (1994). In: Biology of microorganisms. Prentice Hall, Eaglwood Cliffs, New Jersey. 7th eds. p 99-101

Bruchhaus, I., Jacobs, T., Denart, M. and Tannich, E. (1996). Pyrophosphate-dependend phosphofructokinase of *Entamoeba histolytica*: molecular cloning, recombinant expression and inhibition by pyrophosphate analogues. *Biochemical Journal* 316:57-63

Byington, C. L., Dunbrack, Jr., R. L. Whitby, F. G. Cohen, F. E. and Agabian, N. (1997). *Entamoeba histolytica*: computer-assisted modeling of phosphofructokinase for the prediction of broad-spectrum antiparasitic agents. *Experimental Parasitology* 87:194-202.

Byrnes, W. M., Zhu, X., Younathan, E. S. and Chang, S. H. (1994). Kinetic characteristics of phosphofructokinase from *Bacillus stearothermophilus*: MgATP nonallosterically inhibits the enzyme. *Biochemistry* 33:3424-3431

Cagen, L. M. and Friedmann, H. C. (1968). Enzymatic phosphorylation of serine by inorganic pyrophosphate. *Biochemical and Biophysical Research Communications* 33:528-533

Canale-Parola, E. (1977). Physiology and evolution of Spirochetes. *Bacteriological Reviews* 41:181-204

Canganella , F., Andrade, C. M. and Antranikian, G. (1994). Characterization of amylolytic and pullulytic enzymes from thermophilic archaea and from a new *Fervidobacterium* species. *Applied and Microbiological Biotechnology* 42:239-245

Carnal, N. W. and Black, C. C. (1979). Pyrophosphate-dependent 6-phosphofructokinase, a new glycolytic enzyme in pineapple leaves. *Biochemical and Biophysical Research Communications* 86:20-26

Carlisle, S. M., Blakeley, S. D., Hemmingsen, S., Trevanion, S., Hiyoshi, T., Kruger, N. J. and Dennis, T. (1990). Pyrophosphate-dependent phosphofructokinase: conservation of protein sequence between the α- and β- subunits and with the ATP-dependent phosphofructokinase. *The Journal of Biological Chemistry* 265:18366-18371

Chevalier, C., Saillard, C. and Bové, J. M. (1990). Organisation and nucleotide sequences of the *Spiroplasma citri* genes for the ribosomal protein S2, elongation factor Ts, spiralin, phosphofructokinase, pyruvate kinase and an unidentified protein. *Journal of Bacteriology* 172:2693-2703

Cho, Y-K. and Cook, P. F. (1989). pH dependence of the kinetic parameters for the pyrophosphate-dependent phosphofructokinase reaction supports a proton-shuttle mechanism. *Biochemistry* 28:4155-4160

Collins, S. F. and Weissman, S. M. (1984). Directional cloning of DNA fragments at large distances from an initial probe: a circularisation method. *Genetics* 81:6812-6816

Creighton, T. E. (1990). Protein folding. *The Biochemical Journal* 270:1-16

Cronin, C. N. and Tipton, K. F. (1985). Purification and regulatory properties of phosphofructokinase from *Trypanosoma (Trypanozoon) brucei*. *The Biochemical Journal* 227:113-124

Cruden, D. L., Durbin, W. E. and Markovetz, A. J. (1983). Utilisation of PP_i as an energy source by a *Clostridium* sp. *Applied and Environmental Microbiology* 46:1403-1408

Currie, P. D. and Sullivan, D. T. (1994). Structure and expression of the gene encoding phosphofructokinase (PFK) in *Drosophila melanogaster*. *The Journal of Biological Chemistry* 269:24679-24687

Daldal, F. (1984). Nucleotide sequence of gene *pfk*B encoding the minor phosphofructokinase of *Escherichia coli* K-12. *Gene* 28:337-342

Danson, M. J. (1989). Central metabolism of the archaebacteria: an overview. *Canadian Journal of Microbiology* 35:58-64

Danson, M. J. (1993). Central metabolism of the archaea. *The Biochemistry of Archaea* 26:1-24

Danson, M. J., Russell, R. J. M., Hough, D. W. and Taylor, G. L. (1998). Comparative enzymology as an aid to understanding evolution. In "Thermophiles: the key to molecular evolution and the origin of life?" eds. Wiegel J.W. and Adams M.W.W. Taylor and Francis (London) p 255-268

Deckert, G., Warren, P. V., Gaasterland, T., Young, W. G., Lenox, A. L., Grahams, D. E., Overbeek, R., Snead, M. A., Keller, M., Aujay, M., Huber, R., Feldman, R. A., Short, J. M., Olsen, G. J. and Swanson, R. V. (1998). The complete genome sequence of the hyperthermophilic bacterium *Aquifex aeolicus*. *Nature* 392:353-358

Deng, Z., Roberts, D., Wang, S. and Kemp, R. G. (1999). Expression, characterization and crystallization of the pyrophosphate-dependent phosphofructo-1-kinase of *Borrelia burgdorferi*. *Archives of Biochemistry and Biophysics* 371:326-331

Denton, H., Browm, S. M. A., Craig, W. R., Alexander, J., McDonald, V., Thong, K-W. and Coombs, G. H. (1996). Comparison of the phosphofructokinase and pyruvate kinase activities of *Cryptosporidium parvum*, *Eimeria tenella* and *Toxoplasma gondii*. *Molecular Biochemistry and Parasitology* 76:23-29

Denton, H., Thong, K-W. Coombs, G. H. (1994). *Eimeria tenella* contains a pyrophosphate-dependent phosphofructokinase and a pyruvate kinase with unusual allosteric regulators. *FEMS Microbiology Letters* 115:87-92

De Rosa, M., Gambacorta, A., Nicolaus, B., Giardina, P., Poerio, E. and Buonocore, V. (1984). Glucose metabolism in the extreme thermoacidophilic archaebacterium *Sulfolobus solfataricus. The Biochemical Journal* 224:407-414

Deville-Bonne, D., Bourgain, F. and Garel, J-R. (1991a). pH dependence of the kinetic properties of allosteric phosphofructokinase from *Escherichia coli. Biochemistry* 30:5750-5754

Deville-Bonne, D., Laine, R. and Garel, J-R. (1991b). Substrate antagonism in the kinetic mechanism of *E. coli* phosphofructokinase-1. *FEBS Letters* 290:173-176

Ding, J., Su, J-G. J. and Mansour, T. E. (1994). Cloning and characterisation of a cDNA encoding phosphofructokinase from *Schistosoma mansoni. Molecular Biochemistry and Parasitology* 66:105-110

Ding, Y-H. R., Ronimus, R. S. and Morgan, H. W. (1999). Purification and properties of the pyrophosphate-dependent phosphofructokinase from *Dictyoglomus thermophilum* Rt46 B.1. *Extremophiles* 3:131-137

Ding, Y-H. R., Ronimus, R. S. and Morgan, H. W. (2000a). Sequencing, cloning and high-level expression of the *pfp* gene encoding a PP_i-dependent phosphofructokinase from the extremely thermophilic eubacterium *Dictyoglomus thermophilum. Journal of Bacteriology* 182:4661-4666

Ding, Y-H. R., Ronimus, R. S. and Morgan, H. W. (2000b). Phosphofructokinases from *Thermotoga maritima*, two unique enzymes. Accepted by *Journal of Bacteriology*

Dixon, M. and Webb, E. C. (1979) Enzymes. 3rd ed. Webb, Edwin Clifford. Longman Group Ltd., New York.

Doolittle, W. F. (1999). Phylogenetic classification and the universal tree. *Science* 284:2124-2128

Dower, W. J., Miller, J. F. and Ragsdale, C. W. (1988). High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Research* 16:6127-6145

Enomoto, T., Miyatake, K. and Kitaoka, S. (1988). Purification and immunological properties of fructose-2,6-bisphosphate-sensitive pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase from the protist *Euglena gracilis*. *Comparative Biochemistry and Physiology* 90:897-902

Enomoto, T., Ohyama, H. and Sumiyoshi, H. (1994). Role of pyrophosphate:D-fructose-6-phosphate 1-phosphotransferase during germination of rice seeds. *Bioscience, Biotechnology and Biochemistry* 58:464-466

Evans, P. R. and Hudson, P. J. (1979). Structure and control of phosphofructokinase from *Bacillus stearothermophilus*. *Nature* 279:500-504

Fothergill-Gilmore, L. A. and Michels, P. A. M. (1993). Evolution of glycolysis. *Progess in Biophysics & Molecular Biology* 59:105-235

Fraser, C. M., Casjens, S., Huang, W. M., Sutton, G. G., Clayton, R., Lathigra, R., White, O., Ketchum, K. A., Dodson, R., Hickey, E. K., Gwinn, M., Dougherty, B., Tomb, J-F., Fleischmann, R. D., Richardson, D., Peterson, J., Kerlavage, A. R., Quackenbush, J., Salzberg, S., Hanson, M., van Vugt, R., Palmer, N., Adams, M. D., Gocayne, J., Weidman, J., Utterback, T., Watthey, L., McDonald, L., Artiach, P., Bowman, C., Garland, S., Fujii, C., Cotton, M. D., Horst, K., Roberts, K., Hatch, B., Smith, H. O. and Venter, J. C. (1997). Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*. *Nature* 390:580-586

Fraser, C. M., Norris, S. J., Weinstock, G. M., White, O., Sutton, G. G., Dodson, R., Gwinn, M., Hickey, E. K., Clayton, R., Ketchum, K. A., Sodergren, E., Hardham, J. M., McLeod, M. P., Salzberg, S., Peterson, J., Khalak, H., Richardson, D., Howell, J. K., Chidambaram, M., Utterback, T., McDonald, L., Artiach, P., Bowman, C., Cotton, M. D., Fujii, C., Garland, S., Hatch, B., Horst, K., Roberts, K., Sandusky, M., Weidman, J., Smith, H. O. and Venter, J. C. (1998). Complete genome sequence of *Treponema pallidum*, the syphilis spirochete. *Science* 281:375-388

French, B. A., Valdez, B. C., Younathan, E. S. and Chang, S. H. (1987). High-level expression of *Bacillus stearothermophilus* 6-phosphofructo-1-kinase in *Escherichia coli*. *Gene* 59:279-283

Garrett, R. H. and Grisham, C. M. (1995). Biochemistry. International Edition. Saunders College Publishing. USA. p 570-597

Galaev, I. Y., Garg, N. and Mattiasson, B. (1994). Interaction of Cibacron Blue with polymers: implications for polymer-shielded dye-affinity chromatography of phosphofructokinase from baker's yeast. *Journal of Chromatography* 684:45-54

Gekakis, N., Johnson, R. C., Jerkins, A., Mains, R. E. and Sul, H. S. (1994). Structure, distribution and functional expression of the phosphofructokinase C isozyme. *The Journal of Biological Chemistry* 269:3348-3355

Green, P. C., Latshaw, S. P., Ladror, U. S. and Kemp, R. G. (1992). Identification of critical lysyl residues in the pyrophosphate-dependent phosphofructokinase-1-kinase of *Propionibacterium freudenreichii*. *Biochemistry* 31:4815-4821

Greenberg, E. P. and Canale-Parola, E. (1976). *Spirochaeta halophila* sp. nov., a facultative anaerobe from a high salinity pond. *Archives of Microbiology* 110:185-194

Hanahan, D. and Meselson, M. (1983). Plasmid screening at high colony density. *Mehtods in Enzymology* 100:333-343

Hans V., U. Heinemann, C. M. Dobson, and C. V. Robinson. (1998). Detection of a monomeric intermediate associated with dimerisation of protein Hu by mass spectrometry. *Journal of American Chemistry Society* 120:6427-6428.

Hansen, T. and Schönheit, P. (2000). Purification and properties of the first-identified, archaeal, ATP-dependent 6-phosphofructokinase, an extremely thermophilic non-allosteric enzyme, from the hyperthermophilic *Desulfurococcus amylolyticus*. *Archives of Microbiology* 173:103-109

Hardoyo, K. Y., A. Muramatru, Y. Anbe, J. Kato, and H. Ohtake. 1994. Moleclar genetics of polyphosphate accumulation in *Escherichia coli*. Phosphate in microorganisms: cellular and molecular biology. Edit. Annamaria Torriani-Gorini, Ezra Yagil, Simon Silver. U. S. A 209-214

Harold, F. M., (1966). Inorganic polyphophates in biology: structure, metabolism and function. *Bacteriological Reviews* 30:772-794

Harwood, C. S. and Canale-Parola, E. (1984). Ecology of spirochetes. *Annual Review of Microbiology* 38:161-192

Harwood, C. S., Jannasch, H. W. and Canale-Parola, E. (1982). Anaerobic spirochete from a deep-sea hydrothermal vent. *Applied and Environmental Microbiology* 44:234-237

Hatzfeld, W-D., Dancer, J. and Stitt, M. (1989). Direct evidence that pyrophosphate:fructose-6-phosphate phosphotransferase can act as a glycolytic enzyme in plants. *FEBS Letters* 2549:215-218

Häusler, R. E., Holtum, J. A. M. and Latzko, E. (1989). Cytosolic phosphofructokinase from spinach leaves. I. Purification, characteristics and regulation. *Plant Physiology* 90:1498-1505

Heinisch, J. J. (1993). Expression of heterologous phosphofructokinase genes in yeast. *FEBS Letters* 328:35-40

Heinisch, J., Kirchrath, L., Liesen, T., Vogelsang, K. and Hollenberg, C. P. (1993). Molecular genetics of phosphofructokinase in the yeast *Kluyveromyces lactis*. *Molecular Microbiology* 8:559-570

Hellinga, H. W. and Evans, P. R. (1985). Nucleotide sequence and high-level expression of the major *Escherichia coli* phosphofructokinase. *European Journal of Biochemistry* 149:363-373

Hespell, R. B. and Canale-Parola, E. (1973). Glucose and pyruvate metabolism of *Spirochaeta litoralis*, an anaerobic marine spirochete. *Journal of Bacteriology* 116:931-937

Hondmann, D. H. A. and Visser, J. (1990). Screening method for large numbers of dye-adsorbents for enzyme purification. *Journal of Chromatography* 510:155-164

Howard, O., Gerber, A. S. and Hartl, D. L. (1988). Genetic applications of an inverse polymerase chain reaction. *Genetics* 120:621-623

Hsieh, P-C., Shenoy, B. C., Jentoft, J. E. and Phillips, N. F. B. (1993). Purification of polyphosphate and ATP glucose phosphotransferase from *Mycobacterium tuberculosis* H_{37}Ra: evidence that poly(P) and ATP glucokinase activities are catalyzed by the same enzyme. *Protein Expression and Purification* 4:76-84

Huang, M., Albach, R. A., Chang, K-P., Tripathi, R. L. and Kemp, R. G. (1995). Cloning and sequencing a putative pyrophosphate-dependent phosphofructokinase gene from *Entamoeba histolytica. Biochimic et Biophysica Acta* 1260:215-217

Huber, R., Langworthy, T. A., Konig, H., Thomm, M., Woese, C. R., Sleytr, U. B. and Stetter, K. O. (1986). *Thermotogo maritima* sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up to 90°C. *Archives of Microbiology* 144:324-333

Jaenicke, R., Schurig, H., Beaucamp, N. and Ostendorp, R. (1996). Structure and stability of hyperstable proteins: glycolytic enzymes from the hyperthermophilic bacterium *Thermotoga maritima. Advances in Protein Chemistry* 48:181-269

Jannaschk, D., Burgos, M., Centerlles, J. J., Ovadi, J. and Cascante, M. (1999). Application of metabolic control analysis to the study of toxic effects of copper in muscle glycolysis. *FEBS Letters* 445:144-8

Janssen, P. H. and Morgan, H. W. (1992). Glucose catabolism by *Spirochaeta thermophila* RI 19 B1. *Journal of Bacteriology* 174:2449-2453

Johnson, J. L. and Reinhart, G. D. (1992). MgATP and fructose 6-phosphate interactions with phosphofructokinase from *Escherichia coli. Biochemistry* 31:11510-11518

Keister, D. L. and Minton, N. J. (1971). ATP synthesis driven by inorganic pyrophophate in *Rhodospirillum rubrum* chromatophores. *Biochemical and Biophysical Research Communications* 42:932-939

Kemera, V. F., Griffin, C. C. and Brand, L. (1975). Phosphofructokinase from *Escherichia coli. Methods in Enzymology* 9:91-99

Kemp, R. G. (1975). Phosphofructokinase from rabbit liver. *Methods in Enzymology* 9:67-71

Kemp, R. G. and Tripathi, R. L. (1993). Pyrophosphate-dependent phosphofructo-1-kinase complements fructose1,6-bisphosphatase but not phosphofructokinase deficiency in *Escherichia coli. Journal of Bacteriology* 175:5723-5724

Kengen, S. W., de Bok, F. A. M., van Loo, N-D., Dijkema, C., Stams, A. J. M. and de Vos, W. M. (1994). Evidence for the operation of a novel Embden-Meyerhof pathway that

involves ADP-dependent kinases during sugar fermentation by *Pyrococcus furiosus* *The Journal of Biological Chemistry* 269:17537-17541

Kengen, S. W. M., Tuininga, J. E., de Bok, F. A. M., Stams, A. J. M. and de Vos, W. M. (1995). Purification and characterisation of a novel ADP-dependent glucokinase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *The Journal of Biological Chemistry* 270:30453-30457

Kengen, S. W. M., Stams, A. J. M. and de Vos, W. M. (1996). Sugar metabolism of hyperthermophiles. *FEMS Microbiology Review* 18:199-137

Kim, S. S., Choi, I-G. and Kim, S-H. (1999). Molecular cloning, expression and characterization of a thermostable glutamate racemase from a hyperthermophilic bacterium, *Aquifex pyrophilus*. *Extremophiles* 3:175-183

Kolb, E., Hudson, P. J. and Harris, J. I. (1980). Phosphofructokinase: complete amino-acid sequence of the enzyme from *Bacillus stearothermophilus*. *European Journal of Biochemistry* 108:587-597

Kopperschläger, G. (1994). Phosphofructokinase from Baker's yeast. *Methods in Enzymology* 228:144-155

Kornberg, A. (1994). Inorganic polyphosphate: a molecular fossil come to life. In: Phosphate in microorganisms: cellular and molecular biology, eds. Torriani-Gorini, A. Yagil, E. and Silver, S. ASM Press, Washington D.C. USA p 204-208

Kornberg, A. (1995). Inorganic polyphosphate: toward making an forgotten polymer unforgetable. *Journal of Bacteriology* 177:491-496

Kornberg, A. and Fraley, C. D. (2000). Inorganic polyphosphate: a molecular fossil come to life. *American Society of Microbiology News* 66:275-280

Kotlarz, D. and Buc, H. (1981). Regulatory properties of phosphofructokinase 2 from *Escherichia coli*. *European Journal of Biochemistry* 117:569-574

Krahe, M., Antranikian, G. and Märkl, H. (1996). Fermentation of extremophilic microorganisms. *FEMS Microbiology Review* 18:271-285

Kruger, N. J. and Hammond, J. B. W. (1988). Molecular comparison of pyrophosphate- and ATP-dependent fructose 6-phosphate 1-transferases from potato tuber. *Plant Physiology* 86:645-648

Kruger, N. J., Hammond, J. B. and Burrell, M. M. (1988). Molecular characterisation of four forms of phosphofructokinase purified from potato tuber. *Archives of Biochemistry and Biophysics* 267:690-700

Kruger, N. J., Hemmingsen, S. M. and Dennis, D. T. (1986). Distribution of pyrophosphate: fructose 6-phosphate phosphotransferase in maize leaves. *Plant Physiology* 81:12-16

Kulaev, I. S. (1979). The biochemistry of inorganic polyphosphates. John Wileyy & Sons, Inc., New York

Kulaev, I. S. (1994). Polyphosphates and phosphate reserves. In: Phosphate in microorganisms: cellular and molecular biology, eds. Torriani-Gorini, A. Yagil, E. and Silver, S. ASM Press, Washington D.C. p 195-196

Ladror, U. S., Gollapudi, L., Tripathi, R. L., Latshaw, S. P. and Kemp, R. G. (1991). Cloning, sequencing and expression of pyrophosphate-dependent phosphofructokinase from *Propionibacterium freudenreichii. The Journal of Biological Chemistry* 266:16550-16555

Lai, J. C. and Blass, J. P. (1984). Neurotoxic effects of copper:inhibition of glycolysis and glycolytic enzymes. *Neurochemical Research* 9:1699-1710

Lau, F. T-K., Fersht, A. R., Hellinga, H. W. and Evans, P. R. (1987). Site-directed mutagenesis in the effector site of *Escherichia coli* phosphofructokinase. *Biochemistry* 26:4143-4148

Layzer, R. B. (1975). Phosphofructokinase from human erythrocytes. *Methods in Enzymology* 9:110-115

Le Bras, G., Deville-Bonne, D. and Garel, J-R. (1991). Purification and properties of the phosphofructokinase from *Lactobacillus bulgaricus* (a nonallosteric analog of the enzyme from *Escherichia coli*). *European Journal of Biochemistry* 198:683-687

Lee, C-P., Kao, M-C., French, B. A., Putney, S. D. and Chang, S. (1987). The rabbit muscle phosphofructokinase gene. *The Journal of Biological Chemistry* 262:4195-4199

Lemaigre, F. P. and Rousseau, G. G. (1994). Transcriptional control of genes that regulate glycolysis and gluconeogenesis in adult liver. *The Biochemical Journal* 303:1-14

Leschine, B. P. and Canale-Parola, E. (1986). Rifampicin-resistant RNA polymerase in spirochetes. *FEMS Microbiology Letters* 35:199-204

Li, J., Zhu, X., Byrnes, M., Nelson, J. W. and Chang, S. H. (1993). Site-directed mutagenesis of rabbit muscle phosphofructokinase cDNA. *The Journal of Biological Chemistry* 268:24599-24606

Li, Z. and Phillips, N. F. B. (1995). Pyrophosphate-dependent phosphofructokinase from *Giardia lamblia*: purification and characterisation. *Protein Expression and Purification.* 6:319-328

Lipmann, F. (1965). Projecting backward from the present stage of the evolution of biosynthesis. In: "The origins of prebiological systems", (S.W. Fox, Ed.). Academic press, NewYork p 259-280

Liu, C-L., Hart, N. and Peck, H. D. (1982). Inorganic pyrophosphate: energy source for sulfate-reducing bacteria of the genus *Desulfotomaculum. Science* 217:363-364

Love, C. A., Patel, B. K. C., Ludwig, W. and Stackebrandt, E. (1993). The phylogenetic position of *Dictyoglomus thermophilum* based on 16S rRNA sequence analysis. *FEMS Microbiology Letters* 107:317-320

Mahajan, R. and Singh, R. (1989). Properties of pyrophosphate:fructose-6-phosphate phosphotransferase from endosperm of developing wheat (*Triticum aestivum* L.) grains. *Plant Physiology* 91:421-426

Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982). Molecular cloning: A laboratory manual, 2nd edit. Cold Spring Harbor Laboratory

Martin, W. and Müller, M. (1998). The hydrogen hypothesis for the first eukaryote. *Nature* 392:37-41

Martínez-Costa, O. H., Estévez, A. M., Sánchez, V. and Aragón, J. J. (1994). Purification and properties of phosphofructokinase from *Dictyostelium discoideum*. *European Journal of Biochemistry* 226:1007-1017

