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Arginase From *Bacillus caldovelox*

A thesis submitted in partial fulfilment
of the requirements for the degree
of
Doctor of Philosophy in Biochemistry
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by

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ABSTRACT

Nine *Thermus* strains, six thermophilic *Bacillus* species, six extremely thermophilic archaebacteria and other thermophilic bacteria were cultured in arginine-supplemented media and screened for arginase, arginine deiminase and arginine decarboxylase activity by a reversed-phase liquid chromatography method developed to detect these arginine-catabolizing enzymes in cell-free extracts. *Thermus*, *Bacillus* and *Sulfolobus* strains produced arginase, but arginine deiminase and arginine decarboxylase activities were only detected in two archaebacterial anaerobes and *Clostridium thermohydrosulfuricum*. Other enzymes of the arginase and arginine deiminase pathways were also present in these bacteria.

Inducible arginases (EC 3.5.3.1) were purified from *Bacillus caldovelox* (DSM 411) and *Thermus* 4-1A (TRUCC 52) by $(NH_4)_2SO_4$ and protamine sulphate precipitations, DEAE Sepharose CL-6B ion exchange chromatography, Superose 12 GP-FPLC, Mono Q IE-FPLC and phenyl-Superose HI-FPLC. *B. caldovelox* arginase has a M_r of ~176000 and a subunit M_r of 32000, suggesting a hexameric subunit structure. It has an isoelectric point of 5.4, a pH optimum for activity of 9 (at 60°C in 0.1M CAPS/NaOH buffer) and requires a divalent cation for activity. The purified enzyme contains 1.2 Mn^{2+} ions and 0.17 Fe^{2+} ions per subunit and is activated ~30% by Mn^{2+}. The enzyme has a K_m for arginine of 3.4mM at pH9 and 25mM at pH7 and hydrolyses L-canavanine at 4.7% and D-arginine and L-homoarginine at <1% of the rate seen for L-arginine. It is competitively inhibited by L-ornithine with a K_i of 0.55mM at pH9 and 4.4mM at pH7 and has an E_a of 46.3kJ/mole at pH9.

The *B. caldovelox* arginase is thermostable with a half life of 105min at 95°C at pH7 in the presence of 0.5mM Mn^{2+} and 25mM aspartic

acid. Mn^{2+} and chelating agents or proteins have a synergistic stabilizing effect and it is suggested that an excessive $[Mn^{2+}]$ causes inactivation in the absence of a chelating agent. The apoenzyme, prepared by chelation of metal ions at pH7 at 60°C or by incubation at pH4.4 at 20°C, retains its native oligomeric structure but is dissociated into subunits at pH2.5 at 20°C. The subunits reassociate at pH9.5 at 20°C in the absence of Mn^{2+} to form the native oligomer.

The *Thermus* 4-1A arginase has a M_r of ~167000 and a subunit M_r of 33000. It has an isoelectric point of 5.1, a pH optimum of 9.3-9.8 and is activated by VO^{2+}, Mn^{2+}, Ni^{2+} and Cd^{2+} and less strongly by Co^{2+}, Fe^{2+}, Mo(III) and Mg^{2+}. The purified enzyme contains 0.33 Mn^{2+} ions and 0.1 Fe^{2+} ions per subunit and has an E_a of 30.2kJ/mole at pH9.

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LIST OF ABBREVIATIONS

agm	Agmatine
APS	Ammonium persulphate
ATCC	American Type Culture Collection
AUFS	Absorbance units full scale
BSA	Bovine serum albumin
CA	Casamino acids
CAPS	3-(Cyclohexylamino)-1-propanesulphonic acid
CDTA	*trans*-1,2-diaminocyclohexane-*N*,*N*,*N*',*N*'-tetraacetic acid
CHES	2-(Cyclohexylamino)ethanesulphonic acid
cit	Citrulline
DeH	Dehydrogenase
DSM	Deutsche Sammlung von Mikroorganismen
DTT	Dithiothreitol
E_a	Activation energy
EPPS	*N*-(2-Hydroxyethyl)piperazine-*N*'-3-propanesulphonic acid
GP-FPLC	Gel permeation-fast protein liquid chromatography
GP-HPLC	Gel permeation-high performance liquid chromatography
HC	Homocysteic acid
HI-FPLC	Hydrophobic interaction-fast protein liquid chromatography
IEF	Isoelectric focusing
IE-FPLC	Ion exchange-fast protein liquid chromatography
K_{diss}	Dissociation (equilibrium) constant, e.g. $K_{diss}=[E][M]/[EM]$
M&B	May and Baker
MES	2-(*N*-Morpholino)ethanesulphonic acid
MOPS	3-(*N*-Morpholino)propanesulphonic acid
MW	Molecular weight
NBS	National Bureau of Standards
NTA	Nitrilotriacetic acid
OPA-ME	*o*-phthalaldehyde-2-mercaptoethanol
PAGE	Polyacrylamide-gel electrophoresis
PALP	Pyridoxal 5'-phosphate
*p*CMBS	*p*-chloromercuribenzenesulfonic acid
PMSF	Phenylmethanesulphonyl fluoride
RP-LC	Reverse(d)-phase liquid chromatography
rpm	Revolutions per minute
SGE	Scientific Glass Engineering Pty Ltd
std	Standard
syn.	Synonym (this precedes incorrect/old names for microbes)
$t_{1/2}$	Half life
TEMED	*N*,*N*,*N*',*N*'-tetramethylethylenediamine
TP	Trypticase peptone
TRUCC	Thermophile Research Unit Culture Collection
U	Unit of enzyme activity
USB	United States Biochemicals Corporation
YE	Yeast extract

All other abbreviations used conform to the rules given in the "Policy of the Journal and Intructions to Authors" section of the Biochemical Journal. This is updated annually and published in the first volume of each new year, most recently Biochem. J. (1988), **249**, 1-20.

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CHAPTER 1

INTRODUCTION

Preamble

This project was originally conceived as an investigation into the enzymology of arginine catabolism in thermophilic bacteria. Reasons for this choice included the large amount of information that exists on the metabolic physiology and enzymology of arginine catabolism in mesophiles, the variety of degradative pathways (with their member enzymes) that can degrade this amino acid, and the possible evolutionary significance of the distribution of the various pathways in bacteria (Stalon, 1985). There are currently nine enzymes known to catalyse an initial step of arginine catabolism in bacteria and variations in subsequent steps bring the number of distinct catabolic pathways to at least fifteen. The most recent comprehensive review on bacterial arginine catabolism (Cunin *et al.*, 1986) lists 40 enzymes implicated in the various pathways. This diversity of pathways is a reflection of the many functions arginine and its catabolites perform in bacteria. Because the topic is large and new pathways continue to be discovered, a great deal of information is still unavailable even for the more established pathways and enzymes. Considering the opportunities for original work with mesophilic microorganisms, it is perhaps not surprising that reports of arginine catabolism in extremely thermophilic bacteria appear to be limited to the detection of arginase and arginine decarboxylase activities in cell-free extracts of *Thermus aquaticus* (Degryse *et al.*, 1976) and *Clostridium thermohydrosulfuricum*

(Paulin and Pösö, 1983) respectively.

An initial aim of this project was to screen thermophilic bacteria for arginase, arginine deiminase and arginine decarboxylase activities, each activity being unique to a particular catabolic pathway. It was thus hoped to obtain information on the occurrence of these three arginine-degrading pathways as a by-product of the major objective, which was to select a good source of one of the arginine-catabolizing enzymes for biochemical study. The time required to both develop a suitable screening method and culture bacteria meant that this method was not applied as widely as was hoped, but useful results were obtained. The enzyme arginase from *Bacillus caldovelox* (DSM 411) was selected for characterization because of its relatively high yield, possible medical and industrial applications for the enzyme and also because arginase, as a metal-activated enzyme, offered the possibility of additional studies on metal ion-mediated thermostability by other workers in our research group. Subsequently some work was also done on arginase from *Thermus* 4-1A.

This introduction is divided into four sections. The first describes the three pathways of arginine catabolism most relevant to this study by way of their distribution, structure and function in microorganisms (Section 1-1). The other pathways are only discussed briefly although the recent discovery of the arginine succinyltransferase pathway (Stalon, 1985) has altered the interpretation of some earlier results regarding the more established pathways. This section is followed by a brief discussion of pathway distribution (Section 1-2). After a short account of some industrial and medical applications of arginine-catabolizing enzymes (Section 1-3), the introduction concludes with a review of the enzyme arginase with reference to the catalytic and stabilizing functions of metal ions

in enzymes (Section 1-4).

1-1 Arginine Catabolism by Microorganisms

When screening thermophilic bacteria I was primarily concerned with the well characterized arginase, arginine deiminase and arginine decarboxylase pathways of arginine catabolism. A considerable amount of information is available regarding the distribution of these pathways among mesophilic microbes and the properties of the enzymes concerned. Figures 1-1, 1-2 and 1-3 show the reactions occurring in these three pathways. Table 1-1 lists the microbes in which these pathways have been observed. That some bacteria use more than one catabolic pathway does not indicate redundancy, but rather emphasizes the complementary roles of the pathways, the enzymes of which are often induced by different growth conditions.

1-1-1 The arginase pathway

The arginase pathway (Fig. 1-1) is associated with aerobic metabolism and occurs only in aerobes and facultative anaerobes. The pathway supplies carbon and nitrogen, often permitting growth on arginine as a sole nitrogen source or even sole nitrogen and carbon source. Most microbes degrade the urea formed by arginase to ammonia and carbon dioxide (via allophanate in *Saccharomyces cerevisiae* (Whitney and Cooper, 1972)), and ornithine is typically metabolized to glutamate. However, utilization of ornithine as a carbon source in some *Agrobacterium* spp. proceeds via cyclization to proline (Ellis *et al.*, 1979), and ornithine is not metabolized by the cyanobacterium *Synechocystis* sp. PCC 6308 (syn. *Aphanocapsa* sp.) in which the constitutive arginase and urease enzymes probably function to supply only nitrogen by catabolism of arginine (Weathers *et al.*, 1978). Urea

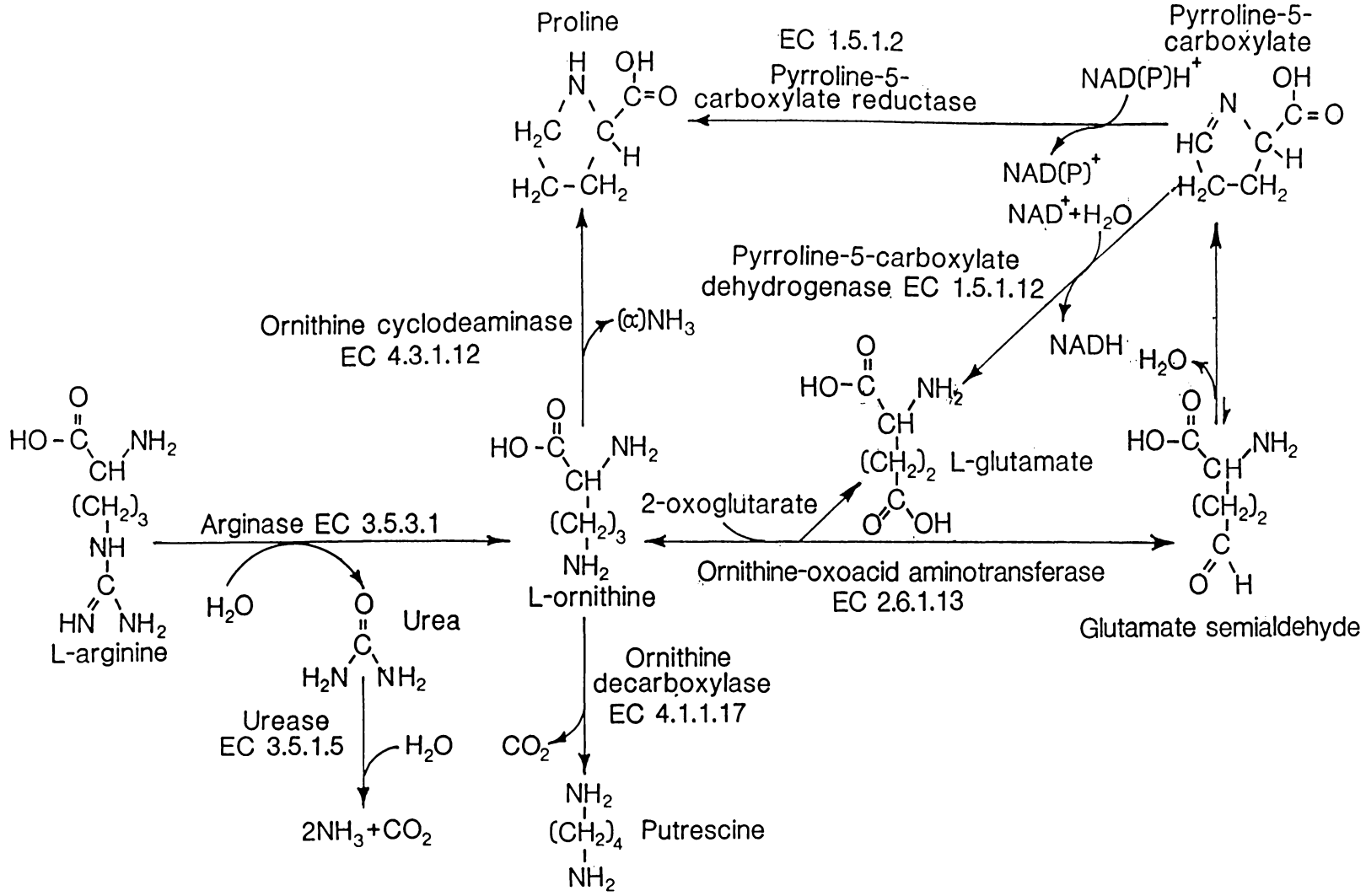

Figure 1-1 The arginase pathway.

A diagram showing the usual three-enzyme arginase pathway (arginase, ornithine aminotransferase and pyrroline-5-carboxylate dehydrogenase) and two alternative fates for ornithine that can apply in some microorganisms. Urease activity is typically present in arginase positive microbes.

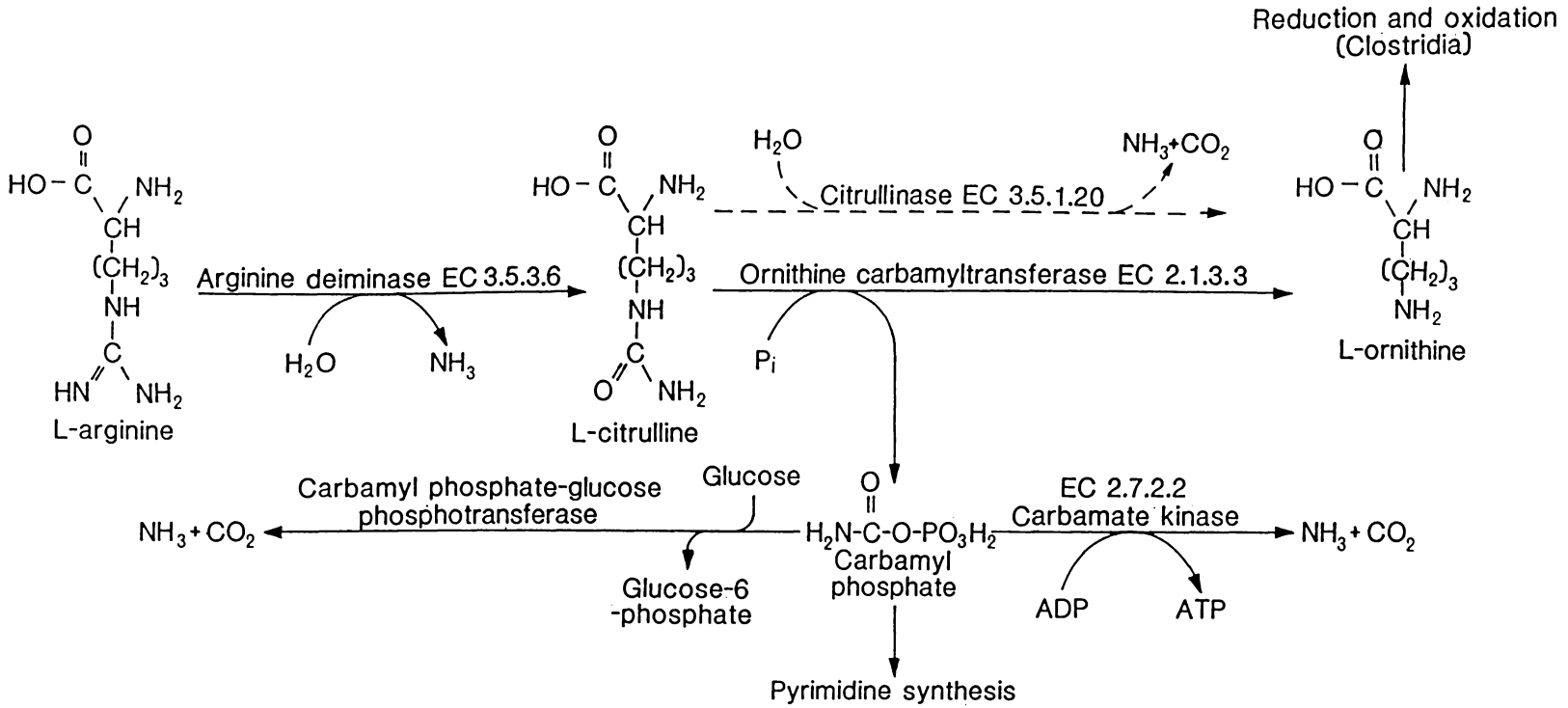

Figure 1-2 The arginine deiminase pathway.

A diagram showing the classical three-enzyme arginine deiminase pathway (arginine deiminase, catabolic ornithine carbamyltransferase and carbamate kinase) and other metabolic options. Citrullinase and catabolic ornithine carbamyltransferase activity cannot be distinguished when assay conditions favour hydrolysis of carbamyl phosphate to NH_3 and CO_2 so it is uncertain if citrullinase occurs in all the studies reporting this activity. Apart from reduction and oxidation, clostridia may decarboxylate ornithine, and the other routes of ornithine utilization indicated in Fig. 1-1 also occur in some deiminase positive microbes, although the enzymes of the different pathways are seldom co-induced.

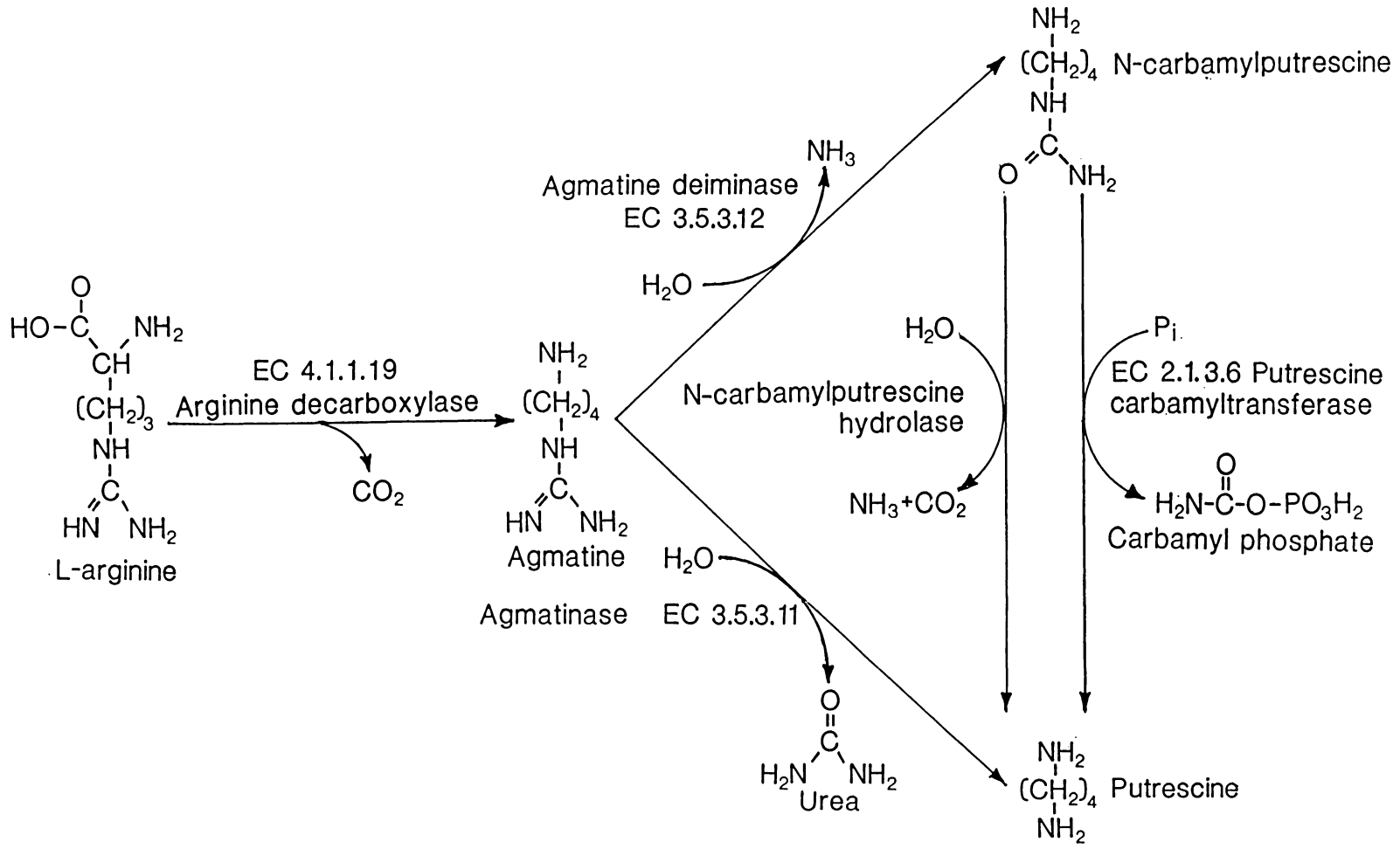

Figure 1-3 The arginine decarboxylase pathway.

This diagram shows the alternative routes by which bacteria transform agmatine to putrescine. The enzyme putrescine carbamyltransferase belongs to the agmatine deiminase pathway (analogous to the arginine deiminase pathway) that occurs in *Streptococcus faecalis* ATCC 11700. This bacterium is devoid of arginine decarboxylase activity.

Table 1-1 Distribution of arginine-catabolizing pathways in microbes

Pathway(s)	Organism	Reference
Arginase	Saccharomyces cerevisiae	Middelhoven, 1964
	Neurospora crassa	Castañeda *et al.*, 1967
	Aspergillus nidulans	Cybis *et al.*, 1972
	Anabena variabilis	Gupta & Carr, 1981
	Agrobacterium tumefaciens	Vissers *et al.*, 1986
	Streptomyces fradiae*	Vargha *et al.*, 1983
	Rhizobium spp.*	Vissers *et al.*, 1986
	Bacillus anthracis (uncapsulated)*	Soru, 1983
	Thermus aquaticus*	Degryse *et al.*, 1976
	Crithidia* & Leptomonas* spp.	Camargo *et al.*, 1987
Arginine deiminase	Pseudomonas spp.	Stalon & Mercenier, 1984
	Enterobacter aerogenes	Chen *et al.*, 1982
	Mycoplasma arthritidis	Schimke *et al.*, 1966
	Mycoplasma hominis	Fenske & Kenny, 1976
	Spiroplasma citri	Townsend, 1976
	Eubacterium lentum	Sperry & Wilkins, 1976
	Lactobacillus buchneri	Manca De Nadra *et al.*, 1984
	Lactobacillus leichmannii	Manca De Nadra *et al.*, 1986
	Clostridium perfringens	Schmidt *et al.*, 1952
	Clostridium botulinum	Mitruka & Costilow, 1967
	Clostridium sporogenes	Venugopal & Nadrarni, 1977
	Lactic streptococci	Crow & Thomas, 1982
	Streptococcus faecalis	Simon *et al.*, 1982
	Streptococcus sanguis	Ferro *et al.*, 1983
	Streptococcus milleri	Rogers *et al.*, 1986
	Streptococcus lactis	Poolman *et al.*, 1987
	Treponema denticola	Blakemore & Canale-Parola, 1976
	Chlorella vulgaris	Shafer & Thompson, 1968
	Crithidia fasciculata	Kidder *et al.*, 1966
	Bacillus spp.*	Ottow, 1974
	Tetrahymena pyriformis*	Hill & Chambers, 1967
	Chlamydomonas reinhardi*	Sussenbach & Strijkert, 1969
	Euglena gracilis*	Park *et al.*, 1983
	Herpetomonas* & Phytomonas* spp.	Camargo *et al.*, 1987
Arginine decarboxylase	Salmonella and Shigella spp.	Chen *et al.*, 1982
	Escherichia coli	Cunin *et al.*, 1986
	Klebsiella pneumoniae	Friedrich & Magasanik, 1978
	Mycobacterium smegmatis	Zeller *et al.*, 1954
	Mycobacterium bovis	Paulin *et al.*, 1987
	Pseudomonas spp.	Stalon & Mercenier, 1984
	Clostridium thermohydrosulfuricum*	Paulin & Pösö, 1983
	Serratia marcescens*	Goldschmidt & Lockhart, 1971
	Enterobacter cloacae*	Goldschmidt & Lockhart, 1971
	Citrobacter spp.*	Goldschmidt & Lockhart, 1971
	Halobacteria spp.*	Kamekura *et al.*, 1987
Arginase and arginine deiminase	Bacillus licheniformis	Broman *et al.*, 1978
	Synechocystis sp. 6308	Weathers *et al.*, 1978
	Acholeplasma spp.*	Salih *et al.*, 1983
	Staphylococcus aureus*	Soru & Zaharia, 1976
		Chen *et al.*, 1982
	Bacillus subtilis*	Baumberg and Harwood, 1979
		Chen *et al.*, 1982
Arginine decarboxylase and arginine deiminase	Pseudomonas aeruginosa	Mercenier *et al.*, 1981
	Pseudomonas spp.	Mercenier *et al.*, 1981
	Aeromonas formicans	Stalon *et al.*, 1982
	Halobacterium salinarium*	Hartmann *et al.*, 1980
		Kamekura *et al.*, 1987

*Only the first enzyme of the pathway(s) was detected.

is not degraded in most *Bacillus* spp., while ornithine utilization as a carbon and nitrogen source proceeds by transamination of the δ-amino group of ornithine to 2-oxoglutarate, forming glutamate and glutamate γ-semialdehyde. The latter compound undergoes spontaneous cyclization to the more stable Δ^1-pyrroline-5-carboxylate, making the reaction physiologically irreversible (Vogel and Kopac, 1959). This intermediate and the final step of oxidation of Δ^1-pyrroline-5-carboxylate to glutamate is common to both the arginase pathway and the -proline catabolic pathway. In *Bacillus licheniformis* (syn. *Bacillus subtilis*) this reaction is catalysed by two distinct pyrroline-5-carboxylate dehydrogenases; one is co-induced with enzymes of arginine catabolism and the other with proline catabolic enzymes (De Hauwer *et al.*, 1964). The *B. licheniformis*, *S. cerevisiae* (Stalon *et al.*, 1987) and *Aspergillus nidulans* (Stevens and Heaton, 1973) ornithine aminotransferases are specific for ornithine and only catalyse the catabolic reaction of the arginase pathway. It would appear that ornithine (and *N*-acetylornithine) aminotransferase activities detected in aerobically grown *Pseudomonas aeruginosa* (Voellmy and Leisinger, 1975), *Aeromonas formicans* (Stalon *et al.*, 1982) and *Klebsiella pneumoniae* (syn. *Klebsiella aerogenes*) (Friedrich and Magasanik, 1978) are actually due to a non-specific N^2-succinylornithine aminotransferase, the third enzyme of the arginine succinyltransferase pathway (Jann *et al.*, 1986; Stalon *et al.*, 1987).

At least the first two enzymes of the arginase pathway are usually induced by arginine and ornithine, and in some *Bacillus* spp. citrulline and proline are also inducers (Laishley and Bernlohr, 1968; Harwood and Baumberg, 1977; Baumberg and Harwood, 1979). Arginine-induced enzymes of the arginase pathway are typically subject to carbon catabolite repression (Laishley and Bernlohr, 1968; Broman *et al.*, 1978) and in

some cases, nitrogen catabolite repression* (Wiame, 1971) although the latter regulation is probably unimportant in most *Bacillus* spp. (Schreier *et al.*, 1982).

1-1-2 The arginine deiminase pathway

The arginine deiminase pathway (Fig. 1-2) degrades arginine to ornithine with the formation of NH_3, CO_2 and one mole of ATP per mole of arginine. The principal function of this pathway is energy generation. It is widely distributed among anaerobes and used by facultative anaerobes to generate ATP under anaerobic conditions. Arginine serves as sole energy source for *Mycoplasma arthriditis* (syn. *M. hominis*) (Schimke *et al.*, 1966), *Treponema denticola* (Blackmore and Canale-Parola, 1976), *Streptococcus faecalis* (Deibel, 1964; Simon *et al.*, 1982), and *P. aeruginosa* (Haas and Kley, 1980; Vander Wauven *et al.*, 1984). This pathway allows the latter organism, usually regarded as a strict aerobe (Krieg, 1984), to grow in anaerobic conditions in the absence of alternative electron acceptors. The energy-generating function is also clear in *Spiroplasma citri* (Igwegbe and Thomas, 1978), *Streptococcus milleri* (Rogers *et al.*, 1986), lactic streptococci (Crow and Thomas, 1982), *B. licheniformis* (Broman *et al.*, 1978), *Halobacterium salinarium* (Hartmann *et al.*, 1980), cyanobacterium *Synechocystis* sp. (Weathers *et al.*, 1978), and other Pseudomonas spp.