Mathrani, I. M. and Ahring, B. K. (1991). Isolation and characterisation of a strictly xylan-degrading *Dictyoglomus* from a man-made, thermophilic anaerobic environment. *Archives of Microbiology* 156:13-17

Mertens, E. (1990). Occurence of pyrophosphate: fructose-6-phosphate 1-transferase in *Giardia lamblia* trophozoites. *Molecular and Biochemical Parasitology* 40:147-150

Mertens, E. (1991). Pyrophosphate-dependent phosphofructokinase, an anaerobic glycolytic enzyme? *FEBS Letters* 285:1-5

Mertens, E. (1993). ATP versus pyrophosphate: glycolysis revisited in parasitic protists. *Parasitology Today* 9:122-126

Mertens, E., De Jonckheere, J. and van Schaftingen, E. (1993). Pyrophosphate-dependent phosphofructokinase from the amoeba *Naegleria fowleri*, an AMP-sensitive enzyme. *The Biochemical Journal* 292:797-803

Mertens, E., Ladror, U. S., Lee, J. A., Miretsky, A., Morris, A., Rozario, C., Kemp, R. G. and Müller, M. (1998). The pyrophosphate-dependent phosphofructokinase of the protist, *Trichomonas vaginalis*, and the evolutionary relationships of protist phosphofructokinases. *Journal of Molecular Evolution* 47:739-750

Mertens, E., Larondelle, Y. and Hers, H-G. (1989a). Induction of pyrophosphate: fructose 6-phosphate 1-transferase by anoxia in rice seedlings. *Plant Physiology* 93:584-587

Mertens, E., van Schaftingen, E. and Müller, M. (1989b). Presence of a fructose-2,6-bisphosphate-insensitive pyrophosphate:fructose-6-phosphate phosphotransferase in the anaerobic protozoa *Tritrichomonas foetus*, *Trichomonas vaginalis* and *Isotricha prostoma*. *Molecular Biochemistry and Parasitology* 37:183-190

Michels, P. A. M., Chevalier, N., Opperdoes, F. R., Rider, M. H. and Rigden, D. J. (1997). The glycosomal ATP-dependent phosphofructokinase of *Trypanosoma brucei* must have evolved from an ancestral pyrophosphate-dependent enzyme. *European Journal of Biochemistry* 250:698-704

Miyatake, K., Enomoto, T. and Kitaoka, S. (1984). Detection and subcellular distribution of pyrophosphate:D-fructose 6-phosphate 1-phosphotransferase (PFP) in *Euglena gracilis*. *Agricultural and Biological Chemistry* 48:2857-2859

Montavon, P. and Kruger, N. J. (1993). Essential arginyl residue at the active site of pyrophosphate:fructose-6-phosphate 1-phosphotransferase from Potato (*Solanum tuberosum*) tuber. *Plant Physiology* 101:765-771

Morgan, H. W. and Ronimus, R. S. (l998). Pyrophosphate-dependent phosphofructokinase in thermophilic and non-thermophilic microorganisms. In "Thermophiles: the key to molecular evolution and the origin of life," eds. Wiegel J.W. and Adams M.W. W. Taylor and Francis (London), p 269-278

Müller, M. (1988). Energy metabolism of protozoa without mitochondria. *Annual Review of Microbiology* 42:465-488

Müller, M. (1992). Energy metabolism of ancestral eukaryotes: a hypothesis based on the biochemistry of amitochondriate parasitic protists. *BioSystems* 28:33-40

Müller, M. (1998). Enzymes and compartmentation of core energy metabolism of anaerobic protists- a special case in eukaryotic evolution? In: Coombs G.H. Vickerman K, Sleigh M.A, Warren A (eds) Evolutionary relationships among protozoa. Chapman & Hall, London, p109-131

Nakajima, H., Noguchi, T., Yamasaki, T., Kono, N., Tanaka, T. and Tarui, S. (1987). Cloning of human muscle phosphofructokinas cDNA. *FEBS Letters* 223:113-116

Nelson, K. E., Clayton, R. A., Gill, S. R., Gwinn, M. L., Dodson, R. J., Haft, D. H., Hickey, E. K., Peterson, J. D., Nelson, W. C., Ketchum, K. A., McDonald, L., Utterback, T. R., Malek, J. A., Linher, K. D., Garrett, M. M., Stewart, A. M., Cotton, M. D., Pratt, M. S., Phillips, D. A., Richardson, D., Heidelberg, J., Sutton, G. G., Fleischmann, R. D., Eisen, J. A., White, O., Salzberg, S. L., Smith, H. O., Venter, J. C. and Fraser, C. M. (1999). Evidence for lateral gene transfer between archaea and bacteria from genome sequence of *Thermotoga maritima*. *Nature* 399:323-329

O'Brien, W. E., Bowien, S. and Wood, H. G. (1975). Isolation and characterisation of a pyrophosphate-dependent phosphofructokinase from *Propionibacterium shermanii*. *The Journal of Biological Chemistry* 250:8690-8695

Ochman, H., Lawrence, J. G. and Grolsman, E. A. (2000). Lateral gene transfer and the nature of bacterial innovation. *Nature* 405:299-304

Pace, N. R. (1997). A molecular view of microbial diversity and the biosphere. *Science* 276:734-740

Paster, B. J, Canale-Parola, E. (1985). *Treponema saccharophilum* sp. nov., a large pectinolytic spirochete from the bovine rumen. *Applied and Environmental Microbiology* 50:212-219

Paster, B. J., Dewhirst, F. E., Weisburg, W. G., Tordoff, L. A., Fraser, G. J., Hespell, R. B., Stanton, T. B., Zablen, L., Mandelco, L. and Woese, C. R. (1991). Phylogenetic analysis of the Spirochetes. *Journal of Bacteriology* 173:6101-6109

Patel, B. K., Morgan, H. W., Wiegel, J. and Daniel, R. M. (1987). Isolation of an extremely thermophilic chemoorganotrophic anaerobe similar to *Dictyoglomus thermophilum* from New Zealand hot springs. *Archives of Microbiology* 147:21-24

Patterson, J. A. (1989). Inorganic pyrophosphate as an energy source for ruminal bacteria. *Current Microbiology* 18:37-39

Peck, H. D., Liu, C-L., Varma, A. K., Ljungdahl, L. G., Szulczynski, M., Bryant, F. and Carreira, L. (1983). The utilization of inorganic pyrophosphate, tripolyphosphate, and tetrapolyphosphate as energy sources for the growth of anaerobic bacteria. Basic life sciences. Plenum Press. New York 25:317-348

Peng, Z-Y. and Mansour, T. E. (1992). Purification and properties of a pyrophosphate-dependent phosphofructokinase from *Toxoplasma gondii*. *Molecular Biochemistry and Parasitology* 54:223-230

Perry, J. M., Zhao, Y. and Marletta, M. A. (2000). Cu^{2+} and Zn^{2+} inhibit nitric-oxide synthase through an interaction reductase domain. *The Journal of Biological Chemistry* 275:14070-14076

Petzel, J. P., Hartman, P. A. and Allison, M. J. (1989). Pyrophosphate-dependent enzymes in walled bacteria phylogenetically related to the wall-less bacteria of the class *Mollicutes*. *International Journal of Systematic Bacteriology* 39:413-419

Pfleiderer, C. and Klemme, J-H. (1980). Pyrophosphate-dependent D-fructose-6-phosphate-phosphotransferase in Rhodospirillaceae. *Z. Naturforsch* 35:229-238

Phillips. N. F. B. and Li, Z. (1995). Kinetic mechanism of pyrophosphate-dependent phosphofructokinase from *Giardia lamblia. Molecular Biochemistry and Parasitology* 73:43-51

Plaxton, W. C. (1996). The organisation and regulation of plant glycolysis. *Annual Reviews of Pant Physiology and Plant Biology* 47:185-214

Pollack, J. D. and Williams, M. V. (1986). PP_i-dependent phosphotransferase (phosphofructokinase) activity in the Mollicutes (Mycoplasma) *Achloeplasma laidlawii. Journal of Bacteriology* 165:53-60

Poorman, R. A., Randolph, A., Kemp, R. G. and Heinrikson, R. L. (1984). Evolution of phosphofructokinase-gene duplication and creation of new effector sites. *Nature* 309:467-469

Pollack, J. D. and Williams, M. V. (1986). PP_i-dependent phosphotransferase (phosphofructokinase) activity in the Mollicutes (Mycoplasma). *Acholeplasma laidlawii.* J. Bacteriol. **165**:53-60

Pribnow, D. (1975). Nucleotide sequence of an RNA polymerase binding site at an early T7 promoter. *Proceedings of the National Academy of Sciences USA* 72:184-188.

Rainey, F. A., Janssen, P. H., Wild, D. J. C. and Morgan, H. W. (1991). Isolation and characterisation of an obligately anaerobic, polysaccharolytic, extremely thermophilic member of the genus *Spirochaeta. Archives of Microbiology* 155:396-401

Ralph, D. and McClelland, M. (1994). Phylogenetic evidence for horizontal transfer of an intervening sequence between species in a Spirochete genus. *The Journal of Biological Chemistry* 176:5982-5987

Rao, B. K., Tyryshkin, A. M., Roberts, A. G., Bowman, M. K. and Kramer, D. M. (2000). Inhibitory copper binding site on the spinach cytochrome b6f complex: implications for Qo site catalysis. *Biochemistry* 39:3285-3296

Reeves, R. E, (1970). Phosphopyruvate carboxylase from *Entamoeba histolytica*. *Biochimica et Biophysica Acta* 220:346-349

Reeves, R. E. and Malin, L. K. (1969). Enzymic assay method for inorganic pyrophosphate. *Analytical Biochemistry* 28:282-287

Reeves, R. E., South, D. J., Blytt, H. J. and Warren, L. G. (1974). Pyrophosphate:D-fructose-6-phosphate 1-phosphotransferase. *The Journal of Biological Chemistry* 249:7737-7741

Reeves, R. E., Serrano, R. and South, D. J. (1976). 6-phosphofructokinase (pyrophosphate), properties of the enzyme from *Entamoeba histolytica* and its reaction mechanism. *The Journal of Biological Chemistry* 251:2958-2962

Resnick., S. M. and Zehnder, A. J. (2000). In vitro ATP regeneration from polyphosphate and AMP by polyphosphate:AMP phosphotransferase and adenylate kinase from *Acinetobacter johnsonii* 210A. *Applied and Environmental Microbiology* 66:2045-2051

Righetti, P. G., Gelfi, C. and Chiari, M. (1996). Isoelectric focusing in immobilized pH gradients. *Methods in Enzymology* 270:235-255

Romano, A. H. and Conway, T. (1996). Evolution of carbohydrate metabolic pathways. *Research Microbiology* 147:448-455

Ronimus R. S. (1993). Investigation into the control of genome topology in the archaeal isolate AN1. PhD Thesis. The University of Waikato

Ronimus R. S., de Heus, E. and Morgan, H. W. (2000). Sequencing, expression, characterisation and phylogeny of the ADP-dependent phosphofructokinase from the hyperthermophilic, euryarchaeal *Thermococcus zilligii*. (submitted)

Ronimus R. S., Koning, J. and Morgan, H. W. (1999a). Purification and characterisation of an ADP-dependent phosphofructokinase from *Thermococcus zilligii*. *Extremophiles* 3:121-129

Ronimus, R. S., Morgan, H. W. and Ding, Y-H. R. (1999b). Phosphofructokinase activities within the order Spirochaetales and the characterisation of the pyrophosphate-dependent phosphofructokinase from *Spirochaeta thermophila*. *Archives of Microbiology* 172:401-406

Rozario, C., Smith, M. W. and Müller, M. (1995). Primary sequence of a putative pyrophosphate-linked phosphofructokinase gene of *Giardia lamblia*. *Biochimica et Biophysica Acta* 1260:218-222

Russell, M. J. and Hall, A. J. (1997). The emergence of life from iron monosulphide bubbles at a submarine hydrothermal redox and pH front. *Journal of Geological Society* 154:377-402

Rypniewski, W. R. and Evans, P. R. (1989). Crystal structure of unliganded phosphofructokinase from *Escherichia coli*. *Journal of Molecular Biology* 207:805-821

Saiki, T., Kobayashi, Y., Kawagagoe, K. and Beppu, T. (1985). *Dictyoglomus thermophilum* gen. nov., sp. nov., a chemoorganotrophic, anaerobic, thermophilic bacterium. *International Journal of Systermatic Bacteriology* 35:253-259

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular cloning: a laboratory manual, 2nd eds. Cold Spring Harbor Laboratory Press, New York

Sanangelantoni, A. M., Cammarano, P. and Tiboni, O. (1996). Manipulation of the *tuf* gene provides clues to the localization of sequence element(s) involved in the thermal stability of *Thermotoga maritima* elongation factor Tu. *Microbiology* 142:2525-2532

Sanangelantoni, A. M., Forlani, G., Ambroselli, F., Cammarano, P. and Tiboni, O. (1992). The *gln* A gene of the extremely thermophilic eubacterium *Thermotoga maritima*: cloning, primary structure and expression in *Escherichia coli*. *Journal of General Microbiology* 138:383-393

Sawyer, M. H., Baumann, P. and Baumann, L. (1977). Pathways of D-fructose and D-glucose catabolism in marine species of *Alcaligenes*, *Pseudomonas marina* and *Alteromonas communis*. *Archives of Microbiology* 112:169-172

Schäfer, T. and Schönheit, P. (1991. Pyruvate metabolism of the hyperthermophilic archaebacterium *Pyrococcus furiosus*. *Archives of Microbiology* 155:366-377

Schäfer, T. and Schönheit, P. (1993). Gluconeogenesis from pyruvate in the hyperthermophilic archaeon *Pyrococcus furiosus*: involvement of reactions of the Emden-Meyerhof pathway. *Archives of Microbiology* 159:354-363

Schirmer, T. and Evans, P. R. (1990). Structural basis of the allosteric behavior of phosphofructokinase. *Nature* 343:140-145

Schröder, C., Selig, M. and Schönheit, P. (1994). Glucose fermentation to acetate, CO_2 and H_2 in the anaerobic hyperthermophilic eubacterium *Thermotoga maritima*: involvement of the Embden-Meyerhof pathway. *Archives of Microbiology* 161:460-470

Schurig, H., Beaucamp, N., Ostendorp, R., Jaenicke, R., Adler, E. and Knowles, J. R. (1995). Phosphoglycerate kinase and triosephosphate isomerase from the hyperthermophilic bacterium *Thermotoga maritima* form a covalent bifunctional enzyme complex. *EMBO Journal* 14:442-451

Scopes, R. K. (1994). Protein purification. Principles and practice. Springer-Verlag, New York

Selig, M. and Schonheit, P. (1994). Oxidation of organic compounds to CO_2 with sulfur or thiosulfate as electron acceptor in the anaerobic hyperthermophilic archaea *Thermoproteus tenax* and *Pyrobaculum islandicum* proceeds via the citric acid cycle. *Archives of Microbiology* 162:286-296

Selig, M., Xavier, K. B., Santos, H. and Schönheit, P. (1997). Comparative analysis of Embden-Meyerhof and Entner-Doudoroff glycolytic pathways in the hyperthermophilic archaea and the bacterium *Thermotoga*. *Archives of Microbiology* 167:217-232

Serre, M-C. and Garel, J-R. (1990). Role of the C-terminal region in the allosteric properties of *Escherichia coli* phosphofructokinase-1. *European Journal of Biochemistry* 189:487-492

Serre, M-C., Teschner, W. and Garel, J-R. (1990). Specific suppression of heterotropic interactions in phosphofructokinase by the mutation of leucine 178 into tryptophan. *The Journal of Biological Chemistry* 265:12146-12148

Shirakihara, Y. and Evans, P. R. (1988). Crystal structure of the complex of phosphofructokinase from *Escherichia coli* with its reaction products. *Journal of Molecular Biology* 204:973-994

Siebers, B., Klenk, H. P. and Hensel, R. (1998). PP_i-dependent phosphofructokinase from *Thermoproteus tenax*, an archaeal descendant of an ancient line in phosphofructokinase evolution. *Journal of Bacteriology* 180:2137-2143

Siebers, B. and Hensel, R. (1993). Glucose metabolism of the hyperthermophilic archaeon *Thermoproteus tenax* . *FEMS Microbiology Letters* 111:1-8

Simpson, C. J. and Fothergill-Gilmore, L. A. (1991). Isolation and sequence of a cDNA encoding human platelet phosphofructokinase. *Biochemical and Biophysical Research Communications* 180:197-203

Smith, E. L. (1968). Some aspects of the evolution of proteins. In: Homologous enzymes and biochemical evolution. Gordon and Breach. New York p 43-68

Smith, T. E., Blamey, J. M. and Adams, M. W. (1994). Pyruvate ferredoxin oxidoreductases of the hyperthermophilic archaeon *Pyrococcus furiosus*, and the hyperthermophilic bacterium, *Thermotoga maritima*, have different catalytic mechanisms. *Biochemistry* 33:1008-1016

Southern, E. (1979). Gel electrophoresis of restriction fragments. *Methods in Enzymology* 68:152-177

Stanier, R. Y., Ingraham, J. L., Wheelis, M. L. and Painter, P. R. (1986). General Microbiology. 5th eds. Prentice-Hall, New Jersey p 87-95

Stellwagen, E. and Harvey, W. (1975). Phosphofructokinase of yeast. *Methods in Enzymology* 9:78-85

Stetter, K. O. (1993). Life at the upper temperature border. In: Tran Thanh Van J, Tran Thanh Van K, Mounolou JC, Schneider J, McKay C (eds) Frontiers of life. Editions Frontieres, Fif-sur-Yvette, p 195-219

Stetter, K. O., Fiala, G. Huber, G., Huber, R. and Segerer, A. (1990). Hyperthermophilic microorganisms. *FEMS Microbiology Reviews* 75:117-124

Stitt, M. (1998). Pyrophosphate as an energy donor in the cytosol of plant cells: an enigmatic alternative to ATP. *Botanic Acta* 111:167-175

Stitt, M., Mieskes, G., Soling, H-D. and Heldt, W. W. (1982). On a possible role of fructose-2,6-bisphosphate in regulating photosynthetic metabolism in leaves. *FEBS Letters* 145:217-222

Su, J-G, J., Mansour, J. M. and Mansour, T. E. (1996). Purification, kinetics and inhibition by antimonials of recombinant phosphofructokinase from *Schistosoma mansoni*. *Molecular Biochemistry and Parasitology* 81:171-178

Su, Y. and Storey, K. B. (1993). Phosphofructokinase from liver of the rainbow trout, *Oncorhynchus mykiss*. *Archives of Biochemistry and Biophysics* 302:49-55

Sung, S-J. S., Xu, D-P., Galloway, C. M. and Black, Jr., C. C. (1988). A reassessment of glycolysis and gluconeogenesis in higher plants. *Physiologia Plantarum* 72:650-654

Todd, J. F., Blakeley, S. D. and Dennis, D. T. (1995). Structure of the genes encoding the α and ß subunits of castor pyrophosphate-dependent phosphofructokinase. *Gene* 152:181-186

Tomlinson, G. A., Koch, T. K. and Hochstein, L. I. (1974). The metabolism of carbohydrates by extremely halophilic bacteria: glucose metabolism via a modified Entner-Doudoroff pathway. *Canadian Journal of Microbiology* 20:105-1091

Tripodi, K. E. J. and Podesta, F. E. (1997). Purification and structure and kinetic characterisation of the pyrophosphate: fructose-6-phosphate 1-phosphotransferase from the crassulacean acid metabolism plant, pineapple. *Plant Physiology* 113:779-786

Tuininga, J. E., Verhees, C. H., van der Oost, J., Kengen, S. W., Stams, A. J. and de Vos, W. M. (1999). Molecular and biochemical characterisation of the ADP-dependent phosphofructokinase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *The Journal of Biological Chemistry* 274:21023-21028

Uryson, S. O. and Kulaev, I. S. (1968). The presence of polyphosphate glucokinase in bacteria. *Doklady Akademii Nauk SSSR* 183:957-959

Uyeda, K. and Kurooka, S. (1975). Phosphofructokinase from *Clostridium pasteurianum*. *Methods in Enzymology* 9:86-91

Uyeda, K. (1979). Phosphofructokinase. In: Advances in enzymology and related areas of molecular biology, eds. Meister, John Wilet and Sons, New York

Valdez, B. C., Chang, S. H. and Younathan, E. S. (1988). Site-directed mutagenegesis at the regulatory site of fructose-6-phosphate-1-kinase from *Bacillus stearothermophilus*. *Biochemical and Biophysical Research Communicatoins* 156:537-542

Valdez, B. C., French, B. A., Yuonathan, E. S. and Chang, S. H. (1989). Site-directed mutagenesis in *Bacillus stearothermophilus* fructose-6-phosphate 1-kinase. *The Journal of Biological Chemistry* 264:131-135

van Schaftingen, E. (1987). Fructose 2,6-bisphosphate. *Advanced in Enzymology* 59:315-396

Varma, A. K. and Peck, Jr., H. D. (1983). Utilisation of short and long-chain polyphosphates as energy sources for the anaerobic growth of bacteria. *FEMS Microbiology Letters* 16:281-285

Wächterhäuser, G. (1990). Evolution fo the first metabolic cycles. *Proceedings of National Academy of Sciences USA* 87:200-204

Wahl, G. M., Meinkoth, J. L. and Kimmel, A. R. (1987). Northern and Southern blots. *Methods in Enzymology* 152:573-581

Walker, I. D., Harris, I., Runswick, M. J. and Hudson, P. (1976). The subunits of rabbit-muscle phosphofructokinase. *European Journal of Biochemistry* 68:255-269

Wallace, D. M. (1987). Large- and small phenol extractions. *Methods in Enzymology* 152:33-48

Wessberg, K. L., Skolnick, S., Marciano-Cabral, J. X. F. and Kemp, R. G. (1995). Cloning, sequencing and expression of the pyrophosphate-dependent phosphofructo-1-kinase from *Naegleria fowleri*. *The Biochemical Journal* 307:143-149

Woese, C. R., Kandler, O. and Wheelis, M. L. (1990). Towards a natural system of organisms: proposal for the domains Archaea, Bacteria and Eukarya. *Proceedings of the National Academy of Sciences USA* 87:4576-4579

Wong, J. H., Kiss, F., Wu, M-X. and Buchanon, B. B. (1990). Pyrophosphate fructose-6-phosphate 1-phosphotransferase from tomato fruit. *Plant Physiology* 94:499-506

Wong, J. H., Kang, T. and Buchanon, B. B. (1988). A novel pyrophosphate fructose-6-phosphate 1-phosphotransferase from carrot roots. *FEBS Letters* 238:405-410

Wood, H. G. and Clark, J. E. (1988). Biological aspects of inorganic polyphosphates. *Annual Review of Biochemistry* 57:235-260

Wood, H. G., O'Brien, W. and Michels, G. (1977). Properties of carboxytransphosphorylase; pyruvate, phosphate dikinase; pyrophosphate-phosphofructokinase and pyrophosphate-acetate kinase and their roles in the metabolism of inorganic pyrophosphate. *Advanced in Enzymology* 45:85-155