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* In this introductory discussion the words induction and repression are used in a descriptive sense only and do not necessarily imply that a particular metabolite is acting directly in the regulatory mechanism. In many cases the evidence available simply relates to changes in enzyme levels in response to the addition of metabolites to the growth medium.

(Mercenier *et al.*, 1981; Stalon and Mercenier, 1984; Vander Wauven *et al.*, 1984). Most of these organisms and others (e.g. *Lactobacillus leichmannii* (Manca de Nadra *et al.*, 1986) and *Aeromonas formicans* (Stalon *et al.*, 1982)) do not metabolize ornithine further. This is in spite of the arginine-induced ornithine succinyltransferase and N^2-succinylornithine aminotransferase activities found in some *Pseudomonas* spp. and *Aeromonas formicans* because the pathway to which these activities belong is aerobic (Stalon *et al.*, 1987). Similarly, ornithine is not metabolized further by *B. licheniformis* in anaerobic cultures due to repression of ornithine aminotransferase activity under these conditions. Both the coordinate induction of deiminase pathway enzymes by arginine under growth conditions that deplete the cellular ATP pool and the coordinate carbon catabolite repression seen in deiminase-positive bacteria are consistent with the energy-generating role of this pathway.

Cyclization of deiminase-formed ornithine to proline can occur in *T. denticola* (Cunin *et al.*, 1986). Clostridia can degrade ornithine by numerous routes including the energy-yielding mutual oxidation-reduction of certain pairs of amino acids, or by single substrate fermentation. Oxidation of L-ornithine in *Clostridium sticklandii* proceeds by racemization then migration of the δ-amino group to C_4 to form D-*threo*-2,4-diaminopentanoate, followed by oxidative deamination at C_4 to yield 2-amino-4-oxopentanoate. This undergoes thiolytic cleavage by reaction with coenzyme A forming acetyl-CoA and D-alanine (Tsuda and Friedman, 1970; Barker, 1981). When L-proline is provided as the "paired" amino acid, this is reduced (after racemization) by D-proline reductase (using the NAD(P)H formed during 2-*threo*-2,4-aminopentanoate oxidation) to yield 5-aminovaleric acid (Seto and Stadtman, 1976). Without a substrate pair ornithine in

C. sticklandii is also reduced (via formation of proline as below), permitting single substrate fermentation. In *Clostridium botulinum* and *Clostridium* PA 3670 (similar to *C. sporogenes*) the use of ornithine as a hydrogen acceptor involves the formation of proline (with retention of the δ-amino group) in a complex cyclodeamination reaction (Muth and Costilow, 1974; Barker, 1981). Mitruka and Costilow (1967) showed that further metabolism of ornithine produced by the arginine deiminase pathway in *C. botulinum* was mainly by single substrate fermentation although ~20% was decarboxylated to putrescine.

In *Mycoplasma arthriditis* (syn. *M. hominis*) it was estimated that ATP produced from arginine catabolism by the arginine deiminase pathway could satisfy the energy requirements for macromolecular synthesis. The high levels of arginine deiminase and ornithine carbamyltransferase (10% and 4% of cytoplasmic protein respectively) found in cells cultured in arginine-supplemented media supported the hypothesis that this pathway is a major route of ATP generation in rapidly growing cultures (Schimke *et al.*, 1966).

Besides ATP synthesis, the deiminase pathway performs other functions in some bacteria. Ammonia generated by the pathway permits utilization of arginine as a sole nitrogen source by *B. licheniformis* and *Aeromonas formicans* grown under anaerobic conditions (Stalon, 1985). Some *Streptococcus* spp. that lack the anabolic enzyme carbamyl phosphate synthetase use carbamyl phosphate synthesized from citrulline and inorganic phosphate by ornithine carbamyltransferase in pyrimidine biosynthesis (Abdelal, 1979) and in *Streptococcus faecium* (syn. *S. faecalis*) which lacks carbamate kinase carbamyl phosphate is used in glucose phosphorylation to initiate glycolysis so preserving the energy-generating role of this pathway (Pendey, 1980). An acid tolerance function has been proposed for the arginine deiminase pathway

in *Streptococcus* spp. and *P. aeruginosa* (Marquis *et al.*, 1987). This pathway continued to function at pH values below those required for growth or glycolysis allowing the bacteria to survive potentially lethal acidification by producing NH_3 to raise the environmental pH.

The arginine deiminase pathway is often referred to as the arginine dihydrolase pathway, a name originally proposed by Hills in 1940 for the enzyme system releasing 2 moles NH_3 and 1 mole CO_2 from arginine in *Streptococcus* spp. (Oginsky and Gehrig, 1952). As the ATP-generating pathway shown in Fig. 1-2 has only one hydrolytic step this name is inaccurate. However, the name arginine dihydrolase is applicable to a pathway that occurs in the protozoan *Tetrahymena pyriformis* in which ornithine carbamyltransferase of the deiminase pathway is apparently replaced by a novel citrullinase activity (Hill and Chambers, 1967; see also Fig. 1-2). Abdelal (1979) proposed that the assay method used for this enzyme favoured the hydrolysis of any carbamyl phosphate formed during the reaction to NH_3 and CO_2 and that the observed activity could be due to a catabolic ornithine carbamyltransferase. The phosphorylytic citrullinase activity in *Crithidia fasciculata* (Kidder *et al.*, 1966) supports Abdelal's suggestion, so despite reports of citrullinase activity in other protozoa (Camargo *et al.*, 1987) and algae (Park *et al.*, 1983) the nature of citrullinase activity (EC 3.5.1.20) in these organisms remains uncertain. It is possible that a true arginine dihydrolase pathway in protozoa would function to provide nitrogen and carbon.

1-1-3 The arginine decarboxylase pathway

The principal function of the arginine decarboxylase pathway is to produce the polyamine putrescine which is used as a precursor in the formation of larger polyamines or as a carbon and nitrogen source by

catabolism to glutamate and succinate (Voellmy and Leisinger, 1976; Cunin *et al.*, 1986). Several lines of evidence show that putrescine or its higher derivatives are required for optimal growth and macromolecular synthesis in prokaryotes (Tabor and Tabor, 1985). As Fig. 1-3 shows, arginine is decarboxylated to form agmatine. Agmatine can be degraded to putrescine either by agmatine deiminase and *N*-carbamylputrescine hydrolase as in *Pseudomonas* (Stalon and Mercenier, 1984) and *Aeromonas* spp. (Stalon *et al.*, 1982), or by agmatinase in *K. pneumoniae* and *Escherichia coli*, the latter organism excreting urea (Cunin *et al.*, 1986). *K. pneumoniae* (Friedrich and Magasanik, 1979) and *P. aeruginosa* (Mercenier *et al.*, 1980) can grow on agmatine as sole carbon and nitrogen source but when grown on arginine the amount metabolized by the decarboxylase pathway need only be sufficient to satisfy the putrescine requirements for polyamine biosynthesis, the bulk of arginine being degraded by the arginine succinyltransferase pathway. *S. faecalis* ATCC 11700 lacks arginine decarboxylase activity but uses agmatine as sole energy source (Simon and Stalon, 1982) by an agmatine deiminase pathway that is analogous to the ATP-generating arginine deiminase pathway also present in this bacterium. The putrescine produced is excreted, sharing the fate of ornithine formed in the arginine deiminase pathway. Some strains of *E. coli* synthesise two distinct arginine decarboxylases. The degradative enzyme, which is induced by arginine and culture conditions that cause acid stress, may function to regulate medium pH by production of CO_2 (Tabor and Tabor, 1976) while the function of the biosynthetic enzyme is to provide putrescine.

Although decarboxylation of ornithine provides a more direct route to putrescine, exogenous arginine typically represses and/or feedback-inhibits the ornithine biosynthetic enzymes of arginine

prototrophic bacteria (i.e. bacteria capable of synthesizing ornithine and arginine from ammonia as a sole nitrogen source). Under these conditions decarboxylation of arginine is the major route to putrescine (Voellmy and Leisinger, 1978). Arginine decarboxylase activity has not been detected in organisms that use the arginase pathway probably because any putrescine requirement could be satisfied by decarboxylation of ornithine formed by arginase.

As a precursor of complex polyamines, putrescine and its synthesis may have special significance in thermophiles. Several novel polyamines have been isolated from extremely thermophilic eubacteria (Paulin *et.al.*, 1983; Oshima and Kawahata, 1983), and from the extremely thermophilic archaebacteria *Caldariella acidophila* (De Rosa *et al.*, 1976) and *Sulfolobus acidocaldarius* (Oshima, 1983). The polyamine composition of the extreme thermophile *Thermus thermophilus* depends on the growth temperature (Oshima and Baba, 1981; Oshima, 1982). Speculation as to a relationship between thermotolerance and unusual polyamines has centered primarily on the stabilization of DNA and RNA (Mahler *et al.*, 1961; Liquori *et al.*, 1967; Cohen, 1982) and (ribosomal) proteins (Stevens and Morrison, 1968) although the stabilization of membrane structures is another possibility (Stevens, 1967). However, Hamana and Matsuzaki (1987) have presented evidence that higher polyamines are not essential for the thermotolerance of all microorganisms and the novel polyamines are now known to occur in mesophilic bacteria and eukaryotes (Oshima, 1983).

1-1-4 Additional pathways and other aspects of arginine catabolism

The remaining pathways of arginine catabolism are either less well characterized or have currently only been identified in a few bacteria. Each is briefly described here by reference to the enzyme that

catalyses the first degradative step. The recently discovered arginine succinyltransferase pathway (Stalon, 1985), which commences with the transfer of a succinyl group from succinyl-CoA to the α-amino group of arginine forming N^2-succinylarginine, provides nitrogen and dissimilates the carbon skeleton of arginine in *Pseudomonas* spp., *Aeromonas formicans* and *K. pneumoniae* (Stalon, 1985; Stalon *et al.*, 1987). Transamination of the α-amino group from arginine to 2-oxoglutarate to produce 2-oxoarginine and glutamate (the arginine aminotransferase pathway) has been observed in *Arthrobacter simplex* (Tochikura *et al.*, 1980), while the arginine oxidase pathway, which is initiated by oxidative deamination of arginine to 2-oxoarginine, occurs in *P. putida*, other *Pseudomonas* spp., and the cyanobacterium *Synecoccocus* sp. PCC 6308 (syn. *Anacystis nidulans*) (Pistorius and Voss, 1982). Arginine 2-monooxygenase oxidatively decarboxylates arginine to guanidinobutanamide (the first step of the arginine oxygenase pathway) in *Streptomyces griseus* (Van Thoai *et al.*, 1966) and other actinomycetes, and possibly in *Brevibacterium helvolum* (Cunin *et al.*, 1986). Arginine transamidinase activity, catalysing the transfer of the guanidine moiety of arginine to suitable acceptors (e.g. hydroxylamine, glycine) was detected in *K. pneumoniae* (Friedrich and Magasanik, 1978) but the presence of the arginine succinyltransferase pathway in this organism has cast doubt on whether the observed transamidinase activity has a function or occurs at all *in vivo*. *C. botulinum* shows transamidinase activity in crude extracts (Mitruka and Costilow, 1967) and an enzyme catalysing several transamidination reactions with arginine as donor has been purified from *Streptomyces bikiniensis* (Walker and Walker, 1970). Most group I Clostridia (Barker, 1981) can degrade arginine directly by a Strickland reaction in which reduction of arginine to δ-aminovaleric acid is coupled to oxidation of

other amino acids, most commonly serine and phenylalanine. The arginine racemase identified in *P. putida* and *P. graveolens* (Yorifuji *et al.*, 1971) allows utilization of D-arginine by L-arginine-specific catabolic pathways in these organisms. More recently Jann *et al.* (1988) reported a D-arginine dehydrogenase activity in *P. aeruginosa* which in combination with arginine racemase may serve to degrade L-arginine via 2-oxoarginine and 4-guanidinobutyrate. *Neurospora crassa* elaborates an extracellular basic L-amino acid deaminase which degrades arginine to 2-oxoarginine (Debusk and Ogilvie, 1984). *Streptomyces* strains use arginine in the synthesis of the antibiotics streptomycin (Walker and Hndica, 1964) and 2-nitro-imidazole (azomycin) (Nakane *et al.*, 1977), while ornithine derived from arginine is incorporated into the cyclic peptide antibiotic bacitracin in post-logarithmic *B. licheniformis* cells (Bernlohr and Novelli, 1963; Ramaley and Bernlohr, 1966).

Some of the mechanisms preventing the energy-wasteful ornithine cycle that could result from the combination of arginine biosynthetic and arginase pathways in eukaryotic microorganisms during a transition from anabolic to catabolic growth are based on compartmentalization of enzymes and metabolites (Davis, 1986). These mechanisms are unavailable to bacteria but epiarginasic control in *B. subtilis* (Issaly and Issaly, 1974) and feedback inhibition of anabolic ornithine carbamyltransferase by arginine in *Agrobacterium tumefaciens* and *Rhizobium* spp. (Vissers *et al.*, 1986) are two ways of inhibiting a futile cycle.

1-2 Distribution of Pathways

Stalon (1985) has discussed the distribution of arginine-catabolizing pathways in bacteria. The importance of the arginine deiminase pathway as a means of generating energy under anaerobic conditions probably accounts for its wide distribution in

prokaryotes, being found in five of the eight groups of the eubacterial line (as defined by Fox *et al.* (1980)) and also in the archaebacterial kingdom (Hartmann *et al.*, 1980). The pathway is present in anaerobic bacteria (e.g. clostridia), facultative anaerobes (e.g. *B. licheniformis*) and aerobic Pseudomonas spp. In the latter two cases the pathway is only used when respiration is limited by lack of oxygen and among the pseudomonads only some species, e.g. *P. aeruginosa*, can employ this pathway for successful anaerobic growth, suggesting that the capacity for anaerobic (fermentative) metabolism has been lost by other pseudomonads (*P. putida*, *P. fluorescens* and *P. mendocina*) during evolution.

Despite the wide distribution of amidinohydrolases and transferases capable of hydrolysing guanidino compounds such as allantoic acid, creatine, agmatine and guanidino- 2, 3, and 4C, or dicarboxylic acids, the hydrolysis of arginine to urea and ornithine is restricted to three of Fox's eubacterial groups. These bacteria are the aerobic bacilli, the *Agrobacterium-Rhizobium* group, and the cyanobacteria. The presence of alternative pathways for the utilization of arginine as a carbon and nitrogen source in other eubacterial groups would account partially for this restricted distribution.

The arginine decarboxylase pathway is a means of synthesizing putrescine from arginine when ornithine is unavailable or when ornithine synthesis is inhibited by arginine. Although the full pathway has only been observed in one of Fox's groups, the enterobacteria-pseudomonads, arginine decarboxylase activity is present in *C. thermohydrosulfuricum* and halobacteria (Kamekura *et al.*, 1987). The relatively narrow distribution of this pathway may be accounted for by the wider distribution of ornithine decarboxylase activity which permits direct synthesis of putrescine from ornithine, coupled with the

fact that ornithine is an intermediate of other arginine-catabolizing pathways.

1-3 Applications of Arginine-Catabolizing Enzymes

Industrial applications of arginase and arginine deiminase lie in the field of amino acid production. The production of L-ornithine and D-arginine from arginine racemates by arginase has received attention (e.g. Veronese *et al.*, 1987), while commercial production of L-citrulline from L-arginine has been accomplished with immobilized *P. putida* cells containing arginine deiminase (Yamamoto *et al.*, 1974). Despite the other arginine-catabolizing pathways that occur in this bacterium and its metabolic potential to process citrulline further to ornithine, L-citrulline was the only product seen. Immobilization of whole cells improved the half-life of the enzyme system. A major use of L-ornithine and L-citrulline is in the treatment of enzyme deficiency and liver disorders.

Applications in medicine include cancer chemotherapy by depletion of amino acids required by tumor cells, and enzyme replacement therapy to treat inherited deficiency diseases in human arginine metabolism. The enzymes may be injected directly into the bloodstream (intracorporeal therapy) or the blood pumped through an extracorporeal shunt containing immobilized enzymes. Several attempts to correct hyperargininemia (arginase deficiency) by enzyme replacement therapy have been reported (Kruse *et al.*, 1981; Adriaenssens, 1984; Sakiyama *et al.*, 1984) but other treatments for this and related urea cycle$^-$ enzyme deficiencies are available (Jackson *et al.*, 1986).

Holcenberg and Roberts (1981) reviewed the enzymes of arginine catabolism that may be of use in treatment of cancer. As arginine and

citrulline are both non-essential amino acids, their depletion in blood is permissible. While many tumor cells are sensitive to a combined depletion of arginine and citrulline, the depletion of arginine alone is usually only effective if combined with antitumor citrulline analogues, as citrulline (but not ornithine) can replace the arginine requirement of most tumors. At present citrulline-degrading enzymes with the properties needed for effective enzyme therapy are not available (Holcenberg. 1982). Arginine deiminase from *Streptococcus faecalis* and chemically modified, immobilized or microencapsulated mammalian arginases (e.g. O'Grady and Joyce, 1981; Veronese *et al.*, 1987) may be of use for the treatment of tumors in which citrulline cannot substitute for arginine. Bacterial arginases from *B. subtilis* and *Staphylococcus aureus* had circulation half-lives in mice of 10min and ~200min respectively. It has been proposed that enzymes from thermophiles may be of greater use in these therapies as the decreased molecular flexibility of these enzymes at 37°C confers a greater resistance to proteolysis (Daniel *et al.*, 1982). It may also confer a lower immunogenicity in comparison to their mesophilic counterparts but it is uncertain if the expected increase in circulation lifetime as a result of these two factors would be sufficient to offset the low specific activity of these enzymes at 37°C (Daniel, 1986).

1-4 Arginase

The properties and regulation of enzymes of the arginase, arginine deiminase and arginine decarboxylase pathways have been reviewed by Abdelal (1979), and additional recent information on arginase and other enzymes is summarised in reviews by Cunin *et al.* (1986) and Davis (1986). Arginase (L-arginine amidinohydrolase, EC 3.5.3.1) catalyses

the hydrolysis of L-arginine to L-ornithine and urea (Fig. 1-1). The enzyme may also hydrolyse L-canavanine and α-*N*-substituted L-arginines (Webb, 1984). Arginase is found in animals, plants (e.g. Desai, 1983, Martin-Falquina and Legaz, 1984) and microorganisms. In the liver of ureotelic animals arginase is one of the four enzymes that comprise the urea cycle. Arginase also occurs in a large number of tissues that lack a functioning urea cycle, for example rat kidney (Kaysen and Strecker, 1973) and human heart (Barańczyk-Kuźma *et al.*, 1980) and in non-ureotelic animals (Reddy and Campbell, 1970). In these cases the enzyme may be involved in the synthesis of proline or utilization of arginine as an energy source, roles which are favoured by the mitochondrial location of arginase in these tissues (Carvajal and Cederbaum, 1986; Carvajal *et al.*, 1987). The arginases of mammalian liver and microbial eukaryotes are typically cytoplasmic (Paulus, 1983; Davis, 1986) whereas plant arginases and arginases from non-hepatic tissues and non-ureotelic species are often associated with mitochondria (Kollöffel and Dijke, 1975; Tsuyama *et al.*, 1980). Arginases isolated from various tissues of the same mammalian species differ in physical and kinetic properties (Kaysen and Strecker, 1973; Reddi *et al.*, 1975; Herzfeld and Raper, 1976; Berüter *et al.*, 1978; Zamecka and Porembska, 1988) as well as in subcellular localization. Immunologically distinct forms of arginase have been isolated in individual mammalian tissues (e.g. Skrzypek-Osiecka *et al.*, 1983; Kedra-Luboińska *et al.*, 1988) with one form typically dominant. A family of arginase genes is apparently responsible for these arginase isoenzymes and their differential expression (Dizikes *et al.*, 1986; Spolarics and Bond, 1988) and the different subunit pI's of distinct gene products combined with *in vivo* proteolysis gives rise to extensive subunit heterogeneity. There are no reports of multiple forms of

bacterial arginases but *N. crassa* may elaborate multiple forms of the enzyme (Borkovich and Weiss, 1987b).

Eukaryotic arginases have been extensively studied, the first major investigation being that of bovine liver arginase (Hellerman and Perkins, 1935-1936; Hellerman, 1937; Hellerman and Stock, 1938). Of all the eukaryotic arginases purified, the mammalian liver enzymes and arginase from *Neurospora crassa* and *Saccharomyces cerevisiae* are the most thoroughly characterized. There have been comparatively few studies on bacterial arginases, the earliest report being that of De Hauwer *et al.* (1964) for *B. licheniformis* (syn. *B. subtilis*) arginase. This enzyme (Simon and Stalon, 1976) and the arginases from *Bacillus anthracis* (Soru, 1983) and *Staphylococcus aureus* (Soru and Zaharia, 1976) are the only bacterial arginases that have been purified to homogeneity. Despite the paucity of information on bacterial arginases those studied possess features typical of eukaryotic arginases. They are homomeric oligomers, are activated by divalent transition metal cations, have alkaline (non-physiological) pH optima and are inhibited by ornithine.

Arginase has an absolute catalytic requirement for a divalent metal ion cofactor. This requirement is evident in oligomeric arginases, monomeric arginases and arginase subunits, and the cation probably participates directly in catalysis (Hirsch-Kolb *et al.*, 1971) rather than simply maintaining the enzyme in a catalytic conformation. A variety of divalent cations will confer activity on (or activate) the arginase apoenzyme (or metal ion-deficient arginase), although the effectiveness varies with the cation, arginase source and activation and assay conditions. Manganese (Mn^{2+}), nickel (Ni^{2+}) and cobalt (Co^{2+}) hydrated ions are effective activators in almost all cases and activation by cadmium (Cd^{2+}), iron (Fe^{2+}) and the vanadyl ion VO^{2+} has

also been reported. This poses the question of which metal ion(s) is the cofactor *in vivo*. Middelhoven (1969) compared the properties of several metallo-arginases with those of the enzyme present in cell-free *S. cerevisiae* extracts and concluded that Fe^{2+} was the *in vivo* cofactor. This form of the enzyme was less active that the Mn^{2+}-activated form, which may account for the observation that the *in vitro* activity of *S. cerevisiae* arginase (assayed after preincubation with Mn^{2+} or with Mn^{2+} in the assay buffer) is much greater than it appears to be in live cells (Davis, 1986). An alternative explanation is that only a fraction of the metal ion-binding sites of the arginase are occupied by a strongly activating metal ion *in vivo*, the remaining sites being filled by poorly activating or non-activating divalent cations, possibly weakly bound Mg^{2+} or Ca^{2+}. Given the apparent lack of specificity of at least some metal ion binding sites of arginases, it is quite plausible that more than one metal ion serves as a cofactor *in vivo*. Immunoprecipitated arginase from *N. crassa* contained negligible Fe^{2+} but significant amounts of Mn^{2+}, which, coupled with comparisons of pH profiles, suggested that Mn^{2+} is the *in vivo* cofactor for this enzyme (Borkovich, 1985). The study by Hirsch-Kolb *et al.* (1971) on rat liver arginase suggests that Mn^{2+} is the native cofactor in at least two of the four metal ion-binding sites although the possibility of substitution of the native metal ion by manganese in the remaining sites cannot be ruled out.

Enzymes requiring metal ion cofactors can be divided into two classes depending on the stability of the metal-protein complex (Vallee and Wacker, 1970). Most Mn^{2+}-dependent enzymes are classed as metal ion-activated enzymes (K_{diss} ~1μM or higher), because Mn^{2+} is bound relatively weakly and can often be removed by simple dialysis. This observation is consistent with the Irving-Williams sequence of the

stability of transition metal ion complexes (Vallee and Wacker, 1970). Metalloenzymes form very stable complexes with metal ions (K_{diss} usually ~0.1nM or lower), and the stoichiometry typically remains unchanged during purification. Pyruvate carboxylase is one of the few Mn^{2+}-dependent enzymes in this class. While a K_{diss} of ~10nM serves as a rough dividing line between metalloenzymes and metal-activated enzymes, enzymes with K_{diss} values between 0.1nM and 1μM can be difficult to classify. This is the case for arginase which normally shows partial retention of metal ions (and therefore activity) during purification. Apparent differences in the stability constants of the four metal binding sites for Mn^{2+} in rat liver arginase which contains two non-dialysable Mn^{2+} ions (K_{diss} 30nM, pH7.5) (Hirsch-Kolb *et al.*, 1971) and in bovine liver arginase with one non-dialysable Mn^{2+} ion (Harell and Sokolovsky, 1972; Ganadu *et al.*, 1984) indicate that a proportion of the sites bind Mn^{2+} too tightly for automatic classification as metal-activated sites. The higher affinity of these sites is a property of the oligomeric structure of arginase as monomeric arginases and immobilized subunits (of oligomeric arginases) bind Mn^{2+} comparatively weakly and are readily inactivated (Aguirre and Kasche, 1983; Iino and Shimadate, 1986). Hirsch-Kolb *et al.* (1971) proposed that the apparent non-equivalence of metal ion-binding sites might be due to a conformational change occurring when 2 Mn^{2+} ions (K_{diss} 50μM, pH7.5) were removed from rat liver arginase, resulting in the remaining ions being bound more tightly, or decreasing the accessibility of these sites. Subsequent work on this enzyme indicated that complete removal of Mn^{2+} was possible without irreversible loss of activity by chelating the ions at 37°C (Dahlig and Porembska, 1977). Ganadu *et al.* (1984) also suggested that loss of a third Mn^{2+} ion (K_{diss}~10μM, pH7.5) from bovine liver arginase resulted in a

conformational change that might decrease the accessibility of metal ion-binding sites thus explaining the slower activation of $1Mn^{2+}$-arginase compared with the $2Mn^{2+}$ form of the enzyme. The rapid activation of monomeric arginase by Mn^{2+} (Carvajal *et al.*, 1984) suggests that this inaccessibility is due to oligomeric structure and it appears that the oligomeric conformation in which metal ion-binding sites are most accessible is destabilized by partial depletion of metal ions.

The catalytic effect of a metal ion on a hydrolytic reaction must be based ultimately on a decrease in the transition state free energy of the rate determining step. Several specific roles proposed for metal ions include:

1. Lewis acid catalysis, involving polarization of bonds in a coordinated substrate ligand to form more positive centres for nucleophilic attack. The metal ion can also stabilize any negative charge that develops in the substrate during catalysis. With the low H_3O^+ concentrations in free solution at pH7, a metal ion represents an alternative electrophilic centre with which the substrate can interact (Buckingham, 1977). However, protein amino acid residues can duplicate this interaction by general acid catalysis. The term "superacid" catalysis is frequently used to describe catalysis by metal ions. However the use of "superacid" is misleading, as the polarizing power of a proton in binding to basic sites is greater than di- or trivalent metal cations. In addition, as most substrates appear to interact with metal ion cofactors in a monodentate fashion, it is not possible to invoke chelation to explain their catalytic efficiency (Page, 1984).

2. Increased acidity of nucleophiles with ionizable protons by

coordination to metal ions. Using H_2O as an example, coordination of H_2O to physiological divalent transition metal ions decreases its pK_a (increases the acidity) from 15.7 to ~7-10 (Dawson *et al.*, 1986), causing increased dissociation of coordinated H_2O to OH^- at neutral pH. The coordinated ionized ligand shows a reactivity (as a nucleophile) intermediate to that of the free ionized and nonionized ligands. If the increase in concentration of the ionized nucleophile due to coordination is sufficient to offset the decreased reactivity then rate enhancement occurs. In the case of OH^- the reactivity of the coordinated nucleophile is almost as great as free OH^-, so base catalysis is facilitated at neutral pH (Vallee and Galdes, 1984). The local environment of a metal ion in a protein can influence its effectiveness in this role: for example, in carbonic anhydrase the local environment of Zn^{2+} (3 neutral histidine ligands in a deep pocket) lowers the pK_a of bound H_2O to ~7 (Lipscomb, 1983). The role of metal ion-coordinated hydroxide and H_2O as nucleophiles and proton donors respectively can also be fulfilled by protein amino acid residues.

3. Facilitation of catalysis by approximation and precise alignment of reactants through coordination to the metal ion as a three-dimensional template (Vallee and Galdes, 1984). In this way metal ions may be able to exert stereochemical control over a reaction (Mildvan, 1970).

In each case a reactant is present in the inner coordination sphere of the metal ion and binding may involve displacement of another ligand, typically H_2O.

For metal-activated enzymes which form 1:1:1 complexes of enzyme,

metal ion and substrate, the four possible coordination schemes (Mildvan, 1970) are:

Complexes of the metal bridge type, illustrated by the latter two diagrams, are most plausible for arginase (Hirsch-Kolb *et al.*, 1971). The concept of a metal bridge complex was introduced to enzymology by Hellerman (1937), who proposed the coordination scheme shown in Fig. 1-4 for bovine liver arginase. Metal bridge type complexes have now been verified for Mn^{2+} forms of many metal ion-dependent enzymes such as pyruvate kinase and carboxylase, carboxypeptidase A, histidine deaminase, D-xylose isomerase and yeast aldolase. If arginine does enter the inner coordination sphere of Mn^{2+}, the complex is most likely to be monodentate, because apart from a non-specific bidentate interaction of α-amino and carboxylate groups, no multidentate interactions forming 5 or 6-membered rings can be formulated. A substrate orienting role for Mn^{2+} in arginase was proposed by Van Thoai *et al.* (1953).