Wood, H. G. and Goss, N. H. (1985). Phosphorylation enzymes of the propionic acid bacteria and the roles of ATP, inorganic pyrophosphate and polyphosphates. *Proceedings of the National Academy of Sciences USA* 82:312-315

Wu, L-F., Reizer, A., Reizer, J., Cai, B., Tomich, J. M. and Saier, Jr., M. H. (1991). Nucleotide sequence of the *Rhodobacter capsulatus fruK* gene, which encodes fructose-1-phosphate kinase: evidence for a kinase superfamily including both phosphofructokinases of *Escherichia coli*. *Journal of Bacteriology* 173:3117-3127

Xiao, Q. and Moore, C. H. (1993). The primary structure of phosphofructokinase from *Lactococcus lactis*. *Biochemical and Biophysical Research Communications* 194:65-71

Xu, J., Green, P. C. and Kemp, R. G. (1994). Identification of basic residues involved in substrate binding and catalysis by pyrophosphate-dependent phosphofructokinase from *Propionibacterium freudenreichii*. *The Journal of Biological Chemistry* 269:15553-15557

Xu, J., Seki, M., Denda, K. and Yoshida, M. (1991). Molecular cloning of phosphofructokinase-1 gene from a thermophilic bacterium *Thermus thermophilus*. *Biochemical and Biophysical Research Communications* 176:1313-1318

Yamagata, Y., Watanabe, H., Saitoh, M. and Namba, T. (1991). Volcanic production of polyphosphates and its relevance to prebiotic evolution. *Nature* 352:516-519

Yan, T-F. J. and Tao, M. (1984). Multiple forms of pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase from wheat seedlings. *The Journal of Biological Chemistry* 259:5087-5092

Yates, J. R. (1996). Protein structure analysis by mass spectrometry. *Methods in Enzymology* 271:351-377

Yoshida, M. (1972). Allosteric nature of thermostable phosphofructokinase from an extreme thermophilic bacterium. *Biochemistry* 11:1087-1093

Yoshida, M., Oshima, T. and Imahori, K. (1971). The thermostable allosteric enzyme: phosphofructokinase from an extreme thermophile. *Biochemical and Biophysical Research Communications* 43:36-39

Yu, J. P., Ladapo, J. and Whitman, W. B. (1994). Pathway of glycogen metabolism in *Methanococcus maripaludis*. *Journal of Bacteriology* 176:325-332

Zheng, Z., Roberts, D., Wang, X. and Kemp, R. G. (1999). Expression, characterisation, and crystallisation of the pyrophosphate-dependent phosphofructo-1-kinase of *Borrelia burgdorferi*. *Archives of Biochemistry and Biophysics* 371:326-331

Zheng, R-L. and Kemp, R. G. (1994). Site-directed mutagenesis of two highly conserved residues near the active site of phosphofructo-1-kinase. *Biochemical and Biophysical Research Communications* 199:577-581

Zhilina, T. N., Zavarzin, G. A., Rainey, F., Kevbrin, V. V., Kostrikina, N. A. and Lysenko, A. M. (1996a). *Spirochaeta alkalica* sp. nov., alkaliphilic anaerobes from the continental soda lakes in central Asia and the East African Rift. *International Journal of Systematic Bacteriology* 46:305-312

Zhilina, T. N., Zavarzin, G. A., Rainey, F., Kevbrin, V. V., Kostrikina, N. A. and Lysenko, A. M. (1996b). *Spirochaeta alkalica* sp. nov., *Spirochaeta africana* sp. nov., and *Spirochaeta asiatica* sp. nov., alkaliphilic anaerobes from the continental soda lakes in central Asia and the East African Rift. *International Journal of Systematic Bacteriology* 46:305-312

Zillig, W., Stetter, O., Schäfer, W., Janekovic, D., Wunderl, S., Holz, I. And Palm, P. (1981). Thermoproteales: a novel type of extremely thermoacidophilic anaerobic archaebacteria isolated from icelandic solfatares. Zentralbl. Bakteriol. Hyg. Abt. Orig. C 2:205-227

PUBICATIONS
CONFERENCE ABSTRACTS
ACADEMIC AWARD

A. PUBLICATIONS

1. Sequencing, cloning and high-level expression of a pyrophosphate-dependent phosphofructokinase from an extremely thermophilic bacterium *Dictyoglomus thermophilum*. ***Journal of Bacteriology*** (2000) 280: 4661-4666
Yan-Huai R. Ding, Ron S. Ronimus and Hugh W. Morgan

2 Purification and properties of the pyrophosphate-dependent phosphofructokinase from *Dictyoglomus thermophilum* Rt46 B.1. ***Extremophiles*** (1999) 3:131-137.
Yan-Huai R. Ding, Ron S. Ronimus and Hugh W. Morgan

3. Phosphofructokinase activities within the Spirochaetales and characterisation of the phosphofructokinase from *S. thermophila*. ***Archives of Microbiology*** (1999) 172(6):401-406
Ron S. Ronimus, Hugh W. Morgan and **Yan-Huai R. Ding**

4. The phosphofructokinases of *Thermotoga maritima*: expression and characterization of two unique enzymes. ***Journal of Bacteriology*** (in press)
Yan-Huai R. Ding, Ron S. Ronimus and Hugh W. Morgan.

B. CONFERENCE ABSTRACTS

5. Phosphofructokinases from hyperthermophilic bacteria, an ancient enzyme?
The Millennium for Microbiology, Joint Scientific Meeting and Exhibition. 8-13, July, 2000; Cairns, Queensland, Australia
Yan-Huai R. Ding, Ron S. Ronimus and Hugh W. Morgan.

6. The biochemical characteristics and phylogeny of ATP-, ADP- and pyrophosphate-dependent phosphofructokinases from thermophilic bacteria and archaea.
International Workshop Enzymology, Molecular Biology and Biogeochemistry of Thermophiles, 6-12 August, 2000, Petropavlovsk-Kamchatsky, Russia
Ron S. Ronimus, **Yan-Huai R. Ding**, Edwin de Heus and Hugh W. Morgan

7. Phosphofructokinases from *Thermotoga maritima*: cloning, over-expression and characterisation of pyrophosphate- and ATP-dependent phosphofructokinases. 3-7, September, 2000, Hamburg-Harburg, Germany
Ron S. Ronimus, **Yan-Huai R. Ding** and Hugh W. Morgan

8. The sequence, phylogeny and expression of ATP-, ADP- and pyrophosphate-dependent phosphofructokinases (PFKs) from thermophilic bacteria and archaea: implication for the evolution of the Embden-Meyerhof pathway. **Conference of Microbiology beyond 2000**. 23-26 Nov. 1999, Dunedin, NZ.
Ron S. Ronimus, **Yan-Huai R. Ding** and Hugh W. Morgan

9. Pyrophosphate-dependent phosphofructokinase activities within Spriocheatales. **IX ICBAM, IXth INTERNATIONAL CONGRESS OF BACTERIOLOGY & APPLIED MICROBIOLOGY**. 16-20, Aug. 1999. Sydney, Australia.
Yan-Huai R. Ding, Ron S. Ronimus and Hugh W. Morgan

10. The purification and characterisation of the pyrophosphate-dependent phosphofructokinases from the phylogenetically divergent thermophilic eubacteria *Dictyoglomus thermophilum* and *Spirochaete thermophila*. **Thermophiles '98**. 6-11, Sept. 1998. Le Quartz-Brest, France.
R. S. Ronimus, **Y. R. Ding** and H. W. Morgan

11. The phylogenetic distribution, biochemical characteristics and comparison of ATP-, ADP- and pyrophosphate-dependent phosphofructokinase sequences: implications for the evolution of the Embden-Meyerhof pathway.
Thermophiles '98. 6-11, Sept. 1998. Le Quartz-Brest, France.
R. S. Ronimus, **Y. R. Ding** and H. W. Morgan

C. ACADEMIC AWARD

An award of the Second Winner in an oral presentation of PhD student from New Zealand. In "The Millennium for Microbiology, Joint Scientific Meeting and Exhibition", 8-13, July, 2000; Cairns, Queensland, Australia.

JOURNAL OF BACTERIOLOGY, Jan. 2001, p. 000–000
0021-9193/01/$04.00+0 DOI: 10.1128/JB.183.2.000–000.2001

Vol. 183, No. 2

Thermotoga maritima Phosphofructokinases: Expression and Characterization of Two Unique Enzymes

YAN-HUAI R. DING*, RON S. RONIMUS, AND HUGH W. MORGAN

Thermophile Research Unit, Department of Biological Sciences, The University of Waikato, Hamilton, New Zealand

Received 5 June 2000/Accepted 25 October 2000

A pyrophosphate-dependent phosphofructokinase (PP_i-PFK) and an ATP-dependent phosphofructokinase (ATP-PFK) from *Thermotoga maritima* have been cloned and characterized. The PP_i-PFK is unique in that the K_m and V_{max} values indicate that polyphosphate is the preferred substrate over pyrophosphate; the enzyme in reality is a polyphosphate-dependent PFK. The ATP-PFK was not significantly affected by common allosteric effectors (e.g., phosphoenolpyruvate) but was strongly inhibited by PP_i and polyphosphate. The results suggest that the control of the Embden-Meyerhof pathway in this organism is likely to be modulated by pyrophosphate and/or polyphosphate.

The Embden-Meyerhof (EM), or glycolytic, pathway is nearly ubiquitous in all life forms, and enzymes of the reaction sequence are highly conserved. One of the key and definitive enzymes of the pathway is phosphofructokinase (PFK). In the majority of organisms, ATP is the phosphoryl donor for the enzyme and the reaction is a nonreversible step in the pathway. Due to its position, PFK is usually allosterically regulated by intracellular metabolites, e.g., phosphoenolpyruvate (PEP), GDP, and/or ADP (27). PFK subtypes utilizing pyrophosphate (PP_i) as the phosphoryl donor, where the reaction becomes more reversible and the enzyme is generally not subject to allosteric control mechanisms, have also been described (16, 18, 25).

Thermotoga maritima is a non-spore-forming, rod-shaped hyperthermophilic bacterium with an optimum growth temperature of 80°C and is phylogenetically classified in the order *Thermotogales*. The phylogeny of the small-subunit rRNA shows that this organism represents one of the deepest and most slowly evolving lineages of bacteria (12). *T. maritima* ferments various carbohydrates, including monosaccharides and polysaccharides, primarily via the EM pathway, and ATP-dependent PFK (ATP-PFK) activity in cell extracts has been reported (23, 24). The genome sequence of this organism indicated the presence of another PFK gene, and sequence comparison showed homology to PP_i-dependent PFK (PP_i-PFK) enzymes (17). If both genes code for functional enzymes, then *Thermotoga* would represent the unusual situation of an organism possessing two distinct PFK activities. Because of its phylogenetic position, the occurrence and origin of these genes are of importance with respect to the origins of the EM pathway. This paper describes the cloning, expression, and characterization of these enzymes, both of which exhibit unusual features.

T. maritima strain 3109 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH and grown in the medium described by Huber et al. (12). *Escherichia coli* DH5α and expression plasmid pPROEX HTb were obtained from Life Technologies. *E. coli* was grown at 30°C with vigorous aeration (200 rpm) in Luria-Bertani broth supplemented with ampicillin (100 μg ml^{-1}) when appropriate. The PFK assay was conducted essentially as described by Ding et al. (6). Preparation of genomic DNA and the alkaline lysis and cesium chloride gradient methods for large scale plasmid DNA purification followed standard procedures (21).

Construction of the PP_i-PFK and ATP-PFK expression clones. The open reading frames representing the full-length sequences of the PP_i- and ATP-PFK genes were amplified directly from genomic DNA from *T. maritima*. Primer design was based on the nucleotide sequences of the 5′and 3′ ends of the putative PFK genes (17). For the PP_i-PFK gene, the forward primer, corresponding to the N terminus, contained an upstream *Sfo*I site (in bold) and 5′-end spacer (5′-**GGAA GGC** GCC ATG GCT GAA AGA TTG GGG ATA CTC G-3′), and the reverse primer, corresponding to the C terminus, contained a flanking *Hin*dIII site (in bold) and a 5′-end spacer (5′-GCTA **AAG CTT** TAT GGA AGC TCT GTC GTA TGC CAG-3′). The primers for the ATP-PFK gene also contained *Sfo*I and *Hin*dIII sites, and their sequences were 5′-**GGCT GGC GCC** ATG AAG AAG ATA GCA GTA TAC-3′ and 5′-**CCA TAA GCT TTA** TGA AAG CAT ATG TGC TAT TTC-3′ for forward and reverse primers, respectively. Ampli*Taq* Gold DNA polymerase was used for PCR (Perkin Elmer). Both PCR products for the two genes (*pfp* and *pfk*) were of the sizes predicted from their nucleotide sequences, approximately 1,200 and 950 bp, respectively (17). These products were sequenced to confirm their identity (1) and then cloned into the expression vector after restriction digestion with *Sfo*I and *Hin*dIII, followed by ligation with T4 DNA ligase using standard protocols (21). The ligation mixture containing restriction enzyme-digested plasmid and PCR product was used to transform *E. coli* strain DH5α by electroporation, according to the manufacturer's instructions (gene pulser; Bio-Rad). Screening of the clones for those with inserts was carried out through alkaline lysis miniprep plasmid isolation (21) followed by restriction enzyme analysis.

Expression, purification, and characterization of the recombinant PP_i- and ATP-PFKs. Flask cultures of the recombinant *E. coli* clones were grown at 30°C in 700 ml of Luria-Bertani

* Corresponding author: Mailing address: Thermophile Research Unit, The University of Waikato, Private Bag 3105, Hamilton, New Zealand. Phone: 64-7-8388266. Fax: 64-7-8384324. E-mail: yrd6@hotmail.com.

TABLE 1. Properties of the cloned *T. maritima* PP_i- and ATP-PFKs

Property	PP_i-PFK[a]	ATP-PFK[b]
pH optima		
Forward reaction	5.6–5.8	7.2–8.0
Reverse reaction	5.6–6.8	ND[c]
$MgCl_2$ optimum concn (mM)	0.5–7.0	1.0–10.0
Sp act (U mg^{-1})	203	432
Thermostability (half-life at 90°C)[d]	>5 h	>5 h
Apparent molecular mass (kDa)		
SDS-PAGE	48	38
Gel filtration	97	200
Phosphoryl donors (%)[e]	Poly-P (157), PPP_i (123), PP_i (100), ATP (0), ADP (0)	ATP (100), GTP (42), UTP (14), CTP (13), TTP (10), PP_i (0), ADP (0)
Cation specificity (%)[f]	Mg^{2+} (100) > Co^{2+} (49) > Mn^{2+} (40) > Ni^{2+} (38)	Mg^{2+} (100) > Mn^{2+} (90) > Fe^{2+} (34)
Sensitivity to cations (% of control activity)[g]		
1.0 μM Cu^{2+}	57	72
1.0 μM Zn^{2+}	56	72

[a] Experimental conditions were 1.0 mM PP_i, 3 mM F-6-P, 5 mM $MgCl_2$, 0.2 mM NADH, 175 mM KCl, and 30 mM bis-Tris (pH 5.8) at 50°C.
[b] Experimental conditions were 0.5 mM ATP, 3 mM F-6-P, 5 mM $MgCl_2$, 0.2 mM NADH, 175 mM KCl, and 30 mM Tris (pH 7.8) at 50°C.
[c] Not detected.
[d] 175 mM KCl, 0.02% Triton-X 100, 0.05 mM dithiothreitol, 3 mM $MgCl_2$, and 50 mM phosphate buffer (pH 7.0).
[e] 0.1 mM phosphoryl donors. 3 mM F-6-P, 5 mM $MgCl_2$, 0.2 mM NADH,, 175 mM KCl, and 30 mM bis-Tris (pH 5.8) at 50°C for PP_i-PFK; 30 mM Tris (pH 7.8) at 50°C for ATP-PFK.
[f] 0.1 mM cations, 3 mM F-6-P, 5 mM $MgCl_2$, 0.2 mM NADH, 175 mM KCl, and 30 mM bis-Tris (pH 5.8) for PP_i-PFK; 30 mM Tris (pH 7.8) at 50°C for ATP-PFK.
[g] Cu^{2+} or Zn^{2} added in standard assay.

broth plus 100 μg of ampicillin ml^{-1} and were induced with 1 mM isopropyl-β-D-thiogalactoside when the culture optical density at 600 nm reached approximately 0.6. After 5 h of induction, the cells were harvested by centrifugation and sonicated, and the cell lysate was incubated for 40 min at 80°C. Further purification of the enzymes from the supernatant was performed using a 3.0-ml column of nickel nitrilotriacetic acid resin and elution following the manufacturer's instructions (Life Technologies). Single bands were obtained for each of the nickel nitrilotriacetic acid resin-purified proteins on denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, indicating a high degree of purity. The estimated molecular weights for the PP_i- and ATP-PFK proteins from SDS-PAGE were approximately 48,000 and 38,000, respectively (Table 1), which is in close agreement with the molecular masses derived from the amino acid sequences (including the N-terminal histidine tag, which is approximately 2 kDa). The conceptual translation masses of the full-length open reading frames of the *pfp* and *pfk* genes are 46,403 Da and 34,447 Da for the PP_i- and ATP-PFKs, respectively. The recombinant PP_i-PFK had a molecular mass of 96 kDa as determined by its elution during gel filtration chromatography, which suggests that the active molecule exists as a homodimer. In contrast, the molecular mass of the ATP-PFK was 200 kDa, and thus a homotetramer is the most probable quaternary structure.

Both recombinant proteins showed enzyme activity specific for their expected phosphoryl donors; thus, the PP_i-PFK was active with PP_i but had no activity with ATP, and vice versa for the ATP-PFK. *Thermotoga* is thus confirmed as the only prokaryote reported which possesses two different functional phosphoryl donor subtypes of PFK. As expected, the enzymes were extremely thermostable, with half-lives for both being greater than 5 h at 90°C in phosphate buffer.

PFKs generally have a requirement for a low concentration (<100 mM) of either sodium or potassium ions for optimal activity (5, 20, 27). For the *Thermotoga* enzymes, the requirement for potassium ions and the stimulation of activity by their presence was more pronounced. The optimum concentration of K^+ for both enzymes was 175 mM, and activity for the ATP- and PP_i-PFKs fell 50 and 60%, respectively, when KCl was omitted from the reaction mixture. It is possible that this requirement reflects the marine environment from which the organism was isolated. Magnesium ions were required for optimal activities of both enzymes with Co^{2+}, Mn^{2+}, and Ni^{2+} being able to substitute for Mg^{2+} with the PP_i-PFK and Mn^{2+} and Fe^{2+} being able to substitute for Mg^{2+} with the ATP-PFK (Table 1). Both *Thermotoga* enzymes were extremely sensitive to Cu^{2+} and Zn^{2+} (Table 1). This sensitivity was also found within the *Dictyoglomus thermophilum* native and recombinant PP_i-PFKs (6, 7) and the archaeal *Desulfurococcus amylolyticus* ATP-PFK (5).

The *Thermotoga* PP_i-PFK was unique in that it exhibited higher activity with tripolyphosphate (PPP_i) and polyphosphate (poly-P) as phosphoryl donors than with PP_i as the donor, and the apparent K_m and V_{max} values (Tables 2 through 4) indicate that the *Thermotoga* PP_i-PFK functions as a poly-P-dependent PFK. This is the first report of a PFK with such characteristics. The PP_i-PFK catalyzes a typically reversible reaction, but with the *Thermotoga* enzyme the pH optima for the forward and reverse reactions are unusually close; pH 5.6 to 5.8 for the forward reaction and pH 5.6 to 6.8 for the reverse reaction (Table 1). In general, other PP_i-PFKs have a pH difference of up to one unit between the forward and reverse

T4

TABLE 2. Kinetics of *Thermotoga* PP_i-PFK

Phosphoryl donor	K_m (mM)	V_{max} (U mg^{-1})	V_{max}/K_m
PP_i	0.067	203	3×10^3
PPP_i	0.010	249	2.5×10^4
Poly-P	0.0038	319	8.4×10^4

TABLE 3. Apparent ATP-PFK K_m values for ATP, F-6-P, and GTP

Phosphoryl donor	K_m (mM)	V_{max} (U mg^{-1})	V_{max}/K_m
ATP	0.009	432	4.8×10^4
F-6-P	0.437	464	1.0×10^3
GTP	1.36	294	2.1×10^2

reactions. Similar to other PP_i-PFKs, the *Thermotoga* PP_i-PFK exhibited essentially no response to traditional allosteric effectors, and presumably the reaction direction and rate (due only to the PP_i-PFK) are dictated simply by the concentrations of intracellular metabolites and the level of the enzyme.

The *Thermotoga* ATP-PFK displayed the highest activity with ATP as the phosphoryl donor but had significant activity when this was replaced by GTP, UTP, CTP, and TTP. No activity was detected with either PP_i, PPP_i, poly-P, or ADP as the phosphoryl donor (Table 1). The pH optimum for the ATP-PFK was between 7.2 and 8.0 (which is more likely to reflect the intracellular pH of the organism). The ATP-PFK showed no significant response to the common allosteric regulators. Thus, activity was only slightly inhibited by citrate at 1.0 mM, and PEP concentrations up to 5 mM did not affect the normal hyperbolic kinetic curve for fructose 6-phosphate (F-6-P). The allosteric response of the ATP-PFKs from *E. coli* and *Bacillus stearothermophilus* is potentially controlled by a glutamic acid residue at position 187 (E^{-187}) via the binding of PEP (2, 8, 22). Sequence alignment shows that the *Thermotoga* ATP-PFK also possesses an equivalent E^{-187} residue, but the biochemical properties from this characterization suggest that PEP is not vital for regulating the *Thermotoga* enzyme and thus probably does not regulate glycolysis in this organism (7). ADP had opposing effects on ATP-PFK activity, as the enzyme was slightly activated at a low concentration of ADP (129% at 0.05 mM) and partially inhibited at higher concentrations (70% at 1.0 mM), but the magnitude of these effects does not reflect allosteric control.