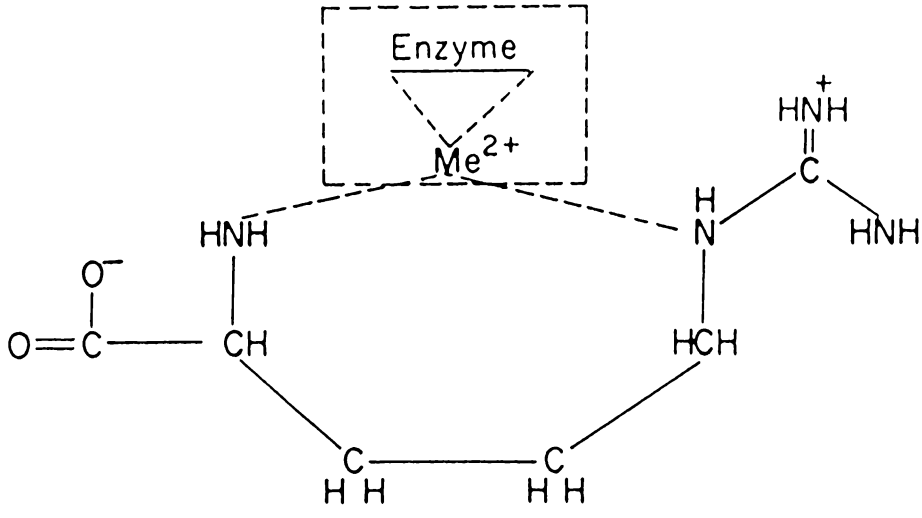

Figure 1-4 Hellerman's (1937) proposed structure for the metal bridge complex of arginase.

A further role of metal ions is the modulation of enzyme activity by metal ions that are not essential for activity. Exposure of Mn^{2+}-activated arginase to non-activating divalent cations such as Mg^{2+}, Ca^{2+} and Zn^{2+} prior to an assay can inhibit arginase activity (Greenberg *et al.*, 1956; Soru and Zaharia, 1976; Carvajal *et al.*, 1984) presumably by exchanging with the activating cation in the active site. Some mammalian arginases did not show this effect (Kayser and Strecker, 1973; Bond *et al.*, 1983) and its significance as a regulatory mechanism *in vivo* is doubtful.

Finally, many enzymes require metal ions for the maintenance and stabilization of tertiary and quaternary structure. Removal of metal ions from these enzymes often causes subunit dissociation which cannot be reversed unless metal ions are present (Vallee and Galdes, 1984). While all arginases require a metal ion cofactor for activity, data regarding the structural function of Mn^{2+} does not permit such a unified conclusion. Mn^{2+} clearly stabilizes oligomeric arginases against thermal inactivation (Rossi *et al.*, 1983) but similar data is not available for monomeric arginases. It is therefore uncertain whether this effect is mediated by stabilization of the quaternary structure or if stabilization of elements of tertiary structure (e.g. active site geometry) alone is responsible.

There are two views on the importance of Mn^{2+} in maintaining the quaternary structure of arginases. The first, which can be formulated from studies on bovine liver arginase (Harell and Sokolovsky, 1972; Dahlig and Porembska, 1977; Rossi *et al.*, 1983; Ganadu *et al.*, 1984), suggests that Mn^{2+} is required for enzyme stability but is not crucial to the maintenance of quaternary structure. Thus inactive apoarginase, prepared by incubation for 6h at room temperature, pH6.3 with 10mM EDTA, possesses the native quaternary structure. With one Mn^{2+} ion

bound the enzyme retained 15-25% of holoenzyme activity and the loss of 2 Mn^{2+} ions did not significantly alter elements of tertiary or secondary structure. The latter result suggests that, in this enzyme at least, conformational changes are not responsible for the observed differences in stability constants of the two weak Mn^{2+}-binding sites and a third site that binds Mn^{2+} more strongly. Studies on this enzyme also demonstrated that the effectiveness of metal ion-chelating treatments is a function of pH and temperature. Thus dialysis for 24h against *o*-phenathroline in Tris/HCl buffer pH7.5 (20°C) at 4°C removed 2 Mn^{2+} ions and a 1h incubation at 37°C followed by extensive dialysis at 4°C was required to remove a third ion. Complete removal of metal ions required treatment at pH6.3. The physiological temperature may be required to form conformations in which the metal ions are more accessible to the chelating agent while the stability of the complex clearly decreases with decreasing pH. As heating at 37°C in the presence of a chelating agent failed to remove the final Mn^{2+} ion from bovine liver arginase, the K_{diss} of this complex is probably lower than the 40nM value found for the two rat liver arginase Mn^{2+} ions removed by this treatment.

The alternative view of the involvement of Mn^{2+} in maintaining arginase quaternary structure is that Mn^{2+} is essential and that apoarginase (which can be prepared by a 1h incubation at 37°C with a suitable chelator at neutral or alkaline pH, followed by extensive dialysis) dissociates into subunits. This is the case for number of arginases (e.g. Carvajal *et al.*, 1971; O'Malley and Terwilliger, 1974) and is exemplified by rat liver arginase (Dahlig and Porembska, 1977). The difference between bovine liver and other arginases possibly reflects the milder conditions (i.e. lower temperature) under which the bovine liver apoarginase can be obtained. It would be interesting to

see if oligomeric rat liver apoarginase could be prepared by treatment at room temperature at pH6.3. Generalizing from the behaviour of bovine liver arginase, it is likely that Mn^{2+} ions are not a prerequisite for the maintenance of quaternary structure but strongly stabilize this structure against dissociation. At physiological temperatures this stabilization is probably essential for many arginases.

Reassociation of subunits prepared by metal chelation or acid treatment apparently has an absolute requirement for Mn^{2+} and a neutral to alkaline pH although bovine liver arginase has not been tested. Acid-dissociated rabbit liver arginase did not reform its native oligomeric structure in the presence of EDTA and the slight activity seen when assayed in the presence of Mn^{2+} was attributed to active subunits rather that reassociation (Vielle-Breitburd and Orth, 1972). Hosoyama (1972) found that reactivation of acid-dissociated rat liver arginase with Mn^{2+} had a pH optimum of pH7. While loss of metal ions precedes dissociation (in the case of acid dissociation this may involve protonation of carboxylate ligands (Rossi *et al.*, 1983)) reactivation experiments suggest that at least some subunits bind a metal ion before reassociation. This binding may cause a conformational change in the monomer that favours reassociation to the oligomeric state (Carvajal *et al.*, 1977). As for inactivation and dissociation by loss of metal ions, the parameters of pH, temperature, ionic strength and additionally the concentration of reactivating metal ion can all influence the degree of reactivation and reassociation.

In addition to the three active monomeric arginases now purified (Reddy and Campbell, 1968; Carvajal *et al.*, 1984; Iino and Shimadate, 1986), there is direct evidence to support the proposal by Vielle-Breitburd and Orth (1972) that subunits of oligomeric arginases are active in the presence of Mn^{2+} ions. Immobilized subunits of human

liver arginase (Carvajal *et al.*, 1977) and rat liver arginase (Aguirre and Kasche, 1983) prepared by metal chelation or acid dissociation of matrix-bound oligomers regained up to ~25% of the oligomeric activity after reactivation/renaturation with Mn^{2+}. The latter study suggested that Mn^{2+}-free subunits were only slightly unfolded. Data on association/dissociation phenomena and involvement of Mn^{2+} in structural and catalytic properties of arginase is summarised in Fig. 1-5.

Arginase exhibits a high degree of specificity for L-arginine. The enzyme also hydrolyses the structurally similar substrate L-canavanine but typically at ~5-40% of the rate found for L-arginine. D-arginine and L-homoarginine are not normally substrates. As is usual for catabolic enzymes in microorganisms, the K_m for L-arginine is quite high at ~2-20mM. Inhibition of bacterial arginases by amino acids has not been extensively studied, but, as for the animal enzymes, competitive product inhibition is seen for ornithine but not urea and inhibition by lysine and branched-chain amino acids also occurs. Carvajal and Cederbaum (1986) proposed that the mixed-type inhibition of human liver arginase by branched-chain amino acids at physiological pH is due to the binding of the inhibitor at a site other than the active site. They also found that the inhibition of human kidney arginase by proline was much stronger than that seen for the liver enzyme, which supports a proposed role in proline synthesis for the kidney enzyme.

Arginases are regarded as stable enzymes and in the presence of optimal concentrations of Mn^{2+} and protein at the appropriate pH the arginases from mesophilic bacteria and eubacterial sources may exhibit stabilities approaching those seen for the enzymes from thermophilic bacteria (e.g. Greenberg *et al.*, 1956; Soru and Zaharia, 1976; Rossi *et*

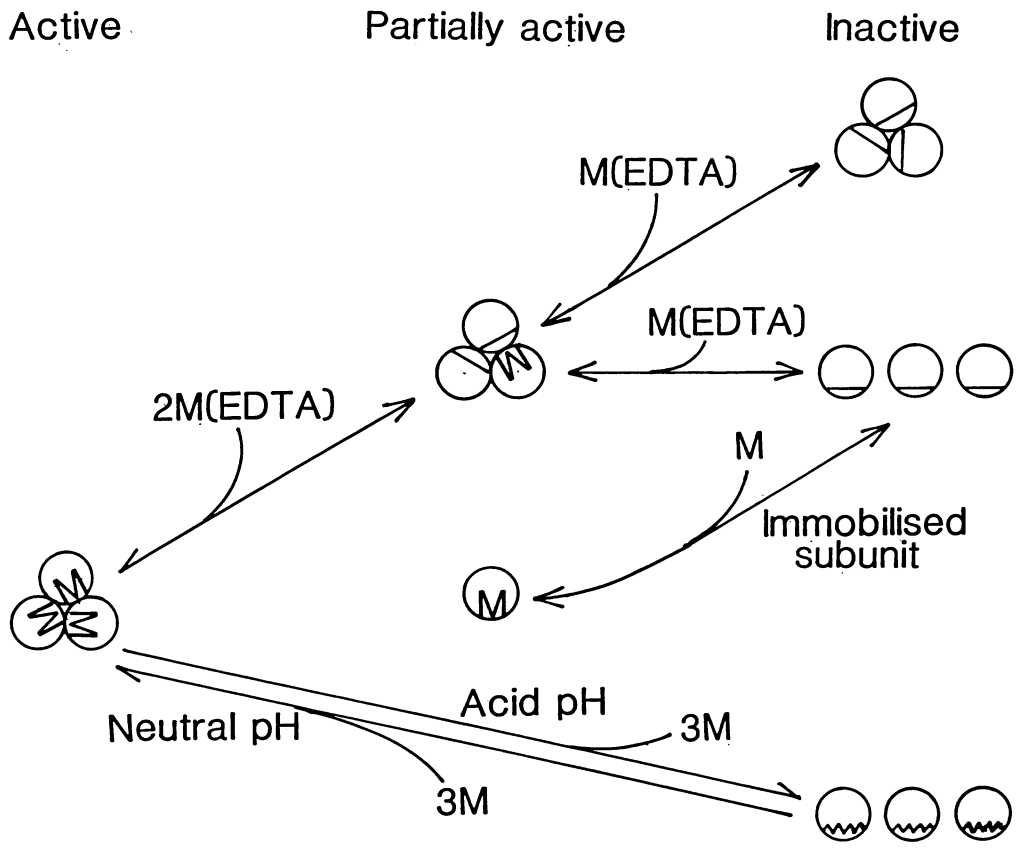

Figure 1-5 A model for Mn^{2+} involvement in arginase structure and activity.

This diagram summarizes the range of behaviour seen for different arginases in several studies. Empty metal ion-binding sites are represented by lines inside the subunits. Subunits prepared by acid dissociation may differ in conformation from those prepared by metal ion chelation (Aguirre and Kasche, 1983). Removal of successive metal ions by chelation at neutral pH may cause conformational changes in the oligomer that hinder both further chelation and rapid reactivation. For some arginases intermediates in the process of subunit dissociation have been observed but normally only native oligomers and monomeric forms are seen. As indicated, most steps appear to be reversible under appropriate conditions.

The subunit structure and metal ion stoichiometry shown in this model is used for clarity only, as experimental data on these properties is scarce and often varies between studies.

al., 1983). Some purification schemes for arginase take advantage of this fact by including a heat-treatment step. Such a procedure has been performed in the presence of Mn^{2+} (Bach *et al.*, 1963; Sakai and Murachi, 1969; Vielle-Breitburd and Orth, 1972; O'Malley and Terwilliger, 1974), ornithine (Simon and Stalon, 1976) or arginine (Penninckx *et al.*, 1974; Borkovich and Weiss, 1987a). A few workers have used affinity chromatography with L-lysine (Tarrab *et al.*, 1974; Skrzypek-Osiecka *et al.*, 1983), L-arginine (Penninckx *et al.*, 1974; Iino and Shimadate, 1986), arginase subunits (Aguirre and Kasche, 1983) or arginase antibodies (Brusdeilins *et al.*, 1985) as the affinity ligand. Improved yields are sometimes obtained if low concentrations of Mn^{2+} are included in buffers used during purification.

The tetrameric structure proposed for mammalian arginases now appears to be incorrect despite clear evidence in its favour (e.g. Hirsch-Kolb and Greenberg, 1968; Carvajal *et al.*, 1982). Rather than oligomeric arginases being grouped as tetramers and octomers (Reddy and Campbell, 1970), it seems likely that most mammalian arginases have a trimeric structure (Brusdeilins *et al.*, 1985) and that oligomeric arginases can be grouped as trimers and hexamers as first proposed by Simon and Stalon (1976). However, it is difficult to reconcile a trimeric structure with observations that bovine liver (Harell and Sokolovsky, 1972; Ganadu *et al.*, 1984) and rat liver (Hirsch-Kolb *et al.*, 1971) arginase oligomers bind four Mn^{2+} ions.

CHAPTER 2

MATERIALS AND METHODS

2-1 Maintenance of Bacteria

All bacteria used in this project are currently held in the Thermophile Research Unit Culture Collection (TRUCC), and either the TRUCC numbers and sample site codes, or overseas culture collection numbers of these bacteria appear in Tables 3-2 and 3-3 and in the associated text. *Thermus* strains were grown at 60°C or 70°C and stored at 5°C on agar slopes of Castenholz D medium (Castenholz, 1969) adjusted to pH7.8 and solidified with 1.75% agar (Coast Biologicals Ltd). *Pseudomonas aeruginosa* was grown at 37°C, and *Bacillus* spp. were grown at 37°C, 60°C or 70°C and stored at 5°C on nutrient agar slopes consisting of 8g/l nutrient broth (BBL) adjusted to pH7 with KOH and solidified with 1.75% agar. Anaerobic archaebacterial strains (*Thermoproteus tenax*, Tok12 S.1, AN1, Well44 S.2) were maintained by fortnightly subculture in Db medium (Zillig *et al.*, 1982; pp. 22-23 Jasperse-Herst, 1984). FjSS3 B.1 inocula were supplied from cultures growing on MSA/CA/YE medium described by Huser *et al.* (1986). *Sulfolobus* strains were maintained at 75°C in DSM media numbers 88 (supplemented with 2g/l sucrose) or 182, and subcultured every 2-4 days.

2-2 Growth and Harvesting of Bacteria

During the course of this work a number of modifications to standard media were assessed for the ability to improve or support the

growth of several bacterial strains. Further alterations were made in an attempt to increase the specific activity of arginine-catabolizing enzymes in cell-free extracts. In general, experimental results of this work have not been given but the media described below are the end results of these experiments. Initially bacteria were grown on a small scale for reversed-phase liquid-chromatography (RP-LC) studies and subsequently large-scale growth of three species was carried out. Media volumes of less than 16 litres were constituted with Milli Q water.

2-2-1 Bacterial growth and harvesting for RP-LC studies

All *Thermus* and *Bacillus* strains and *P. aeruginosa* were cultured on the same basal salts medium typically supplemented with 0.5g/l yeast extract (YE) (Merck) and 20mM L-arginine (free base) to give an "inducing" medium. The basal salts medium contained 5mM $KHSO_4$, 2mM KH_2PO_4, 20mM NaCl, 1mM $MgSO_4$, 0.1mM $CaCl_2$, 50μM Fe(III) citrate with 150μM nitrilotriacetic acid (NTA), and 10ml/l of a stock trace elements solution containing 1.5mM $MnCl_2$, 1mM H_3BO_3 and 0.2mM $CoSO_4$, $CuSO_4$, $ZnSO_4$, Na_2SeO_3, Na_2MoO_4, and 5mM NTA, pH5.2. The medium was adjusted to pH7.8 (*Thermus* spp.) or pH7.4 (*Bacillus* spp.) with HCl and autoclaved. "Standard" media usually consisted of basal salts supplemented with 10mM $(NH_4)_2SO_4$, and 1-3g/l YE and trypticase peptone (TP).

Oxygen limited growth of *Bacillus* spp. and *P. aeruginosa* was carried out in unshaken flasks (filled to top) that had been sealed with tin foil and plastic film on removal from the autoclave and equilibrated at growth temperature without allowing the medium to cool to room temperature. Thermophilic *Bacillus* spp. grown under these conditions were harvested after 13h at an A_{650} of ~0.05 and the cells washed and resuspended in extraction buffer to an A_{650} of ~40. Aerobic growth took place in Erlenmeyer conical flasks shaken at 200rpm in

which the medium occupied not more than 15% of the flask volume. The flasks were closed with cotton wool/gauze plugs. Temperature and oxygen-equilibrated medium was given a 0.5% inoculum of late logarithmic phase cells in the same medium for aerobic cultures and a 1% inoculum (from unshaken cultures in sealed universals) was used for anaerobic cultures. Aerobic cultures were usually sampled at least once before an A_{650} of 0.4 was reached to ensure fully aerobic conditions, and, where applicable, sampled during logarithmic phase so that comparisons of activity would be valid. The pH of culture supernatants was monitored with a combination pH micro-electrode (type U402-M3, Ingold). For a few cultures supernatant samples were retained for measurement of metabolite concentrations. Unless otherwise noted, *Thermus* and *Bacillus* cells were grown at 71±1°C, except for *B. coagulans*, *B. stearothermophilus* and *T. ruber* (60°C) and *B. licheniformis* (37°C). *P. aeruginosa* was also grown at 37°C.

Sulfolobus strains were grown on the appropriate DSM media (DSM, German Collection of Microorganisms, Catalogue of Strains, 3rd edn, 1983) which were slightly modified for "standard" conditions. DSM medium number 88 (*Sulfolobus* medium) for *S. acidocaldarius* was modified by halving the calcium concentration (0.5mM to 0.25mM) and decreasing the Fe(III) level to 50μM. The trace elements consisted of 5μM $MnCl_2$ and H_3BO_3, 1μM $ZnSO_4$, and 0.2μM $CoSO_4$, Na_2SeO_3 and Na_2MoO_4. The medium was also supplemented with 2g/l sucrose. For an "inducing" medium, the "standard" medium was altered as follows: 10mM Na_2SO_4 replaced $(NH_4)_2SO_4$, yeast extract and sucrose concentrations were halved compared to the "standard" medium and 20mM L-arginine (free base) was added. Both "standard" and "inducing" media were adjusted to pH3 with H_2SO_4 before autoclaving. DSM medium number 182 (*S. solfataricus* medium) was altered in its trace element composition as described for

medium 88, to produce the "standard" *S. solfataricus* medium. For the "inducing" medium the yeast extract concentration was halved (to 0.5g/l), 19mM Na_2SO_4 replaced $(NH_4)_2SO_4$, and 1g/l casamino acids was replaced by 20mM L-arginine (free base). The media were adjusted to pH4 with H_2SO_4 and autoclaved. 0.5% inocula from *S. acidocaldarius* cultures growing in DSM medium number 88 supplemented with 2g/l sucrose and from *S. solfataricus* cultures growing in DSM medium number 182 were added to 800ml of "standard" and "inducing" medium in 2l flasks (closed with cotton wool plugs) equilibrated at 75°C and shaken at 200rpm.

Anaerobic archaebacteria were grown in a "standard" medium consisting of 10mM $(NH_4)_2SO_4$, 2mM KH_2PO_4, 1mM $MgSO_4$, 0.25mM $CaCl_2$, 5μM Fe(III) citrate with 15μM NTA, 1ml/l of the *Thermus/Bacillus* stock trace element solution (supplemented with $MnCl_2$ and H_3BO_3 to give a concentration in the medium of 5μM for both, and with $ZnSO_4$ to give a concentration in the medium of 1μM), 0.2g/l yeast extract, 2g/l trypticase peptone, 0.4g/l L-cystine and 1mg/l resazurin. The arginine-supplemented "inducing" medium contained 10mM L-arginine (free base), 0.5g/l trypticase peptone and $(NH_4)_2SO_4$ was replaced with 10mM Na_2SO_4. The complete medium (minus L-cystine) was adjusted to pH7.3 for AN1 and Well44 S.2, pH6.4 for Tok12 S.1 and pH5.4 for *Thermoproteus tenax*. The cystine was then added and the medium autoclaved in open 400ml medical bottles that were each covered with tin foil holding a rubber septum. Immediately after autoclaving the bottles were sealed and 0.15-0.2g/l $Na_2S \cdot 9H_2O$ was added as a sterile 10%(w/v) solution. Headspaces were evacuated and gassed with N_2 and the medium equilibrated at growth temperature and given a 0.25% inoculum. AN1 was grown at 75°C and the other archaebacteria at 88°C. Cells were harvested when the turbidity of the medium indicated sufficient growth had occurred to produce a 0.1-0.2g cell pellet.

The "standard" MSA/CA/YE (2g/l of CA and YE) medium used for FjSS3 B.1 (Huser *et al.*, 1986) was modified for the "inducing" medium by lowering the casamino acids (CA) to 0.4g/l, yeast extract to 0.5g/l, and adding 10mM L-arginine (free base). Growth at 85°C was initiated by a 0.1% inoculum. *Clostridium thermohydrosulfuricum* was grown at 60°C on a modified Zeikus medium (TPYE medium) containing 17mM NH_4Cl, 5.5mM KH_2PO_4, 8.6mM K_2HPO_4, 16mM NaCl, 1mM $MgSO_4$, 3g/l yeast extract, 10g/l trypticase peptone, 1g/l cysteine•HCl, 1ml/l Zeikus trace elements (Zeikus *et al.*, 1979), 5ml/l Wolin's vitamin solution (Wolin *et al.*, 1963) and 1mg/l resazurin. The medium was adjusted to pH6.8 before autoclaving and 5g/l glucose added as a filter-sterilized solution after autoclaving. Growth was started with a 0.5% inoculum from a culture growing in the same medium.

Cultures were cooled to 20°C and cells harvested by centrifugation at 8300*g* ($r_{av.}$ 9.17cm, GSA rotor, Sorvall) for 20min at 5°C. Cells were washed once by resuspension in cold extraction buffer to minimize the carry-over of medium metabolites into enzyme assays, resuspended in extraction buffer to an A_{650} not less than 25, and frozen in liquid N_2. Extraction buffer consisted of 50mM KH_2PO_4, 5mM dithiothreitol, 1mM pyridoxal phosphate (PALP), 1mM $MgSO_4$ and 2%(w/v) glycerol, adjusted to pH7.2 (20°C) with NaOH. All chemicals were of the best quality available as some of this buffer is carried through to the final RP-LC sample.

2-2-2 Large-scale growth of *B. caldovelox*: A 450l batch culture

The growth medium used in this section contained 5mM $KHSO_4^-$, 10mM KCl, 1mM $MgSO_4$, 0.05mM $CaCl_2$, 15μM Fe(III) citrate with 50μM NTA, 10ml/l of the stock solution of trace elements used in the culture of *Bacillus* and *Thermus* spp. (see Section 2-2-1), 1g/l yeast extract

(Merck), and 25mM L-arginine (free base). The medium was adjusted to pH7.2 (20°C) and autoclaved. 20ml/l of a sterile 1M sodium phosphate solution, pH6.8, was added after autoclaving. Colonies of *B. caldovelox* grown at 65°C on an Oshima's medium (Oshima and Imahori, 1974) agar plate were used to inoculate 3x35ml volumes of medium (in 100ml flasks) equilibrated at 70°C and 160rpm. Once growth had started, shaking was increased to 200rpm. After 10h, 10x5ml volumes of these cultures (A_{650}=1.68) were used to inoculate 10x500ml volumes of the same medium in 2l flasks. While this inoculum was growing, an 800 litre fermenter was sterilized by steaming with ~1.5% formalin and then rinsed twice with filter-sterilized boiling tap water. Concentrated neutral solutions of yeast extract, arginine, and salts were passed though the filtration system and the volume made up to ~450l with filtered tap water. The composition of this medium was the same as for the inoculum. After 11h of growth at 70°C and 200rpm, the five litres of *B. caldovelox* culture was used to inoculate the 450l of medium equilibrated at 70°C and pH7.13 (20°C) and stirred with forced aeration.

Cells were harvested 15h after inoculation (A_{650}=1.4) in a continuous flow Sharples centrifuge at 12000*g*, fed at 5l/min with a peristaltic pump. Harvesting took ~85min, during which time the culture was not aerated. The cells (a yield of 1.84kg wet weight) were frozen in liquid N_2 and stored at -70°C.

2-2-3 16 litre cultures of *B. caldovelox* and *Thermus* 4-1A

16 litre cultures of *B. caldovelox* and *Thermus* 4-1A were grown with a view to purifying the arginase from each bacterium for analysis of metal ion composition. The medium was designed to provide all the metal ions that could conceivably function as *in vivo* activators of

arginase.

The growth medium consisted of 5mM $KHSO_4$, 2mM KH_2PO_4, 20mM NaCl, 1mM $MgSO_4$, 0.1mM $CaCl_2$, 50μM Fe(III) citrate (150μM NTA), 1g/l "Bacto" yeast extract (Difco), 20mM L-arginine (free base), 10μM H_3BO_3, 15μM $MnCl_2$, 2μM $VOSO_4$, $CoSO_4$, $NiSO_4$, $CuSO_4$, $ZnSO_4$, Na_2SeO_3, $SrCl_2$, Na_2MoO_4, and 1μM $CdCl_2$. The pH was adjusted to pH7.35 (20°C) with conc. HCl and two 16l volumes autoclaved in 20l glass carboys. The medium was equilibrated to 70°C in a water bath and oxygenated by forced aeration with sterile air using an air compressor. Forced aeration was found to have a cooling effect on the medium, so the water bath was maintained at 78°C to compensate for this. Sterile water equilibrated at 70°C was added during growth to compensate for water evaporation as the condensers were not 100% efficient. Growth was initiated by addition of a 1% logarithmic phase inocula, and monitored at 650nm. *B. caldovelox* was harvested after 10h (A_{650} = 1.04), and *Thermus* 4-1A after 13h (A_{650} = 0.68), in a hollow fibre filtration unit (model DC 10 LA, Amicon) using a hollow fibre cartridge with a nominal cutoff of 0.1μm (type H5MP01-43, Amicon). Before harvesting the cultures were cooled rapidly by placing the carboys in an ice bath while maintaining aeration. Ultrafiltration was started when the culture temperature had dropped below 35°C (20min), and took ~20min to complete. The thick cell slurries were then cooled to 5°C and centrifuged at 8300*g* ($r_{av.}$ 9.17cm, GSA rotor, Sorvall) for 30min at 5°C.

The cell pellets were washed to remove most of the transition metal ions present in the medium by resuspension in 300ml 5mM MOPS/NaOH buffer pH7.5 containing 0.15M KCl and recentrifuged . The washed pellets were frozen in liquid N_2 and stored at -70°C. Washed pellet weights were 21.3g (*B. caldovelox*) and ~50g of sloppy pellet (*Thermus* 4-1A). These yields were low but provided enough material for

purification work (see Section 2-5).

2-2-4 4.4 litre culture of Tok12 S.1

To obtain more cells for further study of enzymes in Tok12 S.1, 4.4 litres of growth medium was prepared in a 5l Duran bottle. The medium consisted of 2mM KH_2PO_4, 10mM Na_2SO_4, 1mM $MgSO_4$, 0.2mM $CaCl_2$, 5μM Fe(III) citrate with 15μM NTA, 0.25g/l yeast extract (Merck), 0.5g/l trypticase peptone, 10mM L-arginine (free base), 0.4g/l L-cystine, 1mg/l resazurin, 0.8ml/l of an autoclavable vitamin solution (200mg riboflavin and p-aminobenzoic acid, 500mg nicotinic acid and pyridoxine•HCl and 100mg biotin per litre) and the same trace elements used to grow this archaebacterium for RP-LC studies (Section 2-2-2), except that $MnCl_2$ was increased from 5μM to 15μM. The medium was adjusted to pH6.4 (20°C) and autoclaved, then reduced with 0.2g/l $Na_2S•9H_2O$ added as a sterile 10% (w/v) solution. The headspace was evacuated and gassed with N_2, the medium equilibrated at 88°C and inoculated with 0.1% Tok12 S.1 culture grown for 22h in Db medium supplemented with 1g/l casein.

Cells were harvested after 19h (A_{650} = 0.022) in late logarithmic phase by centrifugation at 8600g ($r_{av.}$ 9.53, GS3 rotor, Sorvall) for 25min at 5°C. Cells were resuspended and washed twice in cold 25mM MOPS/KOH buffer containing 80mM KCl and 1mM EDTA, pH7 (20°C). This washing step separated a black precipitate of metal sulphides from the cells, with a final yield of ~0.5g (wet weight). The cells were resuspended in 4.5ml of the same buffer minus EDTA, frozen in liquid N_2 and stored at -70°C.

2-3 Assays and Sample Preparation for RP-LC Analysis

0.25-0.35ml of the cell suspensions in extraction buffer were thawed and lysed by sonication on ice with 2-3 2min bursts from a micro-ultrasonic cell disrupter (Kontes) at a power setting of 7, with the probe tip just touching the surface of the suspension. The lysate was centrifuged for 5min in a Runne centrifuge at 12800g and the supernatant transferred to a clean 1.5ml plastic reaction vial and stored at 4°C. The cell-free extracts were used within 1h for enzyme assays and blanks, and within 3h for protein determination.