Surprisingly, the *Thermotoga* ATP-PFK activity was strongly inhibited by PP_i, PPP_i, or poly-P ($n = 15 \pm 3$) at concentrations of less than 0.10 mM and under conditions in which chelation effects on available Mg^{2+} could be excluded (Fig. 1). In particular, activity was strongly inhibited by both PP_i and poly-P at concentrations reported to be common in bacteria (10 to 100 μM) (14). Interestingly, the inhibition of ATP-PFK activity by PP_i could be partially alleviated by the presence of nucleotide diphosphates, i.e., ADP, GDP, or TDP (Table 5). This type of allosteric control has not previously been reported, and it

TABLE 4. Effects of PP_i, PPP_i, Poly-P, and citrate on the apparent K_m for F-6-P

Effector	K_m (mM)	V_{max} (U mg^{-1})	V_{max}/K_m
0.01 mM PP_i	0.436	148	3.4×10^2
0.025 mM PP_i	0.749	136	1.8×10^2
0.05 mM PPP_i	0.983	256	2.6×10^2
0.05 mM poly-P	1.376	278	2.0×10^2
1.0 mM citrate	0.0765	224	2.9×10^2
5.0 mM PEP	0.315	260	8.2×10^2

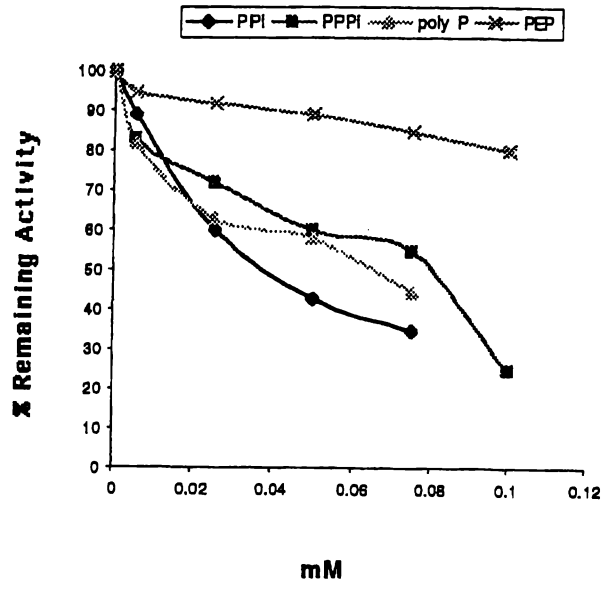


FIG. 1. Effects of PP_i, PPP_i, poly-P, and PEP on *Thermotoga* ATP-PFK activity.

seems that PP_i and/or poly-P might replace, either partially or fully, the function of PEP and other potential modulators within this organism. Although nonallosteric ATP-PFKs have been identified in other organisms, including *D. amylolyticus* (5, 9), *Trypanosoma brucei* (15), and *Lactobacillus bulgaricus* (4), the responses of these enzymes to PP_i and poly-P have not been investigated.

Role of poly-P. Both *Thermotoga* enzymes have unique properties related to poly-P: it is a preferred substrate for the PP_i-PFK and an allosteric regulator for the ATP-PFK. Poly-P is a component of volcanic condensates and deep-oceanic hydrothermal vents, and it is ubiquitously distributed in all living organisms (13) and possibly played a role in the prebiotic evolution of metabolism (3, 29). Significantly, poly-P has been used as an alternate phosphoryl/energy source to ATP for AQ: other enzymes involved with glucose metabolism. For example, poly-P-dependent glucokinase activity has been observed in *Mycobacterium tuberculosis* (11) and *Propionibacterium freudenreichii* (26, 28), and a poly-P-fructokinase has been found in *Mycobacterium phlei* (28). The poly-P-glucokinase from *P. freudenreichii* was particularly responsive to phosphoester chain

TABLE 5. Effects of some compounds on the PP_i inhibition on *Thermotoga* ATP-PFK[a]

Effector	% Activity
None	100
0.1 mM PP_i	20
0.1 mM PP_i and 0.1 mM ADP	57
0.1 mM PP_i and 0.1 mM AMP	22
0.1 mM PP_i, 0.1 mM ADP, and 0.1 mM AMP	57
0.1 mM PP_i and 0.1 mM TDP	69
0.1 mM PP_i and 0.1 mM GDP	68
0.1 mM PP_i and 0.1 mM CDP	27
0.1 mM PP_i and 0.1 mM UDP	47

[a] Experimental conditions were 0.5 mM F-6-P, 0.25 mM ATP, 5 mM $MgCl_2$, 0.2 mM NADH, 175 mM KCl, and 30 mM Tris (pH 7.8) at 50°C.

length, with the apparent K_m declining from 4.3 μM to 0.2 nM for polymer lengths of 30 and 724 residues, respectively (28). The PP_i-PFK from *Thermotoga* demonstrated a similar, though less pronounced, effect, with a decline in K_m values from 67 to 3.8 μM as phosphoester chain length increased from 2 to 18. In contrast, the PP_i-PFKs from *D. thermophilum* and *Spirochaeta thermophila* favor the pyrophosphate substrate (7, 20).

The results presented here indicate that the control of the EM pathway in *Thermotoga* may be mediated by a quite different mechanism than that conventionally found, where the activity of ATP-PFKs is allosterically controlled by either PEP, ADP AMP, F-2,6-P_2, citrate, succinate, or a combination of these. For glycolysis to proceed utilizing the ATP-PFK, the PP_i and poly-P concentrations would have to remain low (<100 μM). If poly-P accumulated and/or the pH fell, then the ATP-PFK would be inhibited and the PP_i-PFK activity would predominate. Poly-P is regarded as ubiquitous in all tested organisms (13, 14) and is present at concentrations above that needed to inhibit the ATP-PFK. Interestingly, no gene encoding a poly-P kinase has been identified in the genome of *Thermotoga*, though in other organisms other enzymes have also been implicated in the synthesis of poly-P, e.g., adenylate kinase in *Acinetobacter johnsonii* (19) and an acetate kinase in *E. coli* (10). Possibly, the PP_i-PFK could produce poly-P by means of the reverse reaction at intracellular pH values between 6.0 and 7.0. The presence of ATP-PFK activity in cell extracts of *Thermotoga* has been reported (23, 24). We found both PP_i-PFK and ATP-PFK activities in cell extracts if the assay pH was adjusted to the optimum for each enzyme (results not shown), so the enzymes appear to be expressed simultaneously. The intracellular concentration of PP_i and poly-P and the internal pH of *Thermotoga* are unknown, but it will be important to determine these if the control of glycolysis in *Thermotoga* is to be understood. In summary, *Thermotoga* appears to be unique in that it contains the genes for two distinct PFKs and both genes can express functional enzymes. Both enzymes have unique properties, in particular, their responses to PP_i and poly-P, and it is likely that these metabolites may play a central role in the control of glucose metabolism in this organism.

We thank the Royal Society Marsden Science Foundation and the University of Waikato for its financial support during the course of this study.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, W. Myers, and D. L. Lipman.** 1997. Basic local alignment search tool. J. Mol. Biol. **215**:403–410.
2. **Auzat, I., G. Le. Bras, P. Branny, F. D. L. Torre, B. Theunissen, and J.-R. Garel.** 1994. The role of Glu 187 in the regulation of phosphofructokinase by phosphoenolpyruvate. J. Mol. Biol. **235**:68–72.
3. **Baltscheffsky, H.** 1996. Energy conversion leading to the origin and early evolution of life: did inorganic pyrophosphate precede adenosine triphosphate? p. ●●●. *In* H. Baltscheffsky (ed.), Origin and evolution of biological energy conversion. VCH Publishers, New York, N.Y.
4. **Branny, P., F. De La Torre, and J.-R. Garel.** 1993. Cloning, sequencing, and expression in *Escherichia coli* of the gene coding for phosphofructokinase in *Lactobacillus bulgaricus*. J. Bacteriol. **175**:5344–5349.
5. **Ding, Y.-H. R.** 2000. Phosphofructokinases from extremely thermophilic microorganisms. Ph.D. thesis. The University of Waikato, Hamilton, New Zealand.
6. **Ding, Y.-H. R., R. S. Ronimus, and H. W. Morgan.** 1999. Purification and properties of the pyrophosphate-dependent phosphofructokinase from *Dictyoglomus thermophilum* Rt46 B.1. Extremophiles **3**:131–137.
7. **Ding, Y.-H. R., R. S. Ronimus, and H. W. Morgan.** 2000. Sequencing, cloning and high-level expression of the *pfp* gene encoding a PP_i-dependent phosphofructokinase from the extremely thermophilic eubacterium *Dictyoglomus thermophilum*. J. Bacteriol. **182**:4661–4666.
8. **Evans, P. R., and P. J. Hudson.** 1979. Structure and control of phosphofructokinase from *Bacillus stearothermophilus*. Nature **279**:500–504.
9. **Hansen, T., and P. Schönheit.** 2000. Purification and properties of the first-identified, archaeal, ATP-dependent 6-phosphofructokinase, an extremely thermophilic non-allosteric enzyme, from the hyperthermophilic *Desulfurococcus amylolyticus*. Arch. Microbiol. **173**:103–109.
10. **Hardoyo, K. Yamada, A. Muramatsu, Y. Anbe, J. Kato, and H. Ohtake.** 1994. Molecular genetics of polyphosphate accumulation in *Escherichia coli*, p. 209–214. *In* A. Torriani-Gorini, E. Yagil, and S. Silver (ed.), Phosphate in microorganisms: cellular and molecular biology. ASM Press, Washington, D.C.
11. **Hsieh, P.-C., B. C. Shenoy, J. E. Jentoft, and N. F. B. Phillips.** 1993. Purification of polyphosphate and ATP glucose phosphotransferase from *Mycobacterium tuberculosis* $H_{37}Ra$: evidence that poly(P) and ATP glucokinase activities are catalyzed by the same enzyme. Protein Expr. Purif. **4**:76–84.
12. **Huber, R., T. A. Langworthy, H. Konig, M. Thomm, C. R. Woese, U. B. Sleytr, and K. O. Stetter.** 1986. *Thermotoga maritima* sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up to 90°C. Arch. Microbiol. **144**:324–333.
13. **Kornberg, A.** 1995. Inorganic polyphosphate: toward making a forgotten polymer unforgettable. J. Bacteriol. **177**:491–496.
14. **Kulaev, I. S.** 1979. The biochemistry of inorganic polyphosphates. John Wiley & Sons, Inc., New York, N.Y.
15. **Michels, P. A. M., N. Chevalier, F. R. Opperdoes, M. H. Rider, and D. J. Rigden.** 1997. The glycosomal ATP-dependent phosphofructokinase of *Trypanosoma brucei* must have evolved from an ancestral pyrophosphate-dependent enzyme. Eur. J. Biochem. **250**:698–704.
16. **Morgan, H. W., and R. S. Ronimus. 1998. Pyrophosphate-dependent phosphofructokinase in thermophilic and non-thermophilic microorganisms, p. ●●●.** *In* M. W. Adams and J. W. Wiegel (ed.), Thermophiles: the key to molecular evolution and the origin of life. Taylor and Francis, London, United Kingdom.
17. **Nelson, K. E., R. A. Clayton, S. R. Gill, M. L. Gwinn, G. R. Dodson, D. D. Haft, K. Hickey, J. D. Peterson, W. C. Nelson, K. A. Ketchum, L. McDonald, T. R. Utterback, J. A. Malek, K. D. Linher, M. M. Garrett, A. M. Steward, M. D. Cotton, M. S. Pratt, C. A. Phillips, D. Richardson, J. Heidelberg, G. G. Sutton, R. D. Fleischmann, J. A. Eisen, O. White, S. L. Salzberg, H. O. Smith, J. C. Venter, and C. M. Fraser.** 1999. Evidence for lateral gene transfer between archaea and bacteria from genome sequence of *Thermotoga maritima*. Nature **399**:323–329.
18. **Reeves, R. E., D. J. South, H. J. Blytt, and L. G. Warren.** 1974. Pyrophosphate:D-fructose 6-phosphate 1-phosphotransferase. J. Biol. Chem. **249**: 7737–7741.
19. **Resnick., S. M., and A. J. Zehnder.** 2000. In vitro ATP regeneration from polyphosphate and AMP by polyphosphate:AMP phosphotransferase and adenylate kinase from *Acinetobacter johnsonii* 210A. Appl. Environ. Microbiol. **66**:2045–2051.
20. **Ronimus, R. S., H. M. Morgan, and Y.-H. R. Ding.** 1999. Phosphofructokinase activities within the order Spirochaetales and the characterization of the pyrophosphate-dependent phosphofructokinase from *Spirochaeta thermophila*. Arch. Microbiol. **172**:401–406.
21. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
22. **Schirmer, T., and P. R. Evans.** 1990. Structural basis of the allosteric behaviour of phosphofructokinase. Nature **343**:140–145.
23. **Schröder, C., M. Selig, and P. Schönheit.** 1994. Glucose fermentation to acetate, CO_2, and H_2 in the anaerobic hyperthermophilic eubacterium *Thermotoga maritima*: involvement of the Embden-Meyerhof pathway. Arch. Microbiol. **161**:460–470.
24. **Selig, M., K. B. Xavier, H. Santos, and P. Schönheit.** 1997. Comparative analysis of Embden-Meyerhof and Entner-Doudoroff glycolytic pathways in the hyperthermophilic archaea and the bacterium Thermotoga. Arch. Microbiol. **167**:217–232.
25. **Siebers, B., H. P. Klenk, and R. Hensel.** 1998. PP_i-dependent phosphofructokinase from *Thermoproteus tenax*, an archaeal descendant of an ancient line in phosphofructokinase evolution. J. Bacteriol. **180**:2137–2143.
26. **Uryson, S. O., and I. S. Kulaev.** 1968. The presence of polyphosphate glucokinase in bacteria. Dokl. Akad. Nauk SSSR **183**:957–959.
27. **Uyeda, K.** 1979. Phosphofructokinase. Adv. Enzymol. **48**:193–241.
28. **Wood, H. G., and J. E. Clark.** 1988. Biological aspects of inorganic polyphosphates. Annu. Rev. Biochem. **57**:235–260.
29. **Yamagata, Y., H. Watanabe, M. Saitoh, and T. Namba.** 1991. Volcanic production of polyphosphates and its relevance to prebiotic evolution. Nature **352**:516–519.

JOURNAL OF BACTERIOLOGY, Aug. 2000, p. 4661–4666 Vol. 182, No. 16
0021-9193/00/$04.00+0

Sequencing, Cloning, and High-Level Expression of the *pfp* Gene, Encoding a PP_i-Dependent Phosphofructokinase from the Extremely Thermophilic Eubacterium *Dictyoglomus thermophilum*

YAN-HUAI R. DING,* RON S. RONIMUS, AND HUGH W. MORGAN

Thermophile Research Unit, Department of Biological Sciences, The University of Waikato, Hamilton, New Zealand

Received 21 March 2000/Accepted 26 May 2000

The sequencing, cloning, and expression of the *pfp* gene from *Dictyoglomus thermophilum*, which consists of 1,041 bp and encodes a pyrophosphate-dependent phosphofructokinase, are described. A phylogenetic analysis indicates that the enzyme is closely related to the pyrophosphate-dependent enzyme from *Thermoproteus tenax*. The recombinant and native enzymes share a high degree of similarity for most properties examined.

Phophofructokinase (PFK) is one of the key enzymes in glycolysis. The textbook version of ATP-dependent PFK (ATP-PFK) is present throughout the domains of *Eucarya*, *Bacteria*, and *Archaea*, although it is not yet known if the single archaeal ATP-PFK recently described is related by sequence (6, 11, 15). Since the first pyrophosphate-dependent PFK (PP_i-PFK) was reported by Reeves et al., who identified it in *Entamoeba histolytica* (21), a large number of PP_i-PFKs have been found in higher plants, primitive eukaryotes, and bacteria, and a single example has been found in archaea (14, 17, 22, 25, 28). It has been suggested previously that PP_i-PFKs may represent the ancestral PFK because PP_i is thought to be an ancient source of metabolic energy utilized before the advent of ATP as the near-universal energy carrier (4, 12, 16). Furthermore, because PP_i-PFKs can function more readily than ATP-PFKs in the gluconeogenic direction, they could also have acted as primitive fructose-1,6-bisphosphatases. *Dictyoglomus thermophilum* Rt46 B.1 is an extremely thermophilic bacterium isolated from a New Zealand hot spring and is deeply rooted near the base of the order *Thermotogales* (18). The *Dictyoglomus* PP_i-PFK has recently been purified and characterized (8). In this paper, we describe the cloning, phylogeny, and overexpression of the first PP_i-PFK from an extremely thermophilic bacterium.

D. thermophilum Rt46 B.1 was obtained from the Thermophile Research Unit Culture Collection, Hamilton, New Zealand, and grown in *Dictyoglomus* medium (18). The *Escherichia coli* strain used for cloning and expression experiments was JM109 (Promega Life Sciences), and it was grown at 37°C with vigorous aeration in Luria-Bertani broth supplemented with ampicillin (100 μg/ml). The expression plasmid pKK223-3 was obtained from Pharmacia Biotech. Genomic DNA from *D. thermophilum* was prepared as described by Sambrook et al. (24). Large-scale plasmid DNA was purified from *E. coli* by using the alkaline lysis method combined with double cesium chloride gradient purification. Restriction digests, electrophoresis, and Southern blotting were carried out according to standard methods (24). In order to clone the full-length *pfp* gene from *D. thermophilum*, a 350-bp fragment was initially amplified, using Ampli *Taq* Gold polymerase (Perkin-Elmer Cetus), by PCR. The sense and antisense degenerate primers were designed according to the N-terminal sequence of the purified native protein and the internal fructose-6-phosphate (F-6-P) binding conserved sequence, respectively. The PCR product was used to probe genomic DNA of *Dictyoglomus* strain Rt46 B.1 digested with 16 restriction enzymes. Analysis of the Southern data identified several bands of appropriate sizes within the *Sau*3AI, *Rsa*I, and *Sau*96I digests. The inverse-PCR technique was employed, and with each enzyme, a single fragment was amplified for sequencing (fragments were 360, 700, and 2,200 bp, respectively) (7).

Identification of ORF encoding the *pfp* gene. The ORF representing the full-length sequence of the *D. thermophilum* PP_i-PFK gene was amplified using a forward primer corresponding to the N terminus (the first seven codons) and containing an upstream *Eco*RI site (in bold) and a 5′-end spacer (5′-GGA **GAA TTC** ATG AGT AAA ATG CGT ATT GGT G-3′), and a reverse primer corresponding to the C terminus (the last seven codons) and containing a flanking *Hin*dIII site (in bold) and a 5′-end spacer (5′-GC **GAG AAG CTT** TAC TTA TTA AAG AAA GTT TTT ACG-3′). The complete sequence of 1,233 nucleotides obtained from overlapping inverse-PCR contigs contains an ORF of 1,041 bp with 346 codons, beginning with an ATG and ending with a TAA stop codon. One hairpin sequence downstream of the stop codon which could act as a transcription terminator was found (20). Potential promoter sites at the 5′ end of the coding region were also identified. For example, a Pribnow-like box sequence (TAAAAT) is located 41 nucleotides upstream from the ATG start codon and is similar to the −10 (TATAAT) promoter sequence. In addition, the TTGTCA sequence located 17 bases upstream from the TAAAAT sequence is similar to the −35 (TTGACA) promoter sequence (20). Finally, a potential ribosome binding site (AGGAGG) was also identified and is located 4 nucleotides upstream of the start codon. The codon usage for *Dictyoglomus* PFK, as expected, reflected the G+C content of the genomic DNA, which is 29.3 mol% (18). For example, among the 43 glycine codons, only 2 were terminated with a C and 3 were terminated with a G. In addition, all codons for phenylalanine, proline, and threonine were terminated with either an A or a U (not shown).

Tree construction and phylogenetic comparison. Ten representative amino acid sequences of PFKs from eukaryotes, bacteria, and the crenarcheaon *Thermoproteus tenax* were re-

* Corresponding author. Mailing address: Thermophile Research Unit, The University of Waikato, Private Bag 3105, Hamilton, New Zealand. Phone: 0064-7-8388266. Fax: 0064-7-8384324. E-mail: yrd2@waikato.ac.nz or yrd6@hotmail.com.

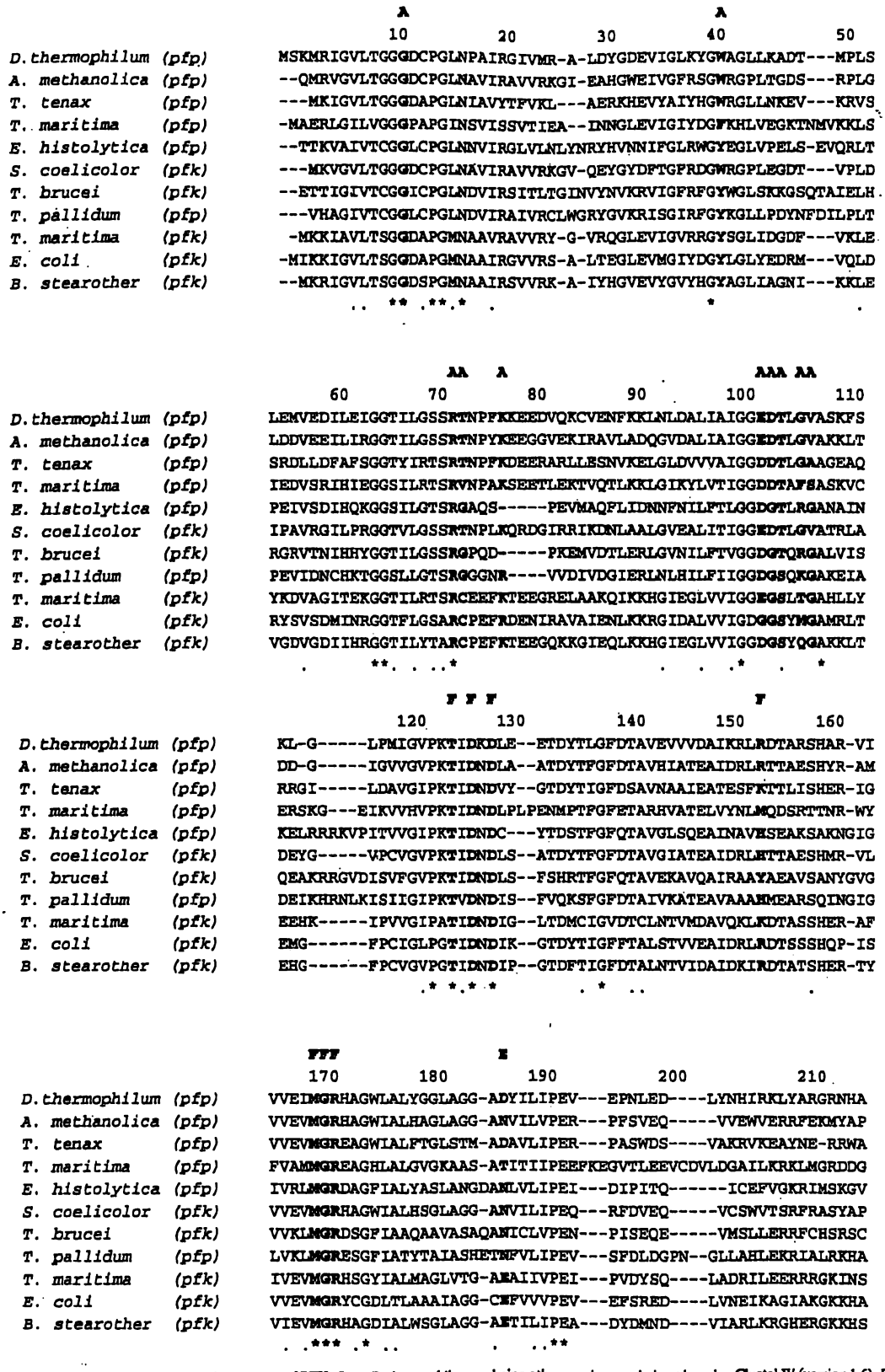


FIG. 1. Multiple alignment of amino acid sequences of PFKs from *D. thermophilum* and nine other species, carried out by using Clustal W (version 1.6). *B. stearother*, *B. stearothermophilus*, *pfp* and *pfk*, genes encoding PP_i-PFK and ATP-PFK, respectively; A, F, and E, residues involved in binding ATP, F-6-P, and phosphoenolpyruvate (PEP), respectively, for the *E. coli* ATP-PFK. Asterisks, identical residues; dashes, gaps; dots, highly conserved residues.

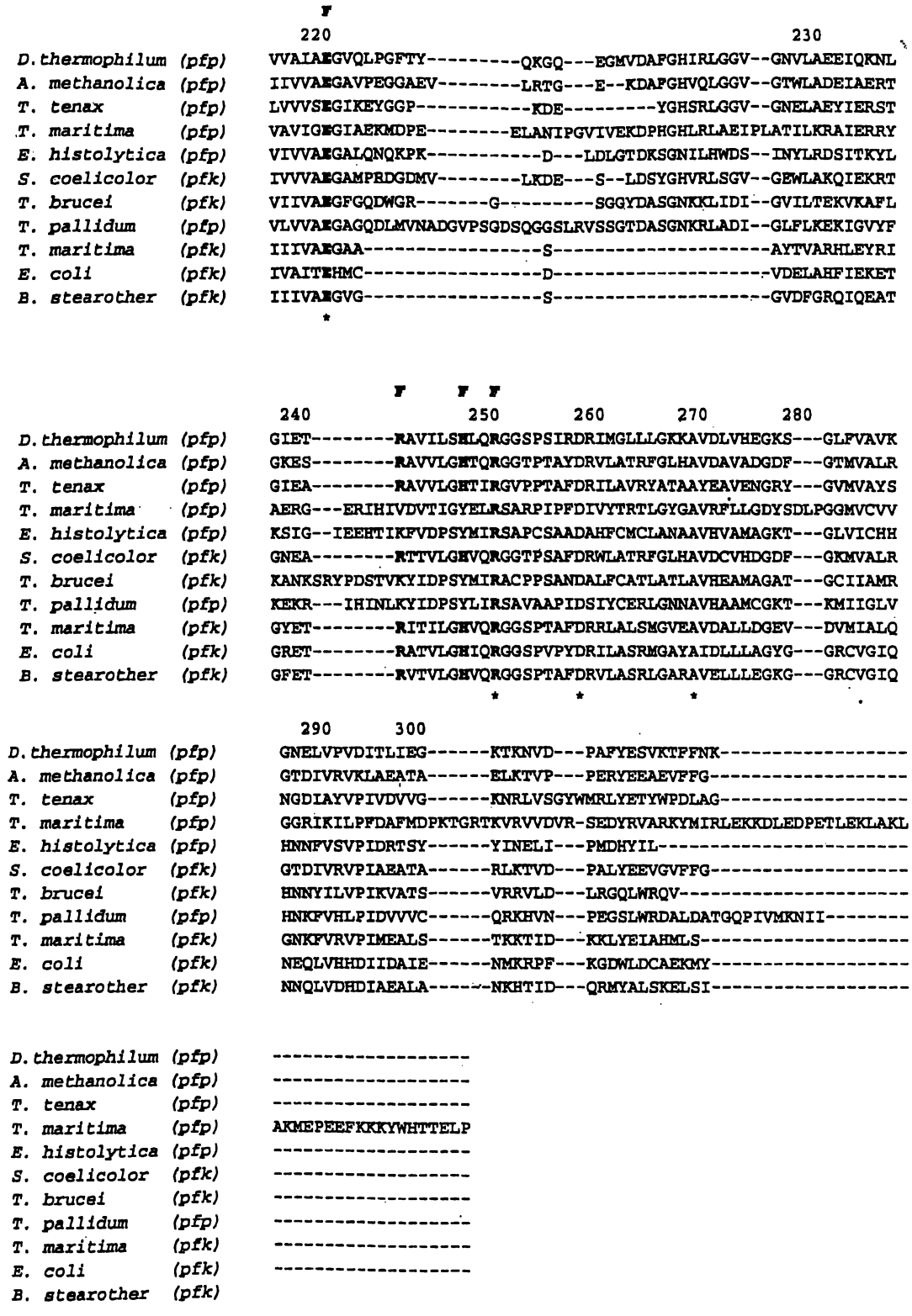


FIG. 1—*Continued.*

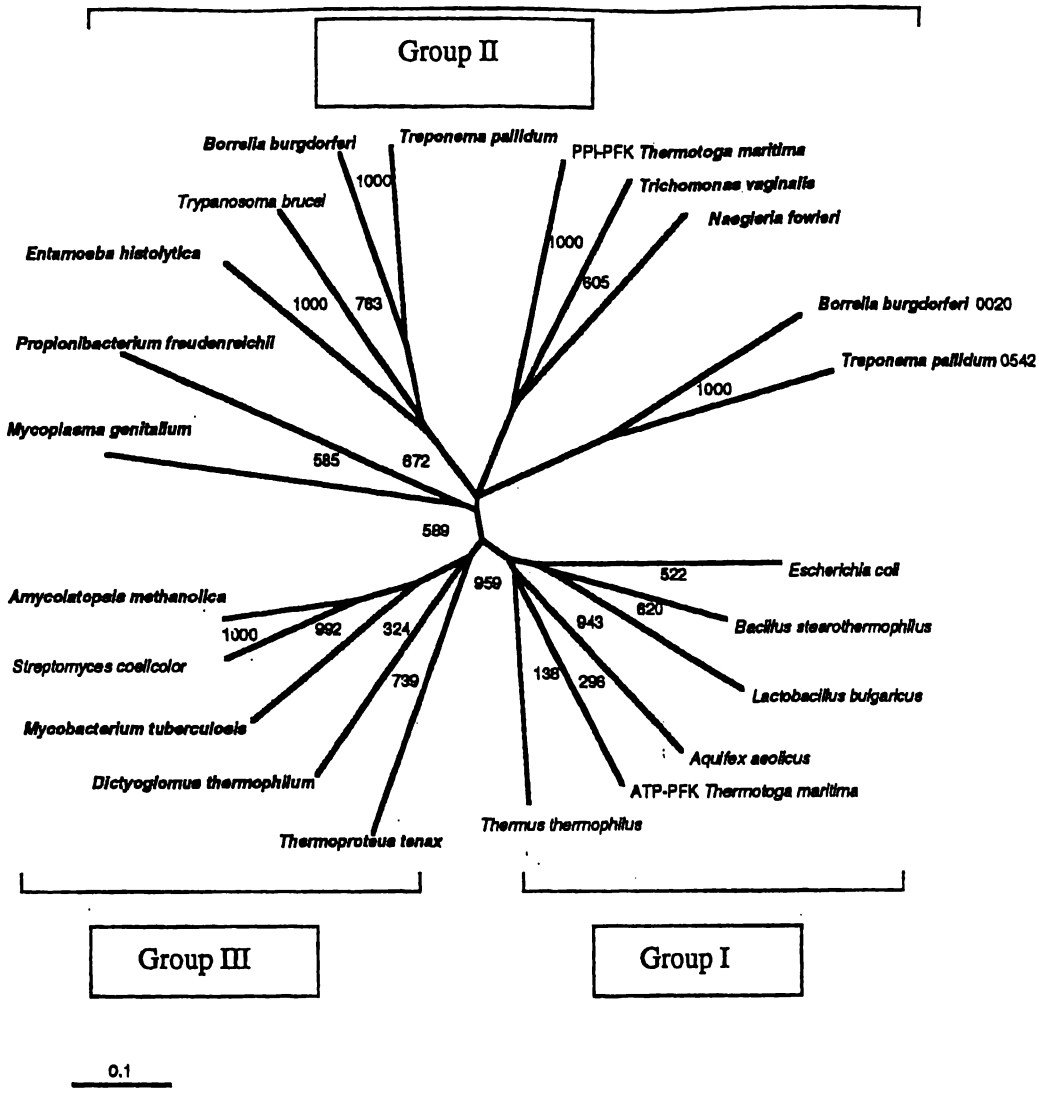


FIG. 2. Phylogenetic relationships of PP_i- and ATP-PFKs. The tree is based on distance analysis (neighbor-joining method) of full amino acid sequences of PP_i- and ATP-PFKs in the EMBL and Swiss Prot databanks. Bootstrap values are based on 1,000 replicates and are given at each node. All of the PP_i-PFKs are bolded. Group I includes *Thermus thermophilus*, *Thermotoga maritima* (ATP-PFK), *Aquifex aeolicus*, *Lactobacillus bulgaricus*, *B. stearothermophilus*, and *E. coli*. Group II includes *Treponema pallidum* 0542, *Borrelia burgdorferi* 0020, *Naegleria fowleri*, *Trichomonas vaginalis*, *Thermotoga maritima* (PP_i-PFK), *Treponema pallidum*, *B. burgdorferi*, *Trypanosoma brucei*, *Entamoeba histolytica*, *Propionibacterium freudenreichii*, and *Mycoplasma genitalium*. Group III includes *Thermoproteus tenax*, *D. thermophilum*, *Mycobacterium tuberculosis*, *A. methanolica*, and *S. coelicolor*.

trieved from sequence databases and aligned (1) with the PP_i-PFK sequence from *D. thermophilum* (Fig. 1). The F-6-P binding sites were highly conserved in all PFKs examined. *Dictyoglomus* PFK has almost the same amino acid residues for F-6-P binding as those from *Amycolatopsis methanolica*, *T. tenax*, *Streptomyces coelicolor*, *Bacillus stearothermophilus*, and *E. coli* (9, 23). The PFK from *Dictyoglomus* also possesses amino acid residues in the phosphoryl binding site identical to those for *A. methanolica*, *T. tenax* (PP_i-PFK), and *S. coelicolor* (ATP-PFK). A phylogenetic tree was generated from 22 representative amino acid sequences (Fig. 2). The *D. thermophilum* sequence is most homologous to the group III PFKs, as defined by Siebers et al. (25), including the PP_i-PFKs from *T. tenax* and *A. methanolica*, the putative PP_i-PFK from *Mycobacterium tuberculosis*, and the ATP-PFK from *S. coelicolor*. The full sequence of the *Dictyoglomus pfp* gene agreed completely with the N-terminal sequence obtained from the purified native protein, except for the lack of the first methionine residue in the native enzyme, which supports our previous suggestion, based on the sequence, that the enzyme has homology with the *T. tenax* enzyme (8). The data from the alignment of 22 sequences and the phylogenetic tree strongly support the contention that the central carbohydrate metabolism of glycolysis was established before the segregation of the three domains of life. The fact that all of the sequences presented in the phylogenetic tree can be aligned, despite their varying phophoryl

TABLE 1. Purification of the recombinant PP_i-PFK from *D. thermophilum* Rt46 B.1

Step	Total activity (U)	Total protein (mg)	Sp act (U/mg)	Purification (fold)	Yield (%)
Heated supernatant	757	2,300	0.33	1	100
Phenyl-Sepharose	516	329	1.6	4.8	68
Q-Sepharose	363	79	4.6	14	48
Red dye 120	328	53	6.2	19	43

donor specificities, demonstrates their homology and therefore, their likely evolution from a single common ancestral sequence (9, 11).

A number of X-ray crystallographic and site-directed mutagenesis studies have investigated the roles of PFK amino acid residues in substrate binding and catalysis (3, 9, 19, 23), as well as those amino acid residues related to allosteric properties (5, 27). For example, when the Glu^{-187} of *E. coli* PFK is replaced by Asp or Leu, the allosteric transition is abolished. The Glu^{-187} of *E. coli* PFK is apparently necessary for the protein to undergo the change from the active into the inactive state induced by phosphoenolpyruvate (PEP) (3). In addition, Valdez et al. (26) reported that Arg^{-25} and Arg^{-211} of the *B. stearothermophilus* ATP-PFK are involved in direct binding of PEP and GDP (26). Sequence analysis of the *Dictyoglomus* PP_i-PFK shows that this enzyme has an Asp^{-187}, a Met^{-25} and an Arg^{-211}, which suggests that this enzyme should be nonallosteric. Biochemical properties reported here and in our earlier study (8) have also confirmed that both the native and recombinant *Dictyoglomus* PFK enzymes are nonallosteric.

Expression, purification, and characterization of recombinant PP_i-PFK. The ligation mixture containing restriction enzyme-digested plasmid pKK223-3 and PCR product with the full-length *Dictyoglomus pfp* gene was used to transform *E. coli* strain JM109 by electroporation (Gene Pulser; Bio-Rad). Potential clones with inserts were examined by restriction digests with *Eco*RI and *Hin*dIII before being grown in 800 ml of Luria-Bertani medium with ampicillin (100 μg/ml) at 30°C. In-frame ligation of the amplified DNA into pKK223-3 plasmid was confirmed by DNA sequencing (not shown). Isopropyl-β-D-thiogalactoside (1.0 mM) was added when the cell culture density reached approximately 0.6 at 595 nm, and enzyme activity was checked hourly after induction for up to 5 h. The conditions for purification and characterization of the recombinant enzyme were essentially the same as those described for the purification of the native enzyme (8). The final yield of the purified recombinant enzyme was 53 mg of protein from 800 ml of induced culture (approximately 5 g [wet weight] of cell pellet), indicating high-level expression of the enzyme (Table 1). A single band was obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) from the purified *Dictyoglomus* recombinant enzyme (Table 1; Fig. 3). The recombinant and native enzymes had the same estimated molecular weights (approximately 37,000) when both were run on the same SDS-PAGE gel (data not shown). In addition, a comparison of the native and recombinant *Dictyoglomus* PP_i-PFKs enzymes demonstrates that they possess a high degree of similarity (Table 2). Most of the biochemical and the kinetic properties of the recombinant enzyme were very similar to those of the native enzyme (8), including, for example, thermostability and the extreme sensitivity to Cu^{2+}. Aliquots of the recombinant and native enzyme preparations were dialyzed against MilliQ water overnight at 4°C to prepare them for molecular mass analysis using mass spectrometry. Enzyme solution (5 μl) was mixed with 50% formic acid (5 μl) to ionize the proteins prior to mass spectrometric detection in the mobile phase, which was 50:50 methyl cyanide/H_2O (continuous flow rate of 0.02 ml/min). The calculated molecular mass for the recombinant enzyme from the gene sequence was 37,445 Da which is similar to the size estimates obtained with SDS-PAGE (estimated molecular weight, 37,000) and mass spectrometry (estimated molecular weight, 38,057). The enzyme is indicated to be a homodimer based on a molecular mass of 74.2 kDa using a large secondary peak from mass spectrometry (10) and 76.7 kDa by gel filtration and comparison with the size estimate obtained by SDS-PAGE.

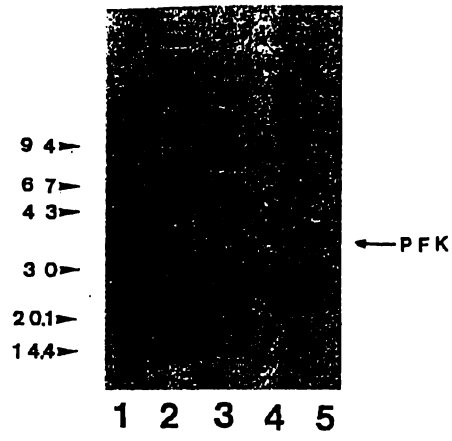


FIG. 3. SDS-PAGE gel (silver-stained) of fractions obtained during purification of the recombinant *Dictyoglomus* PP_i-PFK. Lane 1, molecular mass markers, as follows: phosphorylase b (molecular mass, 94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa); lane 2, cell extract following heat treatment; lanes 3 through 5, fractions obtained after purification through phenyl-Sepharose, Q-Sepharose, and red dye 120 ligand, respectively. Lanes 2, 3, 4, and 5 contained 3.0, 1.0, 0.75, and 0.75 μg of protein, respectively.

PFK is present in most organisms, and its slowly evolving nature makes it a valuable genotypic marker enabling the exploration of the origins of glycolysis and of life. Within this

TABLE 2. Comparison of some properties of native and recombinant *D. thermophilum* PP_i-PFK

PFK	Mg^{2+} (mM)	pI	Mol wt[a]	Forward pH optimum	K_m (mM)		Half-life (min) at 80°C[b]	% Activity in response to 1 μm Cu^{2+}
					F-6-P	PP_i		
Recombinant	1–3	4.3	37,000	5.8	0.136	0.116	165	40
Native	1–3	4.4	37,000	5.9	0.127	0.082	170	38

[a] Determined by SDS-PAGE.

[b] Native and recombinant enzymes (150 μg ml^{-1}) were incubated at 80°C in buffer containing 50 mM MOPS, 2 mM $MgCl_2$, and 0.02% Triton X-100, pH 6.4, under a mineral oil overlay for different lengths of time. Residual activity at 50°C was then assayed and compared to that of the unheated control.

context, Mertens (13) has suggested that the role of PP_i-PFK is that of a glycolytic enzyme adapted to anaerobiosis. However, other investigators have pointed out that PP_i-PFKs (especially those in *Archaea* and extremely thermophilic bacteria) are likely to be more ancient than ATP-PFKs (2, 16). The results from the *Dictyoglomus* PP_i-PFK strongly support the latter hypothesis. As seen with the enzyme from *T. tenax*, the *Dictyoglomus* enzyme is the first biochemically characterized extremely thermophilic bacterial PP_i-dependent enzyme and offers another opportunity to gain insight into the differentiation of PFK substrate specificities and the characteristics of the phenotype of the original ancestral PFK precursor. The group III enzymes are suggested to be of a more ancient origin than group II enzymes, so it follows that the PP_i-PFKs from *T. tenax* and extremely thermophilic bacteria may represent more ancient enzymes than the enzymes in mesophilic bacteria, primitive eukaryotes, and higher plants. In support of this latter contention, in two cases, that of *S. coelicolor* and *Trypanosoma brucei*, there is strong sequence evidence for the evolution of ATP-PFKs from PP_i-PFKs (2, 15). Finally, it is hoped that the X-ray crystallographic determination of the structure of PP_i-PFK from *D. thermophilum* Rt46 B.1, which is in progress, will help clarify the phylogenetic origins of PFKs.

Nucleotide sequence accession number. The *Dictyoglomus pfp* gene sequence data has been submitted to GenBank under accession number AF268276.

We thank the Royal Society Marsden Science Foundation and the University of Waikato for financial support during the course of this study.

We also thank Pat Gread and Cameron Evans for assistance with the mass spectrometry.

REFERENCES

1. Altschul, S. F., W. Gish, W. Miller, W. Myers, and D. L. Lipman. 1997. Basic local alignment search tool. J. Mol. Biol. 215:403–410.
2. Alves, A. M. C. R., G. J. W. Euverink, M. J. Bibb, and L. Dijkhuizen. 1997. Identification of ATP-dependent phosphofructokinase as a regulatory step in the glycolytic pathway of the actinomycete *Streptomyces coelicolor* A3(2). Appl. Environ. Microbiol. 63:956–961.
3. Auzat, I., G. Le Bras, P. Branny, F. D. L. Terre, B. Theunissen, and J.-R. Garel. 1994. The role of Glu 187 in the regulation of phosphofructokinase by phosphoenolpyruvate. J. Mol. Biol. 235:68–72.
4. Baltscheffsky, H. (ed.). 1996. Energy conversion leading to the origin and early evolution of life: did inorganic pyrophosphate precede adenosine triphosphate?, p. 1–9. In Origin and evolution of biological energy conversion. VCH Publishers, New York, N.Y.
5. Berger, S. A., and P. R. Evans. 1990. Active-site mutants altering the cooperativity of *E. coli* phosphofructokinase. Nature 343:575–576.
6. Byrnes, W. M., X. Zhu, E. S. Younathan, and S. H. Chang. 1994. Kinetic characteristics of phosphofructokinase from *Bacillus stearothermophilus*: MgATP nonallosterically inhibits the enzyme. Biochemistry 33:3424–3431.
7. Collins, S. F., and S. M. Weissman. 1984. Directional cloning of DNA fragments at large distances from an initial probe: a circularization method. Genetics 81:6812–6816.
8. Ding, Y.-H. R., R. S. Ronimus, and H. W. Morgan. 1999. Purification and properties of the pyrophosphate-dependent phosphofructokinase from *Dictyoglomus thermophilum* Rt46 B.1. Extremophiles 3:131–137.
9. Evans, P. R., and P. J. Hudson. 1979. Structure and control of phosphofructokinase from *Bacillus stearothermophilus*. Nature 279:500–504.
10. Hans, V., U. Heinemann, C. M. Dobson, and C. V. Robinson. 1998. Detection of a monomeric intermediate associated with dimerization of protein Hu by mass spectrometry. J. Am. Chem. Soc. 120:6427–6428.
11. Hansen, T., and P. Schönheit. 2000. Purification and properties of the first-identified, archaeal, ATP-dependent 6-phosphofructokinase, an extremely thermophilic non-allosteric enzyme, from the hyperthermophilic *Desulfurococcus amylolyticus*. Arch. Microbiol. 173:103–109.
12. Kornberg, A. 1995. Inorganic polyphosphate: toward making a forgotten polymer unforgettable. J. Bacteriol. 177:491–496.
13. Mertens, E. 1991. Pyrophosphate-dependent phosphofructokinase, an anaerobic glycolytic enzyme? FEBS Lett. 285:1–5.
14. Mertens, E., E. Van Schaftingen, and M. Müller. 1989. Presence of a fructose-2,6-bisphosphate-insensitive pyrophosphate:fructose-6-phosphate phosphotransferase in the anaerobic protozoa *Tritrichomonas foetus*, *Trichomonas vaginalis* and *Isotricha prostoma*. Mol. Biochem. Parasitol. 37:183–190.
15. Michels, P. A. M., N. Chevalier, F. R. Opperdoes, M. H. Rider, and D. J. Rigden. 1997. The glycosomal ATP-dependent phosphofructokinase of *Trypanosoma brucei* must have evolved from an ancestral pyrophosphate-dependent enzyme. Eur. J. Biochem. 250:698–704.
16. Morgan, H. W., and R. S. Ronimus. 1998. Pyrophosphate-dependent phosphofructokinase in thermophilic and non-thermophilic microorganisms. *In* M. W. Adam and J. W. Wiegel (ed.), Thermophiles: the key to molecular evolution and the origin of life. Taylor and Francis, London, United Kingdom.
17. O'Brien, W. E., S. Bowien, and H. G. Wood. 1975. Isolation and characterisation of a pyrophosphate-dependent phosphofructokinase from *Propionibacterium shermanii*. J. Biol. Chem. 250:8690–8695.
18. Patel, B. K., H. W. Morgan, J. Wiegel, and R. M. Daniel. 1987. Isolation of an extremely thermophilic chemoorganotrophic anaerobe similar to *Dictyoglomus thermophilum* from New Zealand hot springs. Arch. Microbiol. 147: 21–24.
19. Poorman, R. A., A. Randolph, R. G. Kemp, and R. L. Heinrikson. 1984. Evolution of phosphofructokinase-gene duplication and creation of new effector sites. Nature 309:467–469.
20. Pribnow, D. 1975. Nucleotide sequence of an RNA polymerase binding site at an early T7 promoter. Proc. Natl. Acad. Sci. USA 72:184–188.
21. Reeves, R. E., D. J. South, H. J. Blytt, and L. G. Warren. 1974. Pyrophosphate:D-fructose 6-phosphate 1-phosphotransferase. J. Biol. Chem. 249:7737–7741.
22. Ronimus, R. S., H. M. Morgan, and Y.-H. R. Ding. 1999. Phosphofructokinase activities within the order Spirochaetales and the characterisation of the pyrophosphate-dependent phosphofructokinase from *Spirochaeta thermophila*. Arch. Microbiol. 172:401–406.
23. Rypniewski, W. R., and P. R. Evans. 1989. Crystal structure of unliganded phosphofructokinase from *Escherichia coli*. J. Mol. Biol. 207:805–821.
24. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
25. Siebers, B., H. P. Klenk, and R. Hensel. 1998. PP_i-dependent phosphofructokinase from *Thermoproteus tenax*, an archaeal descendant of an ancient line in phosphofructokinase evolution. J. Bacteriol. 180:2137–2143.
26. Valdez, B. C., S. H. Chang, and E. S. Younathan. 1988. Site-directed mutagenesis at the regulatory site of fructose-6-phosphate-1-kinase from *Bacillus stearothermophilus*. Biochem. Biophys. Res. Com. 156:537–542.
27. Valdez, B. C., B. A. French, E. S. Younathan, and S. H. Chang. 1989. Site-directed mutagenesis in *Bacillus stearothermophilus* fructose-6-phosphate 1-kinase. J. Biol. Chem. 264:131–135.
28. Yan, T.-F. J., and M. Tao. 1984. Multiple forms of pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase from wheat seedlings. J. Biol. Chem. 259:5087–5092.