Enzyme assays were started by adding 50µl aliquots of cell-free extracts (diluted in extraction buffer if necessary) to 1.5ml plastic reaction vials containing 0.45ml buffered substrate equilibrated at the assay temperature. All extracts were assayed at 70°C with the exception of the anaerobic archaebacteria and FjSS3 B.1 (80°C), *T. ruber*, *B. stearothermophilus*, *B. coagulans* and *C. thermohydrosulfuricum* (60°C), and *B. licheniformis* and *P. aeruginosa* (37°C). For arginase, arginine deiminase and arginine decarboxylase assays the buffered substrate was 22mM L-arginine and 1mM $MgSO_4$ in a 60.5mM KH_2PO_4/ 49.5mM K_2HPO_4 buffer giving pH of 7 at 70°C. Because of the low temperature coefficient of phosphate buffer ($\Delta pK_a/°C$ = 0.0028) assay pH did not differ significantly from pH7 between 60 and 80°C, but the pH of assays at 37°C was ~7.3. Buffered substrate for the ornithine aminotransferase assay contained 22mM L-ornithine•HCl, 11mM 2-oxoglutaric acid (K 2000, Sigma), and 50.2mM MOPS (sodium salt, Sigma), resulting in a pH of 7 at 70°C. The catabolic ornithine carbamyltransferase assay mixture contained 22mM L-citrulline and 5.5mM MOPS (sodium salt), also giving a pH of 7 at 70°C. The Tok12 S.1 extract for the ornithine carbamyltransferase assay was prepared from the washed suspension of

Tok12 S.1 cells described in Section 2-2-4, supplemented with 10mM DTT and 50mM potassium phosphate buffer, pH7, before sonication. The assay solution therefore contained 5mM phosphate but no PALP, glycerol or $MgSO_4$.

After incubation (typically for 10min) in sealed tubes, a 100μl sample was withdrawn from the assay mixture with a glass syringe and mixed vigorously with 100μl of 0.2mM DL-homocysteic acid in 4% (w/v) perchloric acid in a plastic reaction vial. After 10min at 4°C this mixture was centrifuged in a Runne centrifuge as above. Supernatants were diluted 10-fold with water in small volume sample inserts (Water Assoc.) and stored at -70°C until analysed. If a further dilution of the sample was required, this was done with 10μM DL-homocysteic acid in 20mM $HClO_4$. Blanks run with selected samples included substrate blanks (incubated substrate + extraction buffer), extract blanks (incubated assay buffer (minus substrate) + extract) and complete blanks (incubated substrate to which extract was added after $HClO_4$). 5μl sample volumes were derivatized with the OPA-ME reagent and the product amines present analysed by RP-LC (see Chapter 3).

2-4 Purification of Fermenter Batch Culture *B. caldovelox* Arginase

Unless otherwise stated, all procedures in this section were carried out at room temperature (17-24°C). Column fractions were stored at 0-5°C between purification steps. With the exception of the 20l volumes used for dialysis, all buffer solutions used for purification procedures were made up with Milli-Q water. This is reagent -grade, deionised water produced by the Milli-Q Water Purification System (Millipore) with a resistivity greater than 15MΩ•cm. All buffers used for column chromatography were partially degassed before use by

filtration through 0.22μm or 0.45μm filters. Sorvall centrifuges (models RC-2B, RC-5B, and SS-3) and Sorvall GSA centrifuge rotors ($r_{av.}$ 9.17) were used for all centrifugation work unless otherwise specified.

2-4-1 Preparation of a cell-free extract and $(NH_4)_2SO_4$ precipitation

B. caldovelox cells (1.5kg wet weight) were thawed from -70°C to 20°C over 6h and transferred to a 10l stainless-steel container. Six litres of extraction buffer containing 7.5ml Triton X-100, 1.5g lysozyme•HCl (L 2879, Sigma), 75mg DNAase (D 8764, Sigma), and 11.2g KCl in 16.6mM MES/NaOH buffer, pH 5.5 (20°C), was added and the slurry stirred while heating to 37°C (15min). Stirring at this temperature was continued for 40min and the lysate cooled to 4°C (15min) and centrifuged at 14700*g* for 30min at 4°C. The supernatant pH was adjusted to 8.25 (20°C) with the addition of 300ml 1.5M EPPS/NaOH, pH9.5, bringing the total volume to 6.85l.

1.203kg of $(NH_4)_2SO_4$ was added gradually with constant stirring over 15min to bring the supernatant to 30% saturation. After further gentle stirring for 35min the solution was centrifuged at 10200*g* for 20min at 20°C. 0.807kg of $(NH_4)_2SO_4$ was added as before to the 6.44 litres of supernatant to bring the solution to 50% saturation. This was centrifuged as above and the pellets drained and redissolved in 300ml of 50mM EPPS/NaOH buffer, pH8.5. The redissolved pellet was placed in a 50cm length of 12000 MW-cutoff dialysis tubing (Union Carbide, inflated diameter $1\frac{7}{8}$ inches) and dialysed at 5°C for 14h against two 20 litre volumes of 5mM EPPS/NaOH pH8.5 (20°C) containing 0.4mM $MnCl_2$. The dialysed 30-50% saturated $(NH_4)_2SO_4$ pellet was centrifuged at 10200*g* for 20min at 20°C and the small pellet discarded. The supernatant volume was 520ml.

2-4-2 Heat treatment with manganous chloride

The pH of the redissolved dialysed pellet was adjusted to pH8.5 after adding 17ml 1.5M EPPS/NaOH, pH9.5 (to increase buffering capacity) then 29ml of a 1M $MnCl_2$ solution was added to give a 50mM concentration of $MnCl_2$. The enzyme solution was heated to 70°C in 15min and maintained at 70°C for 80 min before cooling the solution on ice to 20°C. After centrifuging at 10200*g* for 30min at 20°C, 55ml of glycerol was added to 500ml supernatant. This solution was dialysed against 20l of 50mM Tris/HCl pH8.5 (20°C) at 5°C for 21h then against 20l 5mM Tris/HCl pH8.5 containing 0.1mM $MnCl_2$ at 20°C for 10h. The non-diffusable material was centrifuged at 14700*g* for 25min at 20°C and the supernatant (750ml) freeze-dried to yield 18.4g powder which was stored in a vacuum desiccator at room temperature.

2-4-3 DEAE Sepharose CL-6B anion exchange chromatography

The freeze-dried powder was dissolved in a 0.1M KCl solution (degassed by filtration through a 0.45μm filter) to give a protein concentration of 3.1mg/ml and centrifuged at 14700*g* for 30min at 20°C. The supernatant (1200ml) was loaded onto a DEAE Sepharose CL-6B column (27x5cm) (Pharmacia Fine Chemicals) equilibrated with 0.2M KCl in 50mM Tris/HCl buffer, pH8.4 (20°C), and the column washed with 500ml equilibration buffer. A 2.5l linear salt gradient to 0.35M KCl eluted arginase at ~0.25M KCl. The gravity-fed flow rate was ~3ml/min. Eluent was monitored at 280nm with a 0.1cm pathlength cell in a UV-120-02 spectrophotometer (Shimadzu) and 19.5ml fractions collected by an LKB 2111 Multirac fraction collector. The most active fractions (combined volume 410ml) were concentrated and equilibrated with 25mM EPPS/NaOH buffer pH8.2 (20°C) to 18.5ml by ultrafiltration (PM30 membrane, Amicon) at 5°C.

2-4-4 Preparative GP-HPLC

A TSK-Gel G3000SWG HPLC column (60×2.15cm) (Toyo Soda Manufacturing Co. Ltd) with a void volume of ~90ml was equilibrated with 25mM MOPS/NaOH buffer containing 0.2M KCl, pH7.5, at a flow rate of 5ml/min maintained by an HPLC pump (Waters Assoc.). The 18ml sample (24mg/ml protein) was chromatographed in ten separate runs, 1.8ml volumes of the sample being loaded (2.2ml sample loop) and injected for each run. For each run 5ml fractions were collected using a FRAC-100 fraction collector (Pharmacia) and the eluent absorbance monitored at 280nm by a Lambda-Max UV detector (1cm path length, Model 481, Waters Assoc.). The 5 most active fractions from each run were combined and the 250ml solution concentrated and desalted by ultrafiltration (PM30 membrane, Amicon) to 25ml at 5°C.

2-4-5 Lysine Sepharose CL-6B chromatography

To prepare 100ml of L-lysine Sepharose CL-6B, 100ml Sepharose CL-6B (Pharmacia) was washed with 1.5l Milli-Q water to remove preservatives and the moist cake of drained gel placed in a 500ml Duran bottle with 30ml H_2O. 70ml 0.57M $NaIO_4$ (puriss p.a., Fluka) was then added and the bottle sealed and shaken (130 strokes/min with the length of bottle in line with the direction of shaking) at room temperature for 2h. After this activation by periodate oxidation (Parikh *et al.*, 1974) the gel was washed on a sintered glass funnel with 2l H_2O and allowed to drain. The activated gel (100g) was shaken (as described above) with 100ml 2M L-lysine•HCl (chromatographically homogeneous, BDH), adjusted to pH 4.9 with a few drops of glacial acetic acid (Pronalys, M&B), for 10h. The pH of the gel suspension was adjusted to pH9 (25°C) with solid Na_2CO_3 (AG, Riedel-De Haën) and cooled to 5°C. 1.892g $Na(BH_4)$ (zur synthese, Merck) was dissolved in 20ml H_2O and the

solution (cooled on ice) was added dropwise with gentle stirring at 5°C over 16h using a peristaltic pump. The gel was then washed with 2l of cold 1M NaCl (AR, M&B) and allowed to settle in 300ml of this solution. The washing was repeated and the gel stored in 1M NaCl at 5°C. A small amount of gel was solubilized in 50% (v/v) acetic acid by heating at 75°C and the degree of substitution was found to be ~4.5μmol L-lysine per ml of gel as estimated by the 2,4,6-trinitrobenzenesulfonic acid method (Fields, 1972) using an equimolar solution of DL-2-amino-n-butyric acid and ε-amino-n-caproic acid as a standard. From data presented by Parikh *et al.* (1974) it is probable that between a third and one half of the lysine ligands are attached to the Sepharose matrix through the ε-amino group.

An L-lysine Sepharose CL-6B (prepared as above) column (18.5x2.5cm) (Pharmacia) was equilibrated with 25mM EPPS/NaOH buffer containing 20mM KCl, pH8.2 (20°C) at 1.25ml/min (gravity-fed flow). Sample (25ml) was diluted 5-fold in equilibration buffer, divided into two equal volumes, and each sample chromatographed in a separate run as follows: each sample was loaded at ~1ml/min and after a 120ml wash with equilibration buffer, a 700ml linear salt gradient to 80mM KCl in the same buffer eluted *B. caldovelox* arginase at ~50mM KCl. Monitoring of the eluent (at 220nm) and fraction collection was performed using the equipment described in Section 2-4-3. Active fractions were desalted and concentrated to 3.6ml by ultrafiltration using a PM10 membrane. The concentrated protein solution was frozen in liquid N_2 and stored at -70°C.

2-4-6 Anion exchange FPLC

A Mono Q HR 5/5 anion exchange FPLC column (Pharmacia) was equilibrated in 30mM bis-Tris propane/HCl buffer containing 50mM K_2SO_4,

pH9.5 (20°C) at 1ml/min (HPLC pump, Waters Assoc.). The 3.6ml sample was diluted 3-fold with equilibration buffer and all the sample bound to the column in 7 load (2.2ml injection loop) and inject cycles. After a 10ml wash with equilibration buffer, arginase was eluted with a 40min linear salt gradient to 0.4M K_2SO_4 in the same buffer with a 7min isocratic step after the first 7min of the gradient. Eluent monitoring and collection of 1.5ml fractions was performed as described in Section 2-4-4. The active fractions from each run were pooled and concentrated to ~5ml by ultrafiltration (PM10, Amicon) then dialysed with stirring at 5°C against 3x2l volumes of 0.3mM bis-Tris propane over 12h. The dialysed solution was freeze-dried and the powder (49mg) stored in a vacuum desiccator at room temperature.

2-5 Purification of *B. caldovelox* and *Thermus* 4-1A Arginase for Determination of the Native Cofactor

Arginases from *B. caldovelox* and *Thermus* 4-1A cells harvested in Section 2-2-3 were purified using the steps described below. Although the same steps were used for both enzymes, the detailed application of the steps sometimes differed and this is noted. The comments on enzyme purification made at the start of Section 2-4 apply to this section also.

2-5-1 Preparation of cell-free extracts; protamine sulphate and $(NH_4)_2SO_4$ precipitations

Cell pellets were thawed from -70°C storage and suspended in 84ml 0.1M CHES/NaOH, pH9.5 (20°C) and 102ml 0.12M CHES/NaOH, pH9.5 for *B. caldovelox* and *Thermus* 4-1A pellets respectively. The cells were lysed by sonication on ice with a Dynatech Sonic Dismembranator (Series 300, Artek) for a total time of 40min in 5min bursts at a relative output of

0.5 using a standard 13mm titanium tip. After centrifugation at 16000*g* for 30min at 20°C, the clear supernatant was carefully decanted into a clean centrifuge tube. 41ml and 47ml of filtered 20mg/ml protamine•SO_4 (grade II, P 4380, Sigma) in 0.1M CHES/NaOH buffer, pH10, was then added to *B. caldovelox* and *Thermus* 4-1A cell-free extracts respectively and the solutions stirred for 15min before centrifuging and decanting as above.

$(NH_4)_2SO_4$ (Aristar, BDH) was added to 30% saturation with constant stirring over 15min and the stirring continued for 15min before centrifugation for 20min as above. $(NH_4)_2SO_4$ was then added as before to 55% and 50% saturation for *B. caldovelox* and *Thermus* 4-1A respectively and the solutions treated as before. The $(NH_4)_2SO_4$ pellets were redissolved in 25ml 0.1M bis-Tris propane/HCl buffer, pH9.5 (20°C), and dialysed (12000 MW cutoff membrane prepared by boiling in 25mM Na_2CO_3 followed by thorough rinsing in Milli Q water) separately with stirring against 2x1 litre volumes of 30mM bis-Tris propane/HCl buffer, pH9.4 (20°C) at 5°C for 12h. The solutions were then centrifuged at 28100*g* ($r_{av.}$ 6.98cm, SS-34 rotor, Sorvall) for 20min at 4°C to remove precipitated protein and the supernatants frozen in liquid N_2 for storage at -70°C.