Extremophiles (1999) 3:131–137

ORIGINAL PAPER

Yan-Huai R. Ding · Ron S. Ronimus · Hugh W. Morgan

Purification and properties of the pyrophosphate-dependent phosphofructokinase from *Dictyoglomus thermophilum* Rt46 B.1

Received: July 20, 1998 / Accepted: December 2, 1998

Abstract The distribution of phosphofructokinase phosphoryl donor subtypes (ATP-, ADP-, and pyrophosphate) in the deeply rooted phylogenetic lineages of thermophiles is of interest with regard to the evolution of phosphofructokinase activity and of the Embden–Meyerhof pathway. In this article we present the first biochemical description of a thermostable pyrophosphate-dependent phosphofructokinase from the hyperthermophilic bacterium *Dictyoglomus thermophilum*. The enzyme was not allosterically controlled by traditional modulators of phosphofructokinases and has significant activity with tripolyphosphate and polyphosphate. Kinetic parameters of the enzyme suggest it plays primarily a glycolytic role. The enzyme required Mg^{2+} for optimal activity, was partially activated by some monovalent and divalent cations, and was strongly inhibited by Cu^{2+}. The sequence of the 21 N-terminal residues suggests that the enzyme is most similar to the pyrophosphate-dependent phosphofructokinases from *Amycolatopsis methanolica* and the hyperthermophilic crenarchaeon *Thermoproteus tenax*, enzymes which have been suggested to represent an ancient lineage of phosphofructokinases (Siebers et al. 1998). The unexpected finding of a pyrophosphate-dependent phosphofructokinase in *Dictyoglomus thermophilum*, which is phylogenetically related to *Thermotoga maritima*, previously shown to possess an ATP-dependent phosphofructokinase activity, is discussed.

Key words Phosphofructokinase · Pyrophosphate · *Dictyoglomus thermophilum* · Polyphosphate · Tripolyphosphate

Communicated by G. Antranikian

Y.-H.R. Ding (✉) · R.S. Ronimus · H.W. Morgan
Thermophile Research Unit, Department of Biological Sciences, The University of Waikato, Private Bag 3105, Hamilton, New Zealand
Tel. +64-7-8562889 (ext 8266); Fax +64-7-8384324
e-mail: yrd2@waikato.ac.nz

Introduction

Phosphofructokinase (PFK) is a definitive enzyme of the Embden–Meyerhof pathway and is present within the three domains Bacteria, Eukarya, and Archaea. Three subtypes of PFK, with respect to their phosphoryl donor, have been described: an ATP-, an ADP-, and a pyrophosphate-dependent form. The ATP-PFK is found in most highly evolved organisms (Mertens 1991), in many bacteria (Byrnes et al. 1994), and in the crenarchaeon *Desulfurococcus amylolyticus* (Selig et al. 1997). A unique ADP-PFK has been identified in the genera *Pyrococcus* (*P. furiosus*) and *Thermococcus* (*T. celer*, *T. litoralis*, and *T. zilligii*) of the archaeal kingdom Euryarchaeota (Kengen et al. 1994; Selig et al. 1997; Ronimus et al., in manuscript). This enzyme is likely to represent a new family of PFK sequences as no homologous open reading frame has been identified within the *P. horikoshii* genome sequence (Kawarabayasi et al. 1998) or is identifiable within the *P. furiosus* genome when searched with either ATP- or representative PP_i-PFK encoding sequences (unpublished data; Altschul et al. 1990). In 1974, Reeves et al. reported that there was a PP_i-PFK activity in the amoebic parasite *Entamoeba histolytica*, and subsequently PP_i-PFKs have been shown to be present in many anaerobic bacteria, primitive eukaryotes, and in some plants (Mertens et al. 1993; O'Brien et al. 1975; Yan and Tao 1984). In addition, Morgan and Ronimus (1998) have found that all the thermophilic and nonthermophilic spirochetes possess a PP_i-PFK activity. The only PP_i-PFK from an archaeal species, the crenarchaeon *Thermoproteus tenax*, has been described by Siebers et al. (1998), who also recently reported the sequence of the PP_i-PFK-encoding gene.

Siebers et al. (1998) and Morgan and Ronimus (1998) have suggested that a PP_i-PFK that utilizes pyrophosphate as the phosphoryl donor for PFK activity might represent the ancestral form of the enzyme, although others (e.g., Mertens 1991) have proposed that it represents only an adaptation to anaerobic metabolism and thus would have presumably arisen on multiple occasions from ATP-PFKs.

In support of PP_i possibly representing the original phosphoryl donor for PFK activity, there are at least two examples in which sequence comparisons of ATP-PFKs have strongly suggested that ATP-PFKs are derived from PP_i-PFK ancestors; the ATP-PFK of *Streptomyces coelicolor* and the glycosomal ATP-PFK of *Trypanosoma brucei* (Alves et al. 1997). The number of sequence changes required to convert a PP_i-PFK to an ATP-PFK are not known with certainty, partially because of the lack of a crystal structure for a PP_i-PFK, but may only require subtle changes. However, the ramifications of these changes are significant because the PP_i-PFK-mediated reaction is reversible whereas the ATP-driven reaction is irreversible under physiological conditions. In this context, the biochemical characteristics of the PFKs from Bacteria and Archaea from deeply rooted phylogenetic lineages are of particular interest. The genus *Dictyoglomus* represents a deeply rooted bacterial hyperthermophilic lineage, and this article describes the purification of a PP_i-PFK from *Dictyoglomus thermophilum* Rt46 B.1, an isolate from a New Zealand hot spring (Love et al. 1993; Patel et al. 1987).

Materials and methods

Bacteria and culture conditions

Dictyoglomus thermophilum Rt46 B.1 was obtained from the Thermophile Research Unit Culture Collection and was cultured anaerobically in the medium described by Patel et al. (1987). Large-scale cultures (80 l) for protein purification were carried out in 20-l polypropylene containers. The medium was filter sterilized (0.2 μm) and gassed with 80% N_2 + 20% CO_2 for 60 min before inoculation with 0.5 l of log-phase culture per 20 l fresh medium at 73°C. Cells were harvested by diafiltration with a 0.1-μm hollow fiber system (Amicon, Beverly, MA, USA), recovered by centrifugation, and the resulting cell pellet then stored at −70°C before initiating the purification.

Chemicals and reagents

The following enzymes and reagents were purchased from Sigma (St. Louis, MO, USA): aldolase, α-glycerophosphate dehydrogenase, triosephosphate isomerase, phosphoglucoisomerase, glucose-6-phosphate dehydrogenase, fructose-1,6-diphosphate, fructose-2,6-diphosphate, pyrophosphate, tripolyphosphate, polyphosphate, potential activators and inhibitors, $NADP^+$, NADH, HEPES, and bis-Tris. All other buffers and chemical reagents used were of analytical grade quality.

Determination of forward and reverse PFK reaction rates and protein concentrations

The activity of the *Dictyoglomus* PFK was assayed spectrophotometrically using an Ultrospect 3000, UV/Visible Spectrophotometer (Pharmacia Biotech, Uppsala, Sweden) by a variation of the method of Janssen and Morgan (1992). The final concentration of the reaction mixture in a total volume of 100 μl was 5 mM fructose-6-phosphate (F-6-P); 1.0 mM phosphoryl donor (PP_i, ATP, or ADP); 3.5 mM $MgCl_2$; 0.2 mM NADH; 0.04 U aldolase; 0.5 U α-glycerophosphate dehydrogenase, and 5.0 U triosephosphate isomerase. Coupling enzymes were desalted before use with either Amicon Centricon-3s (α-glycerophosphate dehydrogenase, triosephosphate isomerase) or Centricon-10s (aldolase). Assays were conducted by monitoring the decrease of absorbance at 340 nm at 50°C, the maximum temperature at which the mesophilic coupling enzyme assay can be utilized. The following enzymes were used as positive controls during the investigation: the ATP-dependent PFK-positive control was fructose-6-phosphate kinase (EC. 2.7.1.11) type III from rabbit muscle, and the PP_i-dependent PFK positive control was PP_i-PFK (EC 2.7.1.90) derived from *Propionibacterium freundenreichii*. The ADP-PFK-positive control consisted of a semipurified PFK from *Thermococcus zilligii* (shown to be ADP dependent; Ronimus et al., in manuscript). Other control reactions were performed using the following conditions: no F-6-P, no coupling enzymes, or no phosphoryl donors in the reaction mixture. All assays were conducted in duplicate except those for determining kinetic parameters, which were performed in triplicate and used optimal conditions for at least 2 min. Specific activities are expressed in units mg^{-1} of protein. One unit (U) is defined as that amount of enzyme required to convert 1 μmole of F-6-P into fructose-1,6-diphosphate (F-1,6-P_2) per minute, and for the reverse reaction, the conversion of F-1,6-P_2 into F-6-P per minute. The reverse reaction was assayed at 50°C in a total volume of 100 μl using phosphoglucoisomerase (0.4 U) and glucose-6-phosphate dehydrogenase (0.1 U) as described by Mertens et al. (1993), except that the phosphoglucoisomerase concentration was doubled. The concentrations of other components were 3.5 mM $MgCl_2$, 0.2 mM $NADP^+$, and 50 mM MOPS (pH 7.3 at 50°C). Control experiments were conducted for both forward (1.5 mM F-1,6-P_2) and reverse (1.5 mM F-6-P) reactions to ensure that the coupling enzymes were not rate limiting. Protein was determined by a modification of the method of Bradford using bovine serum albumin as the standard (Bollag and Edelstein 1993).

Purification of the *Dictyoglomus* PP_i-PFK

Step 1. Preparation of cell-free extract

Dictyoglomus cells (120 g wet weight) were suspended in 1.2 l of 0.1 M $MgCl_2$, 0.1 M KCl, 7 mM β-mercaptoethanol, 10% glycerol, 50 mM imidazole buffer, pH 7.5, and lysed by sonication in six 200-ml aliquots for three periods of 2 min each at full power (Heat Systems-Ultrasonics, Farmingdale, NY, USA). Cell debris was removed by centrifugation at 4°C at 15 000 × *g* for 40 min. Ammonium sulfate was added to the supernatant to a final concentration of 1.0 M.

Step 2. Phenyl-Sepharose hydrophobic interaction chromatography
The cell-free extract was applied to a 700-ml phenyl-Sepharose column (Pharmacia Biotech) preequilibrated with starting buffer A [50 mM Tris, 1 mM $MgCl_2$, 1.0 M $(NH_4)_2SO_4$, pH 7.5]. The fractions were eluted with a linear gradient (4.0 l) with buffer B (50 mM Tris, 1 mM $MgCl_2$, pH 7.5) and further eluted with a linear gradient (2.0 l) of buffer C (50 mM Tris, 1 mM $MgCl_2$, 50% ethanediol, pH 7.5). Active fractions were pooled and concentrated with a YM 30 ultrafiltration membrane (Amicon) and dialyzed overnight against buffer B.

Step 3. Q-Sepharose anion exchange chromatography
The dialyzed enzyme solution was applied to a 55-ml Q-Sepharose column 26/10 (Pharmacia Biotech) that was equilibrated with buffer B. The activity was eluted with a gradient (400 ml) of 50 mM Tris, 1 mM $MgCl_2$, 1 M NaCl, pH 7.5, at a flow rate of 4 ml min^{-1}. Active fractions were pooled, concentrated by ultrafiltration with a YM30 membrane (Amicon), and dialyzed overnight against buffer B.

Step 4. Chromatofocusing
The recovered fractions from the Q-Sepharose step were loaded onto a Mono P HR 5/20 column (Pharmacia Biotech). Proteins were eluted with a 40-ml pH gradient (6.5–5.5) in buffer D and E with a flow rate of 1.0 ml min^{-1}. Buffer D contained 0.025 M bis-Tris, 1 mM $MgCl_2$, at pH 6.7, and buffer E contained 25 mM bis-Tris, 1 mM $MgCl_2$, 4.0 ml polybuffer 96 (Pharmacia), and 6.0 ml polybuffer 74 in a total volume of 100 ml (Pharmacia Biotech), pH 5.5. Active fractions were pooled and concentrated by ultrafiltration and dialyzed overnight against buffer F (25 mM HEPES, 50 mM NaCl, 1 mM $MgCl_2$, pH 7.8).

Step 5. Gel filtration chromatography
The concentrated and dialyzed sample was applied to a BIOSEP-SEC3000 column (60 × 0.78 cm; Phenomenex, Torrance, CA, USA), which was equilibrated with buffer F as the mobile phase. Active fractions were pooled, concentrated, and dialyzed against buffer G containing 25 mM bis-Tris and 5 mM $MgCl_2$, pH 6.0.

Step 6. Red dye-120 dye-ligand chromatography
Dialyzed active fractions following gel filtration were loaded onto a red dye-120 column that was equilibrated with buffer G. The enzyme was eluted by three linear gradients of $MgCl_2$, pH, and PP_i utilizing buffers G and H. Buffer H contained 25 mM Tris, 1 mM $MgCl_2$, and 10 mM PP_i, pH 7.5. The purified PFK was concentrated and extensively dialyzed against 20 mM bis-Tris and 1 mM $MgCl_2$, pH 6.8, to remove PP_i from the final purification step. Aliquots of the purified enzyme were then stored in 50% glycerol, 7 mM β-mercaptoethanol, 0.1 mM EDTA, and 1 mM $MgCl_2$ at −70°C.

SDS-PAGE gel electrophoresis

Determination of the subunit molecular weight was carried out using 10%–15% SDS Phast Gels (Pharmacia Biotech). The isoelectric point was derived from comparison of the purified enzyme on pH 3–9 isoelectric focusing (IEF) gels (Pharmacia Biotech) with a pH 3.0–10.0 isoelectric focusing standard (Pharmacia Biotech). The SDS-PAGE and IEF separations and subsequent silver stainings were performed according to the manufacturer's instructions.

Determination of the native molecular weight and thermostability

The molecular mass of the purified PP_i-PFK was determined by gel filtration using a BIOSEP-SEC3000 column (Phenomenex) equilibrated with 25 mM bis-Tris, 200 mM NaCl, and 1 mM $MgCl_2$, pH 6.7. The standard proteins used for the calibration of the column were thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), lactate dehydrogenase (140 kDa), carbonic anhydrase (29 kDa), and α-lactate albumin (14 kDa), which were compared to the elution volume of the purified PFK. All injections were of constant size (25 µl), and the proteins were eluted at a flow rate of 1.0 ml min^{-1} in the buffer used for equilibration. Blue-dextran 2000 (2000 kDa) was used to determine the column void volume. The thermostability of the purified *Dictyoglomus* PFK was investigated by incubating the enzyme (150 µg ml^{-1}) at 80°C or 90°C in buffer containing 50 mM MOPS, 2 mM $MgCl_2$, and 0.02% Triton X-100, pH 6.4, under a mineral oil overlay for different incubation times. Residual activity was then assayed at 50°C and compared to the unheated control.

N-Terminal sequencing

Sequencing was carried out using Edman degradation chemistry on an Applied Biosystem Procise 492 protein sequencer. The first 21 residues were used for homology searching and incorporated the BLASTP search at the National Centre for Biotechnology Information, National Library of Medicine, National Institute of Health, Bethesda, MD, USA (Altschul et al. 1990).

Determination of kinetic parameters

Forward reaction kinetic parameters were determined at 50°C and pH 6.0 by varying the concentration of either F-6-P or PP_i (or other phosphoryl donors) in the presence of saturating quantities of PP_i (1 mM) or F-6-P (5 mM), respectively. Reverse reaction parameters were determined at 50°C and pH 7.2 by varying the concentration of F-1, 6-P_2 or phosphate (P_i) in the presence of saturating quantities of P_i (10 mM) or F-1,6-P_2 (10 mM), respectively. Possible activators or inhibitors of PP_i-PFK were added

separately (each adjusted to pH 6.0) to the assay mixture with the purified enzyme, using near-apparent K_m concentrations of the substrates F-6-P (0.5 mM) and PP_i (0.3 mM).

Results

Purification of the *Dictyoglomus* PP_i-PFK

The PP_i-PFK was purified to homogeneity as indicated by SDS-PAGE (Fig. 1), after elution from the red dye-120 column, and had a specific activity of 24.4 U mg^{-1} protein. The enzyme was not sensitive to oxygen and was purified 231 fold with a final yield of 2.4% of the activity in the cell-free extract (purification summarized in Table 1). A single band was also seen during IEF, which indicated that the pI of the enzyme was 4.4 (not shown). Based on the percent yield of activity and the quantity of enzyme recovery after the red dye-120 step, the PFK represents approximately 0.4% of the total soluble cell protein.

Subunit structure of the enzyme and thermostability

The subunit molecular mass of the PP_i-PFK was estimated to be 37 kDa by SDS-PAGE. Gel filtration showed that the native enzyme had a molecular weight of approximately 65 kDa (Fig. 2). Thus, the PP_i-PFK from *Dictyoglomus thermophilum* Rt46 B.1 is indicated to be a homodimer. The half-life for the enzyme was determined to be 10 min at 90°C and 150 min at 80°C.

Substrate specificities, cation effects, and kinetic properties

The enzyme had optimal activity in the forward reaction at pH 5.7–6.3 at 50°C. The pH optimum for the reverse reaction was 7.0–7.5. Optimal activity was obtained with 0.5–3.5 mM $MgCl_2$ (with a PP_i concentration of 1.0 mM; not shown). The apparent K_m and V_{max} values for the forward and reverse reactions, as determined by double-reciprocal plotting, are summarized in Table 2a. The apparent K_m values for both substrates for the reverse reaction were

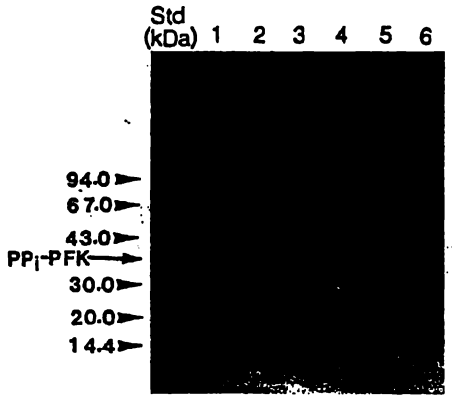


Fig. 1. SDS-PAGE gel (silver-stained) of fractions obtained during purification of the PP_i-PFK. *Lane 1* contained 0.3 µg purified *Dictyoglomus* PP_i-PFK and the remaining lanes contained (purification step, quantity in µg): *lane 2* (step 5, 1.0); *lane 3* (step 4, 1.0); *lane 4* (step 3, 1.0); *lane 5* (step 2, 4.0); and *lane 6* (step 1, 8.0). The standard (*Std*) contained the following molecular weight markers: phosphorylase b (molecular weight, 94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.0 kDa), and α-lactalbumin (14.4 kDa)

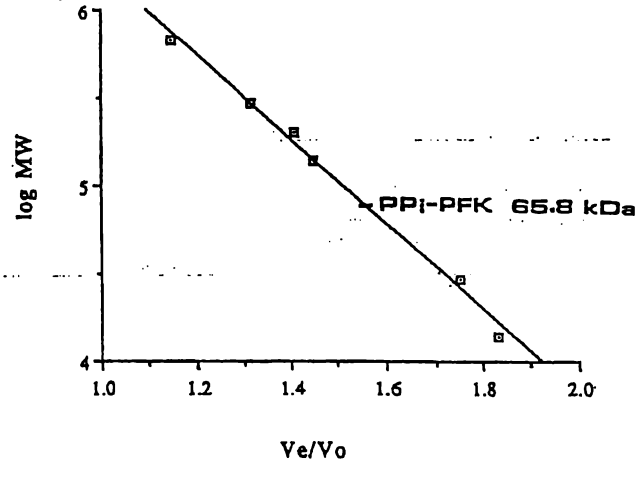


Fig. 2. Native molecular weight of *Dictyoglomus* PP_i-PFK. The standard proteins used for the calibration of the column were thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), lactate dehydrogenase (140 kDa), carbonic anhydrase (29 kDa), and α-lactate albumin (14 kDa), which were compared to the standard elution volume of purified PFK (25 µl). Blue dextran 2000 was used to determine the column void volume

Table 1. Purification of the PP_i-PFK from *Dictyoglomus thermophilum* Rt46B.1

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
(1) Cell-free extract	1374	13230	0.104	1	100
(2) Phenyl-Sepharose	955	927	1.03	10	70
(3) Q-Sepharose	730	633	1.10	11	53
(4) Mono P	103	58.7	1.75	17	7.5
(5) Gel filtration	72.8	30.3	2.4	23	5.3
(6) Red dye-120	32.9	1.35	24.4	231	2.4

Table 2. Biochemical characteristics of the PP_i-PFK from *Dictyoglomus thermophilum*

a. Summary of kinetic parameters:

Substrate	K_m (mM)	V_{max} ($U mg^{-1}$)	V_{max}/K_m
F-6-P[a]	0.228	4.62	20.3
PP_i[b]	0.022	5.30	241
PPP_i[b]	0.220	2.86	13.1
F-1,6-P_2[c]	2.900	0.15	0.05
P_i[d]	4.300	0.60	0.14

b. Summary of cation effects on PP_i-PFK activity:[e]

Cation	mM	% activity
Mg^{2+}[f]	3.5	100
Mn^{2+}	0.1	54
Fe^{3+}	0.1	16
Se^{4+}	0.1	25
	0.01	38
Cu^{2+}	1.0	0
	0.1	8
	0.01	38
	0.001	80

[a] With 3 mM PP_i and 3.5 mM $MgCl_2$
[b] With 5 mM F-6-P and 3.5 mM $MgCl_2$
[c] With 20 mM P_i and 3.5 mM $MgCl_2$
[d] With 10 mM F-1,6-P_2 and 3.5 mM $MgCl_2$
[e] Experiments conducted using the following: 5 mM F-6-P, 1 mM PP_i, 3.5 mM $MgCl_2$, 0.2 mM NADH, 50 mM bis-Tris, pH 6.0, 50°C, with cations at the stated concentrations
[f] Control assay with $MgCl_2$

much higher than the substrates for the forward reaction and the V_{max} values much lower. For example, the K_m for F-1,6-P_2 was 23 fold higher than the K_m for F-6-P and the ratio of K_m (P_i) to K_m (PP_i) was 195 fold. In addition, the ratios of V_{max}/K_m for PP_i and F-6-P for the forward reaction were much higher than the V_{max}/K_m ratios for P_i and F-1,6-P_2 for the reverse reaction. The apparent K_m and V_{max} values for tripolyphosphate were an order of magnitude higher and approximately half that determined than those for PP_i, respectively.

In addition to the enzyme using PP_i as the phosphoryl donor, the enzyme could also utilize tripolyphosphate and polyphosphate ($n = 15 \pm 3$) at 82% and 75% of the control reaction rates, respectively (not shown). In contrast, no activity was detected with either ATP, ADP, AMP, TTP, TDP, GTP, GDP, CTP, CDP, UTP, UDP, phosphoenolpyruvate, phosphoarginine, or phosphocreatine as the phosphoryl donors or D-glucose-6-phosphate in place of F-6-P (not shown). The *Dictyoglomus* PP_i-PFK enzyme activity was not affected by the traditional allosteric effectors ATP, ADP, AMP, GDP, phosphoenolpyruvate, or citrate (all at 1.0 mM). In addition, the *Dictyoglomus* PP_i-PFK activity was not affected by either 0.1 or 1.0 mM fructose-2,6-diphosphate (not shown). The enzyme activity was strongly inhibited by Cu^{2+}, Se^{4+}, Mn^{2+}, and Fe^{3+} (Table 2b). For example, a concentration of only 10.0 µM of Cu^{2+} resulted in a decrease of 62% of PP_i-PFK activity, but in control reactions only a slight inhibition of the coupling enzyme system was found at a Cu^{2+} concentration of 100 µM. In contrast to the inhibitory effects of Cu^{2+}, the enzyme activity was partially activated by K^+ (10 mM, 140%), Na^+ (10 mM, 135%), Ca^{2+} (0.1 mM, 120%), Fe^{2+} (0.1 mM, 120%), and Co^{2+} (0.1 mM, 120%).

Determination of N-terminal sequence

The first 21 amino acid residues of the PP_i-PFK were SKMRIGVLTGGGDCPGLNPAI. The N-terminal sequence is 84% and 78% identical to the N-termini of the PP_i-PFKs from *Amycolatopsis methanolica* and *Thermoproteus tenax*, respectively, but only 58% identical to the PP_i-PFK of the primitive eukaryote, *Trichomonas vaginalis* (Altschul et al. 1990).

Discussion

We have presented here the first biochemical desciption of a PP_i-PFK from a hyperthermophilic bacterium, *Dictyoglomus thermophilum* strain Rt46 B.1. The properties of this thermostable enzyme can now be compared to other ATP- and PP_i-PFKs that have been characterized from other sources.

The *Dictyoglomus thermophilum* PP_i-PFK is indicated, by the SDS-PAGE (37 kDa) and gel filtration results (65 kDa), to be a homodimer. Homodimeric PP_i-PFKs have also been identified in the bacterium *Propionibacterium freundenreichii* (O'Brien et al. 1975), *Entamoeba histolytica* (Reeves et al. 1974), *Rhodospirillum rubrum* (Pfleiderer and Klemme 1979), *Acholeplasma laidlawii* (Pollack and Williams 1986), and in the anaerobic protozoa *Isotricha prostoma*, *Toxoplasma gondii*, and *Trichomonas vaginalis* (Mertens et al. 1989). The acidic pH optimum for the forward reaction and neutral to slightly alkaline optimum for the reverse reaction are similar to the PP_i-PFKs from some primitive eukaryal organisms, e.g., *Entamoeba histolytica* (Reeves et al. 1974), *Trichomonas* spp. (Mertens et al. 1989), *Tritrichomonas foetus* (Mertens et al. 1989), *Toxoplasma gondii* (Peng and Mansour 1982), *Eimeria tenella* (Denton et al. 1994), and *Naegleria fowleri* (Mertens et al. 1991, 1993). In contrast, the pH optima of PP_i-PFK from *P. freundenreichii* and higher plants are near neutral for the forward and the reverse reactions (Mertens 1991).

The lack of effect of traditional eukaryal or bacterial allosteric effectors on the *Dictyoglomus* enzyme, or by fructose-2,6-diphosphate, which is a potent glycolytic effector for most higher eukaryotes but not in either prokaryotes or most anaerobic protozoa, suggests that one of the major control points of the Embden–Meyerhof pathway is not operational (Mertens 1991). The lack of allosteric control is a general feature of PP_i-PFKs (Mertens 1991) and implies that the rate of the PP_i-PFK catalyzed reaction must instead be controlled by a combination of the level of activity of the enzyme, its kinetic properties, and the cellular concentrations of reactants and products. In support of the kinetic parameters having a significant effect on controlling the

glycolytic flux are the much lower apparent K_m values for the forward reaction, the higher V_{max} values compared to the reverse reaction, and the much higher V_{max}/K_m ratios seen with either PP_i or F-6-P for the forward reaction. These results taken together suggest that the glycolytic direction of the reaction is favored in this organism.

The *Dictyoglomus* PP_i-PFK has a requirement for Mg^{2+} ions for optimal activity and was partially activated by some monovalent cations, both of which are common biochemical attributes of PFKs (Uyeda 1979). The enzyme is sensitive to Se^{4+} and Fe^{3+} and, interestingly, extremely sensitive to Cu^{2+}. The extreme sensitivity of the enzyme to Cu^{2+} ions could have an important role in determining the ecological distribution of *D. thermophilum* strains. The organism must have cellular detoxifying mechanisms for maintaining the intracellular concentration of Cu^{2+} at extremely low levels.

The ability of the *D. thermophilum* PP_i-PFK to utilize pyrophosphate, tripolyphosphate, and polyphosphate is potentially relevant with regard to the origins of PFK activity. This is because pyrophosphate and tripolyphosphate are known to be formed under hydrothermal conditions (Yamagata et al. 1991) and are considered as relics of ancient metabolism (Kornberg 1995). These substrates may have provided a continuing energy source to drive metabolism during primeval conditions (Baltscheffsky 1996). In such a scenario, the earliest life forms would be expected to have possessed a PP_i-PFK activity (Morgan and Ronimus 1998). The reversible nature of the reaction with PP_i as phosphoryl donor with its $\Delta G^{\circ\prime}$ value of $-2.08\,kcal\,mol^{-1}$ ($8.7\,kJ\,mol^{-1}$) would be compatible with either a glycolytic or gluconeogenic origin of the Embden–Meyerhof pathway (Mertens 1991). In support of this scenario, Siebers et al. (1998) have recently inferred that the PP_i-PFKs from the hyperthermophilic crenarchaeon *Thermoproteus tenax* and the mesophilic bacterium *Amycolatopsis methanolica* represent a unique lineage of PFK, and possibly the most ancient form of the PFK enzyme. The PP_i-PFK from *D. thermophilum* Rt 46 B.1 is similar to the *T. tenax* enzyme in respect to its nonallosteric nature, subunit size, and N-terminal amino acid sequence.

It was an unexpected finding to discover a PP_i-PFK in *Dictyoglomus* because this organism branches at the base of the phylum Thermotogales (Love et al. 1993). Both *Thermotoga maritima* (Selig et al. 1997), and *Fervidobacterium nodosum*, which also branches within the Thermotogales (Love et al. 1993), possess an ATP-PFK activity (unpublished data). *Aquifex aeolicus*, the deepest branching thermophilic bacterium, has also been indicated by sequence analysis to contain an ATP-PFK (Deckert et al. 1998), and this enzyme clusters with the ATP-PFKs of *Thermus thermophilus* and *Bacillus stearothermophilus* (unpublished data). It will be interesting to see if the full sequence of the *Dictyoglomus* PP_i-PFK-encoding gene confirms a phylogenetic linkage with the *T. tenax* enzyme or whether it is more closely linked to the ATP-PFKs of *Aquifex*, *Thermotoga*, and *Fervidobacterium*. The presence of a PP_i-PFK activity in the deeply branching *D. thermophilum* lineage and the detailed properties of the enzyme are likely to play significant roles in the development of our understanding of the evolution of the PFKs and of the Embden–Meyerhof pathway itself.

Acknowledgments The authors thank the Royal Society Marsden Science Foundation and the University of Waikato for financial support during the course of this study. We also acknowledge the assistance of Colin Monk with large-scale cultivation, Jurre Koning for semi-purified *Thermococcus zilligii* (*Thermococcus* strain AN1) ADP-PFK preparations, and Catriona Knight for N-terminal sequencing from the School of Biological Sciences, Auckland University, New Zealand.

References

Altschul SF, Gish W, Miller W, Myers W, Lipman DL (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Alves AMCR, Euverink GJW, Bibb MJ, Dijkhuizen L (1997) Identification of ATP-dependent phosphofructokinase as a regulatory step in the glycolytic pathway of the Actinomycete *Streptomyces coelicolor* A3(2). Appl Environ Microbiol 63:956–961

Baltscheffsky H (1996) Energy conversion leading to the origin and early evolution of life: did inorganic pyrophosphate precede adenosine triphosphate? In: Baltscheffsky H (ed) Origin and evolution of biological energy conversion. VCH Publishers, New York

Bollag DM, Edelstein SJ (1993) Protein methods. Wiley, New York

Byrnes WM, Zhu X, Younathan ES, Chang SH (1994) Kinetic characteristics of phosphofructokinase from *Bacillus stearothermophilus*: MgATP nonallosterically inhibits the enzyme. Biochemistry 33:3424–3431

Deckert G, Warren PV, Gaasterland T, Young WG, Lenox AL, Grahams DE, Overbeek R, Snead MA, Keller M, Aujay M, Huber R, Feldman RA, Short JM, Olsen GJ, Swanson RV (1998) The complete genome sequence of the hyperthermophilic bacterium *Aquifex aeolicus*. Nature (Lond) 392:353–358

Denton H, Thong K-W, Coombs GH (1994) *Eimeria tenella* contains a pyrophosphate-dependent phosphofructokinase and a pyruvate kinase with unusual allosteric regulators. FEMS Microbiol Lett 115:87–92

Janssen PH, Morgan HW (1992) Glucose catabolism by *Spirochaeta thermophila* RI 19.B1. J Bacteriol 174:2449–2453

Kawarabayasi Y, Sawada M, Horikawa H, Haikawa Y, Hino Y, Yamamoto S, Sekine M, Baba S-i, Kosugi H, Hosoyama A, Nagai Y, Sakai M, Ogura K, Otsuka R, Nakazawa H, Takamiya M, Ohfuku Y, Funahashi T, Tanaka T, Kudoh Y, Yamazaki J, Kushida N, Oguchi A, Aoki K-i, Yoshizawa T, Nakamura Y, Robb FT, Horokoshi K, Masuchi Y, Shizuya H, Kikuchi H (1998) Complete sequence and gene organization of the genome of a hyper-thermophilic archaebacterium, *Pyrococcus horikoshii* OT3. DNA Res 5:55–76

Kengen SW, de Bok FAM, van Loo N-D, Dijkema C, Stams AJM, de Vos WM (1994) Evidence for the operation of a novel Embden-Meyerhof pathway that involves ADP-dependent kinases during sugar fermentation by *Pyrococcus furiosus*. J Biol Chem 269:17537–17541

Kornberg A (1995) Inorganic polyphosphate: toward making a forgotten polymer unforgettable. J Bacteriol 177:491–496

Love CA, Patel BKC, Ludwig W, Stackebrandt E (1993) The phylogenetic position of *Dictyoglomus thermophilum* based on 16S rRNA sequence analysis. FEMS Microbiol Lett 107:317–320

Mertens E (1991) Pyrophosphate-dependent phosphofructokinase, an anaerobic glycolytic enzyme? FEBS Lett 285:1–5

Mertens E, Van Schaftingen E, Müller M (1989) Presence of a fructose-2,6-bisphosphate-insensitive pyrophosphate:fructose-6-phosphate phosphotransferase in the anaerobic protozoa *Tritrichomonas foetus*, *Trichomonas vaginalis* and *Isotricha prostoma*. Mol Biochem Parasitol 37:183–190

Mertens E, De Jonckheere J, Van Schaftingen E (1993) Pyrophosphate-dependent phosphofructokinase from the amoeba *Naegleria fowleri*, an AMP-sensitive enzyme. Biochem J 292:797–803

Morgan HW, Ronimus RS (1998) Pyrophosphate-dependent phosphofructokinase in thermophilic and non-thermophilic microorganisms. In: Adam MW, Wiegel JW (eds) Thermophiles: the key to molecular evolution and the origin of life. Taylor & Francis, London

O'Brien WE, Bowien S, Wood HG (1975) Isolation and characterisation of a pyrophosphate-dependent phosphofructokinase from *Propionibacterium shermanii*. J Biol Chem 250:8690–8695

Patel BK, Morgan HW, Wiegel J, Daniel RM (1987) Isolation of an extremely thermophilic chemoorganotrophic anaerobe similar to *Dictyoglomus thermophilum* from New Zealand hot springs. Arch Microbiol 147:21–24

Peng Z-Y, Mansour TE (1992) Purification and properties of a pyrophosphate-dependent phosphofructokinase from *Toxoplasma gondii*. Mol Biochem Parasitol 54:223–230

Pfleiderer C, Klemme J-H (1979) Pyrophosphate-dependent D-fructose-6-phosphate-phosphotransferase in *Rhodospirillaceae*. Z Naturforsch 35:229–238

Pollack JD, Williams MV (1986) PP_i-dependent phosphotransferase (phosphofructokinase) activity in the Mollicutes (Mycoplasma) *Acholeplasma laidlawii*. J Bacteriol 165:53–60

Reeves RE, South DJ, Blytt HJ, Warren LG (1974) Pyrophosphate:D-fructose 6-phosphate 1-phosphotransferase. J Biol Chem 249:7737–7741

Selig M, Xavier KB, Santos H, Schönheit P (1997) Comparative analysis of Embden-Meyerhof and Entner-Doudoroff glycolytic pathways in the hyperthermophilic archaea and the bacterium *Thermotoga*. Arch Microbiol 167:217–232

Siebers B, Klenk HP, Hensel R (1998) PP_i-dependent phosphofructokinase from *Thermoproteus tenax*, an archaeal descendant of an ancient line in phosphofructokinase evolution. J Bacteriol 180:2137–2143

Uyeda K (1979) Phosphofructokinase. Adv Enzymol 48:193–241

Yamagata Y, Watanabe H, Saitoh M, Namba T (1991) Volcanic production of polyphosphates and its relevance to prebiotic evolution. Nature (Lond) 352:516–519

Yan T-F J, Tao M (1984) Multiple forms of pyrophosphate:D-fructose-6-phosphate 1-phosphotransferase from wheat seedlings. J Biol Chem 259:5087–5092

Arch Microbiol (1999) 172:401–406

ORIGINAL PAPER

Ron S. Ronimus · Hugh W. Morgan
Yan-Huai Richard Ding

Phosphofructokinase activities within the order Spirochaetales and the characterisation of the pyrophosphate-dependent phosphofructokinase from *Spirochaeta thermophila*

Received: 9 June 1999 / Accepted: 7 September 1999

Abstract The subtype of phosphofructokinase activity, either ATP-, ADP- or pyrophosphate-dependent, present in members of three genera from the Spirochaetales was investigated. The individual species/strains examined included *Spirochaeta alkalica*, *S. asiatica*, *S. halophila*, *S. isovalerica*, *S. litoralis*, *S. zuelzerae*, *S. thermophila*, two thermophilic spirochetes, *Treponema bryantii*, *T. denticola*, *T. pectinovorum*, *Leptospira biflexa* and *L. interrogans*. All of the Spirochaeta strains, regardless of their phenotype, possessed primarily a pyrophosphate-dependent phosphofructokinase. In contrast, *T. bryantii*, *T. denticola* and *L. biflexa* had predominantly an ATP-dependent activity, whereas no activity was detected in *T. pectinovorum* or *L. interrogans*. The results suggest that pyrophosphate-dependent phosphofructokinase activity may be a reliable phenotypic marker for the genus *Spirochaeta* and that there are potentially interesting differences in how the catabolism of saccharides is controlled among members of genera within the Spirochaetales. The pyrophosphate-dependent phosphofructokinase from *S. thermophila* strain RI 19.B1 was purified (303-fold) to homogeneity and biochemically characterised. The *S. thermophila* enzyme displayed hyperbolic kinetics with respect to both the forward and reverse cosubstrates and was not significantly affected by traditional activators or inhibitors of phosphofructokinase. The biochemical characterisation represents the first spirochete phosphofructokinase to be described.

Key words Spirochetes · Phosphofructokinase · *Treponema* · *Leptospira* · *Spirochaeta* · Glycolysis · Pyrophosphate · Thermophile

R. S. Ronimus (✉) · H. W. Morgan · Y.-H. R.. Ding
Thermophile Research Unit, Department of Biological Sciences, University of Waikato, Private Bag 3105, Hamilton, New Zealand
e-mail: ronimus@waikato.ac.nz,
Tel.: +64-7-8384029, Fax: +64-7-8384324

Introduction

Phosphofructokinase is a well-known and definitive enzyme of the Embden-Meyerhof pathway and is present within the three domains Eukarya, Bacteria and Archaea (Selig et al. 1997; Morgan and Ronimus 1998). The "classic" ATP-dependent phosphofructokinase (EC 2.7.1.11) is found in almost all higher eukaryotes and most bacteria and catalyses an important, allosterically controlled rate-limiting step of glycolysis. Although most highly evolved organisms use ATP, other phosphoryl donors such as pyrophosphate (PP_i) and ADP (in some Archaea) are also utilised (Mertens 1991; Selig et al. 1997; Ronimus et al. 1999). The utilisation of a PP_i-phosphofructokinase can conserve ATP, which is particularly significant for anaerobes, and negates the need for a fructose-1,6-bisphosphatase (Mertens 1991). Reeves et al. (1974) were the first to identify a PP_i-phosphofructokinase (EC 2.7.1.90) activity in the amoebic parasite *Entamoeba histolytica* and since then other PP_i-phosphofructokinases have been identified in higher plants, primitive eukaryotes and in other anaerobic, bacterial genera (Mertens 1991; Selig et al. 1997).

The order Spirochaetales is phylogenetically interesting as it contains a diverse array of phenotypes that includes strictly anaerobic, facultatively anaerobic, halophilic, alkaliphilic, thermophilic and both free-living and obligately parasitic species (Canale-Parola 1997). Molecular phylogenies based on small ribosomal subunit RNA sequences have generally supported the cohesiveness of the diverse species and genera (Paster et al. 1991; Rainey et al. 1992). The extremely thermophilic *Spirochaeta thermophila* strain RI 19.B1, which grows optimally at 65 °C (and between 44 °C and 73 °C), has been shown to possess a PP_i-phosphofructokinase and to contain all the enzyme activities for a fully functional glycolytic pathway (Rainey et al. 1991; Aksenova et al. 1992; Janssen and Morgan 1992). *S. thermophila* is able to degrade a diverse array of saccharides but is not able to utilise a variety of alcohols, TCA cycle intermediates, organic acids or amino acids, suggesting that the glycolytic direction is predominant during

normal growth conditions (Aksenova et al. 1992; Janssen and Morgan 1992). Because parasitic spirochete PP_i-phosphofructokinases may represent promising enzymes to target with pharmaceutically appropriate inhibitors, it was of interest to determine if the presence of this PP_i-phosphofructokinase simply reflected its thermophilic habitat or whether it was common throughout the genus. A directly related goal was to also characterise the biochemical activity from at least one species of *Spirochaeta*. In this communication, we describe both the screening of a variety of representative organisms from *Spirochaeta*, *Treponema* and *Leptospira* for the subtype of phosphofructokinase activity and the purification and characterisation of the PP_i-phosphofructokinase from *S. thermophila*. This represents the first biochemical description of a PP_i-phosphofructokinase from the Spirochaetales and the second from a thermophilic bacterium (Ding et al. 1999).

Materials and methods

Bacterial strains, growth conditions and preparation of cell-free extracts

All of the strains in this study, the media used for cultivation and incubation temperatures are listed in Table 1. Bovine rumen fluid (Dairying Research, New Zealand) was centrifuged at 15,000 × g for 40 min and rabbit serum (Rapid Rabbits, New Zealand) was sterilised by filtration (0.2 μm). Cells were harvested by centrifugation (15,000 × g for 20 min at 4 °C) and the cell pellets washed twice and resuspended with 0.1 M $MgCl_2$, 0.1 M KCl, 1.4 mM β-mercaptoethanol, 10% glycerol (v/v); 50 mM imidazole buffer, pH 7.4. Cells were sonicated, centrifuged at 15,000 × g for 20 min and the supernatants used either immediately for activity measurements or stored at −70 °C after flash-freezing in liquid N_2. *S. thermophila*, strain RI 19.B1 (DSM 6192) was obtained from the Thermophile Research Unit Culture Collection (TRUCC) and was grown in large scale culture (80 l) using CBM4 media prepared according to Rainey et al. (1991). Cells were harvested by diafiltration (Amicon; 0.1 μm).

Reagents and determination of enzyme activities

The activity of extracts was assayed in duplicate at the optimal temperature for each strain by a modification of the method of Janssen and Morgan (1992) using coupling enzymes and substrates from Sigma-Aldrich. The reaction concentrations were: 5 mM fructose 6-phosphate; 1.0 mM phosphoryl donor (PP_i, ATP or ADP); 3.5 mM $MgCl_2$; 0.2 mM NADH; 0.04 units (U) aldolase; 0.5 U α-glycerophosphate dehydrogenase and 5.0 U triosephosphate isomerase in 50 mM imidazole, pH 7.4. The protein concentrations of extracts varied between 2.95 and 0.41 mg/ml and the amount used was never more than 26 μl in a 600 μl reaction. Assays were conducted for at least 2 min and specific activities are expressed in U/mg protein. Protein was assayed by the method of Bradford (1976) with bovine serum albumin as the standard.

S. thermophila PP_i-phosphofructokinase assays were conducted at 50 °C to prevent the premature denaturation of coupling enzymes and contained either 30 mM histidine or Bis-Tris, pH 6.0 (at 50 °C) with otherwise identical conditions for analysis of cell-free extracts. Kinetic parameters for the forward reaction were obtained by extrapolation of results obtained by either varying the concentration of fructose 6-phosphate or PP_i (or tripolyphosphate or polyphosphate) in the presence of saturating quantities of PP_i (1.0 mM) or fructose 6-phosphate (5 mM), respectively. Determination of allosteric effects were conducted with substrate concentrations close to their respective K_m values (0.5 mM fructose 6-phosphate and 200 μM PP_i). The ability of other phosphoryl donors, or Mn^{2+} to replace Mg^{2+}, to support activity was examined using 1.0 mM of the relevant cation with otherwise standard conditions. Activity with polyphosphate ($n = 15$) used 30.5 mM $MgCl_2$ to overcome complexing of Mg^{2+} (compared to a control with PP_i, also with 30.5 mM $MgCl_2$).

The reverse reaction was conducted (50 °C) using phosphoglucoisomerase and glucose-6-phosphate dehydrogenase as described by Mertens et al. (1993) except the phosphoglucoisomerase concentration was doubled. Kinetic parameters for the reverse reaction were determined by varying the concentration of fructose 1,6-diphosphate or inorganic phosphate (P_i) with saturating quantities of either P_i (10 mM) or fructose 1,6-diphosphate (5 mM), respectively.

Purification procedures for isolating the PP_i-phosphofructokinase from *S. thermophila*

Cells (80.0 g wet weight) were lysed by sonication in 600 ml of 50 mM imidazole, pH 7.4, 5 mM EDTA, 50 mM KCl, 0.1% β-mer-

Table 1 Sources of strains and conditions of growth. The media number or type listed as being used for each species or strain are those of the designated supplier of that species or strain. *SE* Spirochete enrichment medium (g/l): $(NH_4)_2SO_4$, 0.5 g; KH_2PO_4, 0.28 g; $MgSO_4 \cdot 7H_2O$, 0.247 g; $CaCl_2 \cdot 2H_2O$, 0.074 g; yeast extract, 0.5 g; Tryptone, 0.5 g; maltose, 3.0 g; HEPES buffer, 2.4 g; resazurin (0.1%), 1 ml; distilled water 1000 ml, pH 7.2

Strains	Sources	Media	Temperature (°C)	Aerobic/ anaerobic	Carbon source
Spirochaeta					
S. alkalica	DSM 8900	700	37	Anaerobic	Sucrose
S. asiatica	DSM 8901	700	37	Anaerobic	Sucrose
S. halophila	ATCC 29478	937	37	Facultative	Maltose
S. isovalerica	DSM 2461	273	30	Anaerobic	Glucose
S. litoralis	DSM 2029	173	30	Anaerobic	Glucose
S. zuelzerae	DSM 1903	169	30	Anaerobic	Glucose
S. thermophila	TRUCC	SE	68	Anaerobic	Maltose
Rt 118. B.1	TRUCC	SE	55	Anaerobic	Maltose
GAB 76	TRUCC	SE	55	Anaerobic	Maltose
Treponema					
T. bryantii	ATCC 33254	602	37	Anaerobic	Glucose+maltose
T. denticola	ATCC 33520	1357	37	Anaerobic	Glucose
T. pectinovorum	ATCC 33768	1223	34	Anaerobic	Pectin
Leptospira:					
L. biflexa	ATCC 23582	1470	30	Aerobic	Rabbit serum
L. interrogans	ATCC 23605	1470	30	Aerobic	Rabbit serum

captoethanol and 500 μM phenylmethylsulfonyl fluoride. Cell debris was removed by centrifugation at 4 °C at 12,000 × g for 40 min. The supernatant was fractionated by $(NH_4)_2SO_4$ precipitations according to standard methods, first with a 0–35% saturation followed by a centrifugation step then by 35–75% saturation and centrifugation. The 35–75% precipitate was resuspended in 50 mM imidazole, 1.5 M $(NH_4)_2SO_4$, pH 7.4, and applied to a phenyl-Sepharose column (300 ml bed volume; Pharmacia) at 20.0 ml/min equilibrated in 1.5 M $(NH_4)_2SO_4$, 50 mM imidazole, pH 7.4. The fractions were eluted in a linear gradient (total of 3.0 l) starting with column-equilibration buffer and ending in 50 mM imidazole, pH 7.4. Fractions were collected and active fractions pooled and dialysed against 20 mM imidazole, pH 7.4. The dialysed samples were split into six near-equal portions and each sequentially loaded onto a Mono Q (HR 16/10) column (Pharmacia) equilibrated in starting buffer (20 mM imidazole, pH 7.4). Fractions were eluted with a 140 ml gradient at 2.0 ml/min (0–35% for 60 min then 35–100% for 10 min) starting with 20 mM imidazole, pH 7.4, and ending with 20 mM imidazole, pH 7 4, 1.0 M NaCl. Active fractions were pooled, concentrated and diafiltered with Centricon-10s (Amicon). The phosphofructokinase was chromatographed by gel-filtration (20 injections, each ≤ 80 μl) through a BIOSEP-SEC3000 column equilibrated in 100 mM Na_2HPO_4, pH 7 4, at 1.0 ml/min.

Results

Phosphofructokinase activities within spirochetes

All six mesophilic *Spirochaeta* species, *S. thermophila* and the two thermophilic strains of *Spirochaeta* were found to possess primarily a PP_i-dependent phosphofructokinase activity (Table 2). The specific activities for eight of the nine *Spirochaeta* samples varied between 5.25 and 1.1 U/mg, while that of *S. litoralis* was significantly lower (0.1 U/mg). A low level of ATP-dependent background activity was present in some *Spirochaeta* cell-free extracts, but in all cases the PP_i-based activity was a minimum of 3.1-fold (*S. isovalerica*) and up to between 50-fold (*S. halophila*) and 55-fold higher (strain GAB 76). A similar low-level background activity was seen with extracts of *S. thermophila*, which was almost entirely removed on further purification of the PP_i-phosphofructokinase. ADP-dependent activity (likely due to adenylate kinase activity within the extracts) was either absent or a maximum of 20% (*S. litoralis*) of the PP_i-based activity when screening the *Spirochaeta* cell-free extracts and is most likely not of physiological significance.

In contrast to the above findings with members of the genus *Spirochaeta*, *T. bryantii*, *T. denticola* and *L. biflexa* possessed primarily an ATP-dependent phosphofructokinase activity, whereas no phosphofructokinase activity (either ATP-, ADP- or PP_i-based) was found in either *T. pectinovorum* or *L. interrogans*, despite repeated attempts at detection. Similar to the results obtained with *Spirochaeta* strains, some background activity (either PP_i- or ADP- or both) was present in some cases but the ATP-based activity was 13.7-fold higher than the next highest activity (ADP-) in the case of *T. bryantii*, nearly 3-fold higher than the ADP-based activity for *T. denticola* and 2.4-fold higher than that of the PP_i- based *L. biflexa* activity.

Table 2 Phosphofructokinase activities in spirochetes. *ND* Not detected

Strains	Specific activity (units/mg)			PFK subtype
	PP_i	ATP	ADP	
Spirochaeta				
S. alkalica	5.25	0.73	ND	PP_i
S. asiatica	2.27	0.63	ND	PP_i
S. halophila	3.02	0.06	0.09	PP_i
S. isovalerica	3.23	1.04	ND	PP_i
S. litoralis	0.10	0.01	0.02	PP_i
S. zuelzerae	1.40	0.19	0.16	PP_i
S. thermophila	1.47	0.31	0.19	PP_i
Rt 118. B1	3.90	0.18	ND	PP_i
GAB 76	1.10	0.02	0.02	PP_i
Treponema				
T. bryantii	0.15	6.29	0.46	ATP
T. denticola	ND	0.86	0.29	ATP
Leptospira:				
L. biflexa	0.38	0.9	0.16	ATP

Characterisation of the purified *S. thermophila* PP_i-phosphofructokinase

The spirochete phosphofructokinase, purified with a yield of 13% by the protocol as outlined in Table 3, migrated as a single band during denaturing electrophoresis with an estimated molecular mass of 57 ± 1.2 kDa ($n = 5$; not shown) and had a specific activity of 446 U/mg. The native molecular mass from gel filtration was estimated to be 95.5 kDa ± 0.2 kDa ($n = 3$). The enzyme had a *p*I of approximately 5.3 and was very thermostable, with a half-life of 24 h at 65 °C and 4 h at 85 °C (not shown). The effect of temperature on reaction rates was examined at 15, 25, 35, 45 and 55 °C and reaction rates increased by a constant factor of approximately 1.5 (not shown).

The pH optimum for activity for the forward reaction was 5.0–6.0 and pH 7.0–7.5 for the reverse reaction. The spirochete enzyme required Mg^{2+} ions for optimal activity (3.5–5.0 mM) and these could only be partially replaced by Mn^{2+}, with Mn^{2+} supporting 16% of control activity with Mg^{2+}. There was a slight enhancement of activity when either Na^+ (115% of control activity) or K^+ (114%) was also present at 100 mM in the reactions. In contrast, most

Table 3 Purification of the PP_i-phosphofructokinase from *Spirochaeta thermophila*

Purification step	Protein (mg)	Activity (U)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
1. Cell-free extract	2800	4100	1.5	1	100
2. 35–75% $(NH_4)_2SO_4$	2200	3700	1.7	1.2	90
3. Phenyl-Sepharose	570	1900	3.4	2.3	46
4. Mono-Q	6.3	1550	251	171	38
5. Gel filtration	1.2	535	446	303	13

Table 4 Kinetic parameters of the *S. thermophila* phosphofructokinase

Substrate	K_m (μM)	V_{max} (U mg^{-1})
Forward reaction		
PP_i	110	393
Tripolyphosphate	170	350
Polyphosphate	430	178
Fructose 6-phosphate	240	438
Reverse reaction		
P_i	400	193
Fructose 1,6-diphosphate	38	239

other cations (in the presence of 3.5 mM $MgCl_2$) were associated with a reduction or no effect and included (mM concentration; % relative activities): $CaCl_2$ (0.1; 84%); $CaCl_2$ (1.0; 96%); $MnCl_2$ (0.1; 69%); $FeCl_2$ (0.1; 86%); $FeCl_3$ (0.1; 64%); $CoCl_2$ (0.1; 99%); $NiCl_2$ (0.1; 101%); $ZnCl_2$ (0.1; 84%); $SeCl_4$ (0.1; 98%).

Activity with other phosphoryl donors (1.0 mM) was examined and the rates, relative to PP_i, were (substrate; rate): (ATP; 3%); (ADP; 2%); (tripolyphosphate; 95%); (polyphosphate; 56%); (adenosine 5′-tetraphosphate; 13%); and (acetyl-phosphate; 13%).

The ability of traditional and other effectors of phosphofructokinases to affect the spirochete phosphofructokinase was examined, and none of these had an effect large enough for them to be considered as a true effector. These included (at 1.0 mM): pyruvate, succinate, phosphoenolpyruvate, citrate, fructose 2,6-diphosphate, ATP, ADP, AMP, CTP, GTP, TTP, UTP, cis-aconitate, isocitrate, malate, fumarate, acetyl-CoA, coenzyme A, oxaloacetate, acetate, glucose 6-phosphate, 2-phosphoglycerate, 3-phosphoglycerate and acetyl-phosphate.

Apparent K_m and V_{max} parameters were determined for the forward and reverse reactions (Table 4) with all of the reactions being hyperbolic with respect to the appropriate substrates (not shown). Interestingly, the apparent K_m for fructose 6-phosphate was approximately sixfold higher than that for fructose 1,6-diphosphate. Overall, the maximum forward reaction rates (V_{max}) were approximately twice those for the reverse reactions. In addition, the apparent K_m values for tripolyphosphate and polyphosphate were higher and their V_{max} values lower than those for PP_i, respectively.

The N-terminal of the protein was analysed for 35 cycles with Edman-degradation chemistry and gave the following residues: MTRISPLQKARYGYVPKLPPV(S)L-(N)QD(E)AIARIQV(A)G(E)LG(K)Q(L). In some later cycles, secondary minor signals were detected and these are shown in parentheses after the primary residue. Matches were found with putative phosphofructokinases from *Treponema pallidum* (42% identity with Tp 0108) and *Borrelia burgdorferi* and with the α-subunits of *Ricinus communis* and *Solanum tuberosum* PP_i-phosphofructokinases (Altschul et al. 1997; Fraser et al. 1998).

Discussion

Distribution of phosphofructokinase subtypes within the Spirochaetales

The first goal of this study was to investigate the distribution of phosphofructokinase subtypes within the Spirochaetales and in particular whether all *Spirochaeta* species contained a PP_i-phosphofructokinase. The fact that PP_i-phosphofructokinase activity was the dominant activity type in all of the *Spirochaeta* tested in this study and has also been found in *S. stenostrepta* suggests that this may represent a reliable phenotypic marker for the genus which is apparently not related to their diverse physiologies (Morgan and Ronimus 1998). We cannot, however, discount that another subtype of phosphofructokinase is not also present but the low or undetectable levels of ATP- and ADP-dependent activities, especially in some extracts suggests that this is not the case. A small but significant portion of these background activities may be due to the inherent substrate specificities of their phosphofructokinases as even the highly purified phosphofructokinase from *S. thermophila* still possessed some activity with ATP, ADP and polyphosphates.

One reason for why an ATP-dependent activity was found in *T. bryantii* and *T. denticola* (which are host-associated spirochetes) and *L. biflexa* may be that the normall supply of growth substrates for these organisms may not be either as limiting or random as would presumably be the case for free-living spirochetes. A reduction in the nett ATP produced per glucose by using an ATP-dependentt phosphofructokinase may not be so critical for these organisms. The ATP-PFK activity detected in *Treponema* species is interesting in that the recently sequenced *T. pallidum* and *B. burgdorferi* genomes putatively contain PP_i-phosphofructokinase encoding genes based on sequence comparisons (Fraser et al. 1997, 1998). However, neither of these two phosphofructokinases, which represent potentially promising enzyme activities to target with inhibitors, have been isolated yet nor the enzymes characterised. Interestingly, no activity was found in either *T. pectinovorum* or *L. interrogans* despite repeated attempts at detection. It is possible that these organisms may degradte sugars via the Entner-Doudoroff pathway, as has been suggested for *T. saccharophilum* (Paster and Canale-Parola 1985). Thus, the overall results presented here suggest that significant differences do exist in how species within genera of the order Spirochaetales degrade saccharides.

Characterisation of the *S. thermophila* PP_i-phosphofructokinase

In addition to the identification of PP_i-phosphofructokinase activity within cell-free extracts of numerous spirochetes, described above, we have also reported here a biochemical description of a thermostable PP_i-phosphofructokinase from *S. thermophila*. The enzyme, to our knowll-

edge, has the highest specific activity recorded to date for a prokaryotic or primitive eukaryotic PP_i-phosphofructokinase, and this would be expected to be approximately two-fold higher (i.e. approximately 892 U/mg) at 65 °C assuming a constant Q_{10} of 1.5 between 50 °C and 65 °C. Other characterised PP_i-phosphofructokinases with relatively high specific activities have been found in *Giardia lamblia* (146 U/mg), *Propionibacterium freundenreichii* (258 U/mg) and *Hexamita inflata* (300 U/mg; O'Brien et al. 1975; Li and Phillips 1995; Phillips et al. 1997). The specific activity of the PP_i-phosphofructokinase within cell-free extracts of *S. thermophila* (Table 2), interestingly, is in the lower range of those observed for most other members of the *Spirochaeta tested* (1.1–5.25 U/mg). It remains to be seen whether the high specific activity and other biochemical characteristics of the *S. thermophila* enzyme are similar to the phosphofructokinases of other *Spirochaeta*. In partial support of a close relationship is that antibodies made in rabbits using the purified *S. thermophila* phosphofructokinase cross-reacted with a protein of a similar size derived from a *S. halophila* cell-free extract, indicative of a similar structure (unpublished data). The molecular mass under denaturing conditions, however, is in the range of what is predicted for the phosphofructokinases from *T. pallidum* and *B. burgdorferi*, respectively and for the β-subunits of *R. communis* (60.1 kDa) and *S. tuberosum* (60.0 kDa; Carlisle et al. 1990; Todd et al. 1995; Fraser et al. 1997, 1998). The native molecular mass estimated from the gel filtration data would be equivalent to 0.84 of the size of a homodimer based on the molecular mass of the protein obtained after denaturing electrophoresis and is suggestive of a dimeric structure. Interestingly, both the *T. pallidum* and *B. burgdorferi* genome sequences contain two open reading frames coding for putative phosphofructokinase subunits and *R. communis* and *S. tuberosum* phosphofructokinases each contain an α- and a β-subunit. Evidence against a heterodimeric structure for the *S. thermophila* enzyme (as opposed to a homodimeric) are the single bands routinely obtained under denaturing conditions and the N-terminal sequence which both partially support the presence of only a single type of subunit.

The spirochete PP_i-phosphofructokinase was virtually unaffected by traditional allosteric effectors and displayed hyperbolic kinetics for both the forward and reverse directions and is thus not likely to be allosterically regulated. This lack of apparent regulation is similar to what has been found with other bacterial and most primitive eukaryotic PP_i-phosphofructokinases, except those from *Naegleria fowleri and Euglena gracilis*, which have been shown to be either activated by AMP or fructose 2,6-diphosphate, respectively (Enomoto et al. 1988; Mertens et al. 1993). Reaction rates in vivo in *S. thermophila* are thus likely to be in near-equilibrium, controlled simply by the level of enzyme activity and the concentration of the intracellular reactants and products. This scenario is similar to that suggested for the protist *E. histolytica* (Reeves et al. 1974). The apparent K_m and V_{max} values for the spirochete phosphofructokinase for polyphosphate (and secondarily tripolyphosphate) suggests that this substrate could support substantial glycolytic flux in the presence of intracellular polyphosphates compared to PP_i. We are unsure about any physiological relevance of activities with either adenosine 5′-tetraphosphate or acetyl-phosphate. This could be due to the structure of the enzyme, which may allow access of these "high"-energy phosphate residues permitting some catalysis. No attempt was made to determine kinetic parameters for these substrates.

The spirochete phosphofructokinase has sequence similarity, as expected, with open reading frames from *T. pallidum* and *B. burgdorferi*. A recent phylogenetic analysis suggests that the *Treponema* and *Borrelia* PP_i-phosphofructokinases are most closely related to *Giardia lamblia*, *Entamoeba*, *R. communis* and *S. tuberosum* PP_i-phosphofructokinases (Mertens et al. 1998). It will be interesting to determine whether a phylogenetic comparison with the complete *S. thermophila* sequence suggests it to be more ancestral than those phosphofructokinases sequences derived from mesophilic spirochetes. This would provide some support for our suspicions that the first spirochete-like organisms to have evolved and which later gave rise to the order Spirochaetales were free-living thermophiles.

References

Aksenova HY, Rainey FA, Janssen PH, Zavarin GA, Morgan HW (1992) *Spirochaeta thermophila* sp. nov, an obligately anaerobic, polysaccharolytic, extremely thermophilic bacterium. Int J Syst Bacteriol 42:175–177

Altschul SF, Madden TL, Schäffer AA, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. Anal Biochem 72:248–254

Canale-Parola E (1997) Physiology and evolution of spirochetes. Bacteriol Rev 41:181–204

Carlisle SM, Blakeley SD, Hemmingsen SM, Trevanion SJ, Hiyoshi T, Kruger NJ, Dennis DT (1990) Pyrophosphate-dependent phosphofructokinase. J Biol Chem 265:18366–18371

Ding Y-HR, Ronimus RS, Morgan HW (1999) Purification and properties of the pyrophosphate-dependent phosphofructokinase from *Dictyoglomus thermophilum* Rt46 B.1. Extremophiles 3:131–137

Enomoto T, Miyatake K, Kitaoka S (1988) Purification and immunological properties of fructose 2,6-biphosphate-sensitive pyrophosphate: D-fructose 6-phosphate 1-phosphotransferase from the protist *Euglena gracilis*. Comp Biochem Physiol 90B:897–902

Fraser CM, Casjens S, Huang WM, Sutton GG, Clayton R, Lathigra R, White O, Ketchum KA, Dodson R, Hickey EK, Gwinn M, Dougherty B, Tomb J-F, Fleischmann RD, Richardson D, Peterson J, Kerlavage AR, Quackenbush J, Salzberg S, Hanson M, van Vugt R, Palmer N, Adams N, Gocayne J, Weidman J, Utterback T, Watthey L, McDonald L, Artiach P, Bowman C, Garland S, Fujii C, Cotton MD, Horst K, Roberts K, Hatch B, Smith HO, Venter CJ (1997) Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*. Nature 390:580–586

Fraser CM, Norris SJ, Weinstock GM, White O, Sutton GG, Dodson R, Gwinn M, Hickey EK, Clayton R, Ketchum KA, Sodergren E, Hardham JM, McLeod MP, Salzberg S, Peterson J, Khalak H, Richardson D, Howell JK, Chidambaram M, Utterback T, McDonald L, Artiach P, Bowman C, Cotton MD, Fujii C, Garland S, Hatch B, Horst K, Roberts K, Sandusky M, Weidman J, Smith HO, Venter CJ (1998) Complete genome sequence of *Treponema pallidum*, the syphilis spirochete. Science 281:375–388

Janssen PH, Morgan HW (1992) Glucose catabolism by *Spirochaeta thermophila* RI 19.B1. J Bacteriol 174:2449–2453

Li Z, Phillips NFB (1995) Pyrophosphate-dependent phosphofructokinase from *Giardia lamblia*: purification and characterisation. Protein Expr Purif 6:319–328

Mertens E (1991) Pyrophosphate-dependent phosphofructokinase, an anaerobic glycolytic enzyme? FEBS Lett 285:1–5

Mertens E, De Jonckheere J, Van Schaftingen E (1993) Pyrophosphate-dependent phosphofructokinase from the amoeba *Naegleria fowleri*, an AMP-sensitive enzyme. Biochem J 292:797–803

Mertens E, Ladror US, Lee JA, Miretsky A, Morris A, Rozario C, Kemp RG, Müller M (1998) The pyrophosphate-dependent phosphofructokinase of the protist, *Trichomonas vaginalis*, and the evolutionary relationships of protist phosphofructokinases. J Mol Evol 47:739–750

Morgan HW, Ronimus RS (1998) Pyrophosphate-dependent phosphofructokinases in thermophilic and nonthermophilic microorganisms? In: Wiegel J, Adams M (eds) Thermophiles: the keys to molecular evolution and the origin of life. Taylor and Francis, Philadelphia, pp 269–277

O'Brien WE, Bowien S, Wood HG (1975) Isolation and characterization of a pyrophosphate-dependent phosphofructokinase from *Propionibacterium shermanii*. J Biol Chem 250:8690–8695

Paster BJ, Canale-Parola E (1985) *Treponema saccharophilum* sp. nov., a large pectinolytic spirochete from the bovine rumen. Appl Environ Microbiol 50:212–219

Paster BJ, Dewhirst FE, Weisburg WG, Tordoff LA, Fraser GJ, Hespell TB, Stanton TB, Zablen L, Mandelco L, Woese CR (1991) Phylogenetic analysis of the Spirochetes. J Bacteriol 173:6101–6109

Phillips NFB, Li Z, Lindmark DG (1997) Isolation of a pyrophosphate-dependent phosphofructokinase from *Hexamita inflata*. Mol Biochem Parasitol 90:377–380

Rainey FA, Janssen PH, Wild DJC, Morgan HW (1991) Isolation and characterization of an obligately anaerobic, polysaccharolytic, extremely thermophilic member of the genus *Spirochaeta*. Arch Microbiol 155:396–401

Rainey FA, Dorsch M, Morgan HW, Stackebrandt E (1992) 16S rDNA analysis of *Spirochaeta thermophila*: its phylogenetic position and implications for the systematics of the order *Spirochaetales*. System Appl Microbiol 15:197–202

Reeves RE, South DJ, Blytt HJ, Warren LG (1974) Pyrophosphate:D-fructose 6-phosphate 1-phosphotransferase. A new enzyme with the glycolytic function of 6-phosphofructokinase. J Biol Chem 249:7737–7741

Ronimus RS, Koning J, Morgan HW (1999) Purification and characterization of an ADP-dependent phosphofructokinase from *Thermococcus zilligii*. Extremophiles 3:121–129

Selig M, Xavier KB, Santos H, Schönheit P (1997) Comparative analysis of Embden-Meyerhof and Entner-Doudoroff glycolytic pathways in the hyperthermophilic archaea and the bacterium *Thermotoga*. Arch Microbiol 167:217–232

Todd JF, Blakeley SD, Dennis DT (1995) Structure of the genes encoding α the β subunits of castor pyrophosphate-dependent phosphofructokinase. Gene 152:181–186