2-5-2 DEAE Sepharose CL-6B chromatography

B. caldovelox (33.6ml) and *Thermus* 4-1A (30ml) solutions were thawed and diluted with 101ml 30mM bis-Tris propane/HCl containing 0.2M KCl (AR, Merck), pH9.4 (20°C), and 90ml 0.1M KCl in the same buffer respectively. Samples equilibrated with the appropriate sample dilution buffer were loaded onto a DEAE Sepharose CL-6B column (25x5cm) (Pharmacia) at a gravity-fed flow rate of ~2ml/min and washed with 750ml dilution buffer. The arginases were eluted at ~5ml/min with 2.5l

linear salt gradients to 0.4M KCl, the eluent being monitored at 280nm with a 1cm pathlength flow cell (model UA-5 absorbance/fluorescence monitor with type 6 optical unit, ISCO) and 20ml fractions collected as described in Section 2-4-3. Active fractions, eluted at ~0.24M KCl for both enzymes, were combined and concentrated by ultrafiltration (PM10, Amicon) to 1.45ml and 3.6ml for *B. caldovelox* and *Thermus* 4-1A arginases respectively and stored at -70°C.

2-5-3 Gel permeation and anion exchange FPLC

A Superose 12 prep grade HR 16/50 column (30μm bead size, bed volume ~100ml, void volume ~35ml) (Pharmacia) was equilibrated with 30mM bis-Tris propane/HCl buffer, pH9.5 (20°C) at 1ml/min using an HPLC pump (Waters Assoc.). 1.4ml of the *B. caldovelox* sample was thawed and mixed with 2ml equilibration buffer and loaded (2.2ml injection loop) and injected in two successive chromatographic runs. 3.4ml of the *Thermus* 4-1A sample was also chromatographed in two cycles. The eluent was monitored and 1ml fractions collected as described in Section 2-4-4. Active fractions were pooled and 8ml solutions of the two enzymes frozen at -70°C.

A Mono Q HR 5/5 FPLC column (Pharmacia) was equilibrated as described in Section 2-4-6 and thawed 8ml arginase samples were loaded (2.2ml sample loop) and injected in four cycles. The bound protein was then eluted with a 30min linear salt gradient to 250mM K_2SO_4 (Aristar, BDH) in the same buffer, the eluent being monitored at 280nm and 2ml fractions being collected as described in Section 2-4-4. Chromatography of *B. caldovelox* arginase included a 4min isocratic step at 8min 30sec, a second isocratic step at 16min 50sec for 3min 10sec and a final isocratic step at 27min 45sec for 3min 15sec. Chromatography of *Thermus* 4-1A arginase included two isocratic steps in

the gradient, the first starting at 7min and lasting 3min 30sec and the second for 4min starting at 20min. The two most active fractions from the *B. caldovelox* run and the three most active fractions from the *Thermus* 4-1A sample were combined and solid K_2SO_4 added to give K_2SO_4 concentrations of 0.4M and 0.5M respectively.

2-5-4 Hydrophobic interaction FPLC

A Phenyl-Superose HR 5/5 hydrophobic interaction FPLC column (Pharmacia) was equilibrated with 30mM bis-Tris propane/HCl buffer containing 0.5M K_2SO_4 , pH9.5 (20°C), at 0.5ml/min (HPLC pump, Waters Assoc.). The samples prepared above were loaded onto the column by repeated loading and injection from the 2.2ml sample loop and after washing with 3ml of equilibration buffer the arginases were eluted with 45min (*B. caldovelox*) and 40min (*Thermus* 4-1A) linear gradients to 30mM bis-Tris propane/HCl, pH9.5. A single isocratic step was inserted in both elution gradients at 34min for 4min 30sec, and at 23min for 3min 30sec for *B. caldovelox* and *Thermus* 4-1A arginases respectively. The eluent was monitored at 280nm and 1.5ml fractions collected as described in Section 2-4-4.

Two fractions from each run were combined, made up to 0.35M K_2SO_4 (*B. caldovelox*) and 0.5M K_2SO_4 (*Thermus* 4-1A) and rechromatographed on the Phenyl-Sepharose column with 40min linear gradients from 0.5M to 0mM K_2SO_4 in 30mM bis-Tris propane/HCl, pH9.5. A single 2-2.4ml fraction was collected for each arginase and these were dialysed with stirring against 5x1 litre volumes of 0.1mM bis-Tris propane over 13h at 5°C. Spectra/Por 1 membrane tubing (Spectrum Medical Industries Inc., Los Angeles, USA) with a nominal M_r cutoff of 8000 and a wet cylindrical diameter of 6.4mm (part no. 132645) was used after extensive washing under hot running water followed by soaking in a 5mM

trisodium EDTA (ED3SS, Sigma) solution and exhaustive rinsing with Milli Q water. During dialysis the solution volumes doubled. 10% and 21% of the *B. caldovelox* and *Thermus* 4-1A arginase solutions respectively, were frozen in liquid N_2 as small aliquots and stored at -70°C. These samples were referred to as "native metals" arginases. The remainder of each solution was freeze-dried and stored in a vacuum desiccator at room temperature. The freeze-dried powders were later reconstituted for attempts to determine the native metal ions by electron "probe" analysis and carbon rod atomic absorption spectrophotometry.

2-6 Methods for Determination of Molecular Weight

All standards used for M_r determinations are listed in Table 5-2-2. All reagents used for these methods were of analytical/reagent grade, or where applicable electrophoresis grade, unless otherwise specified. Milli Q water was used for all solutions.

2-6-1 Polyacrylamide gel electrophoresis

The discontinuous buffer system of Laemmli (1970) was used with or without the inclusion of sodium dodecyl sulphate (SDS) in the gel, reservoir, and sample buffers. In non-denaturing experiments 2-mercaptoethanol was also omitted from the sample buffer. Unpolymerised resolving gel solutions were maintained at room temperature while being degassed with stirring for 10min then 0.016% (v/v) *N,N,N',N'*-tetramethylethylenediamine (TEMED) and 0.24% (v/v) of a fresh 10% (w/w) ammonium persulphate (APS) solution were stirred into the solution without introducing bubbles. The concentration of both catalysts in the gel is ~1.05mM. After pouring, the resolving gels were overlaid with water-saturated 2-methyl propan-1-ol (AR, Ajax) and

allowed to set for 50-60min at 18-23°C. The top of the resolving gel was rinsed thoroughly with dilute stacking gel buffer to remove the overlay and unpolymerised acrylamide and the gel was then drained of excess liquid by setting the casting stand on its side for ~8min while degassing the stacking gel solution as above. Stacking gels were polymerised with 0.08% (v/v) TEMED and 0.4% (v/v) of a fresh 10% (w/w) APS solution and ~1h later the wells were rinsed with sample buffer and drained before mounting the gel on the cooling core and loading the samples. 0.75mm thick gels with total acrylamide percentages between 4 and 10% in the resolving gel, 3 or 4% in the stacking gel and 3% bisacrylamide concentrations in all gels were run on a Protean II slab cell system (Biorad) at ~18°C with a current of 13mA while proteins were stacking (initial voltage ~70V for SDS gels), and a current of 18mA in the resolving gel, with a voltage limit of ~200V. SDS and non-denaturing gels typically took 4-5 hours to run under these conditions. Unless otherwise indicated protein samples in the Laemmli SDS sample buffer were prepared by heating for 10min at 100°C in an oil bath.

Protein bands were detected on polyacrylamide gels by a Coomassie Blue R250 stain or a more sensitive silver stain method. Although the stains were used to detect protein bands in both denaturing and non-denaturing gels, denaturing gels required more extensive washing prior to the silver stain or during Coomassie Blue R250 staining as SDS decreases the sensitivity of these stains (pg. 127, Andrews, 1986). Occasionally the stains were used sequentially on the same gel (Hallinan, 1983). The Coomassie Blue R250 staining mixture contained 1g of the dye (B 0630, Sigma) dissolved in 500ml methanol to which 100ml glacial acetic acid and 400ml water were added. Gels were stained for 1-2h (non-denaturing) or for at least 8h (SDS) before destaining in the

staining solvent and were stored in 5% acetic acid (pg. 29, Andrews, 1986). The silver staining procedure used is based on that of Merril *et al.* (1982) and is described below. The times given in this silver staining procedure are suitable for 0.75mm gels.

Step

1. Fix in 200ml of 40% (v/v) methanol (AR, BDH) and 10% (v/v) glacial acetic (AR, M&B), for 2h (overnight for SDS gels).

2. Rinse for 15min in 10% (v/v) ethanol (AR, Ajax Chemicals) and 5% (v/v) glacial acetic acid. Repeat.

3. Immerse in 250ml oxidiser solution for 8min. Solution contains 3.4mM $K_2Cr_2O_7$ (Pronalys, M&B) and 3.2mM HNO_3 (Aristar, BDH).

4. Two 8min washes in at least 500ml Milli Q H_2O.

5. Immerse in 200ml of a 12mM $AgNO_3$ solution for 30min under a bright (fluorescent) light.

6. A 1min wash in Milli Q H_2O to remove excess Ag^+ ions.

7. Wash gel with 100ml of developer (0.05% (v/v) formaldehyde (AR, Ajax) in 0.28M Na_2CO_3 (AR, BDH)) and immediately replace with 200ml fresh developer. Continue development, changing the developing solution if it begins to take a brown colour.

8. When the bands are dark enough or before the background begins to yellow, the gel is immersed in 5% (v/v) glacial acetic acid. After 15min this can be exchanged for water.

For both staining methods best results were obtained if the gels were agitated gently in the solutions. For the silver staining procedure all steps were carried out in a glass dish. Silver-stained polyacrylamide gels were stored in sealed plastic bags with a small amount of water. The Coomassie Blue G250 dye-based method of Reisner (1984) was used when rapid visualization of protein bands was required.

Gels were mounted on a light-box and photographed with Polaroid 665 film using a Polaroid Cu-5 camera (F value 5.6, $\frac{1}{30}$ second exposure). Densiometric scans of photographic negatives were performed on an LKB 2202 Ultroscan laser densitometer and the raw data processed (GS-365 Electrophoresis Data Reduction System, Hoefer Scientific

Intruments) and displayed using a Commodore PC20II.

2-6-2 Analytical gel-permeation: FPLC and HPLC

A TSK-Gel G3000SW HPLC column (600x7.5mm) (Toyo Soda Manufacturing Co. Ltd) was equilibrated with various running buffers at 1ml/min. A Superose 12 HR 10/30 FPLC column (Pharmacia) was equilibrated in 0.01M sodium tetraborate containing 7mM NaOH and 50mM K_2SO_4, pH9.5 (20°C) or 26.7mM KH_2PO_4/ 13.3mM H_3PO_4 containing 50mM K_2SO_4, ~pH2.7 (20°C) at a flow rate of 0.7ml/min. For both columns 10-100μl samples were loaded into a 200μl sample loop and injected onto the column. The eluent was monitored at 205nm and 0.5ml fractions collected when necessary using the equipment described in Section 2-4-4. The columns were calibrated using a M_r (HPLC) marker protein kit (item 30180, USB). At pH2.7 not all the standard proteins ran as expected and the additional standards yeast alcohol dehydrogenase and carbonic anhydrase were used to construct calibration curves.

3-6-3 Sucrose density gradient centrifugation

Sucrose density gradient centrifugation for M_r determinations was performed in a TL-100 benchtop ultracentrifuge using a TLS-55 swinging bucket rotor (both from Beckman Intruments) at 55000rpm (max. 260000*g*) and 5°C with acceleration and deceleration programs 9. Linear 5-20% (w/w) sucrose (AR, Fisons) gradients were formed in 11x34mm (2.2ml) polyallomer tubes (Beckman) by diffusion of 0.5ml layers of 20, 15, 10 and 5% (w/w) sucrose for 1h at 20-25°C. The procedure is outlined in application note 1 (Bioresearch DS•640, 1984) for the TL-100 ultracentrifuge. To form reproducible gradients it was necessary to layer the sucrose solutions of decreasing density using a peristaltic pump (P1, Pharmacia) at a flow rate of ~0.2ml/min. The boundaries between layers thus formed were clearly visible. After 1h the filled

centrifuge tubes were transferred to a 5°C cold room for 1-2h. Sample volumes of ~50μl containing up to 3mg/ml protein were layered onto the gradients and the tubes loaded into the pre-cooled rotor. Constant buffer and salt concentrations were maintained in individual gradients by using the same volume of a concentrated buffer solution to make up the sucrose solutions. The buffers and salts used in gradients at pH9.5 and 2.7 were those used as eluting buffers for the analytical GP-FPLC experiments described in the Section 2-6-2. The 5-20% gradients were assumed to be isokinetic with respect to the centrifugation conditions applying in these experiments.

After centrifugation the base of each tube was pierced with a 19 gauge 38mm needle (blunted at the point to ~45° angle) using a home-made needle guide. The plastic sleeve of the needle was largely cut away to minimise mixing between successive drops. Two drop fractions (~30μl per drop) were collected (a total of 33-34.5 fractions) and diluted 10-fold with water. Protein was determined by absorption at 205nm as sucrose and the buffers/salts used did not absorb strongly at this wavelength. Catalase activity was measured by following the decrease in A_{240} at 25°C in a thermostated cuvette holder with pre-equilibrated buffered substrate as described by Sigma (pg. 383, Sigma catalogue, 1988) and arginase activity was assayed with 20mM L-arginine in the presence of 5μM $MnSO_4$ at pH9 at 60°C using the acid-ninhydrin method to detect ornithine. When fractions contained acid-inactivated arginase, reactivation was performed in 0.1M MOPS/NaOH buffer, pH7 (60°C) at 60°C in the presence of 1mg/ml bovine serum albumin (BSA) and 0.5mM $MnSO_4$. The distances travelled by the proteins were determined from plots of fraction number vs activity or A_{205} with the location of the meniscus being measured to within ±0.25 fractions.

2-7 Isoelectric Focusing

To prepare a 0.5mm thick 1% agarose gel 2.52g glycerol, 0.2g Agarose IEF (Pharmacia), and 16.7g water were mixed and boiled to solubilize the agarose. The solution was equilibrated at 70°C and 1.5ml of 40% (w/v) Servalyt ampholytes (AG 3-10, Serva), or 1.3ml Pharmalyte ampholytes (4-6.5, Pharmacia) added slowly with constant stirring. A mould consisting of a 200x125x0.2mm polyester backing sheet (Gel-Fix for Agarose, 42961, Serva) held to a glass supporting plate by a few drops of water and a second glass plate separated from the supporting plate by two 0.75mm Teflon spacers was clamped and heated at ~65°C for 30min. The agarose solution was poured onto the backing sheet and the top glass plate lowered carefully to exclude bubbles and clamped in place (i.e. the flap technique of Radola (1980)). The mould was allowed to cool to room temperature and the gel was hardened for at least 1h at 4°C before use.

A Pharmacia flatbed apparatus (FBE-3000) for supporting and cooling the gel was connected to an Isco Electrophoresis power supply (Model 494). 0.5M NaOH and 0.05M H_2SO_4 solutions were used for the cathode and anode electrolytes respectively. The anode wick was well blotted before use so that it would absorb the excess water that accumulates at this end of the gel during focusing. 10cm long gels were prefocused for 40min at 5W and then samples applied in squares of filter paper for large volume samples (up to 25μl) and applicator strips for small samples (up to ~4μl). Focusing was continued at 5W and the sample applicators removed after ~1h at which time the anode wick was blotted. After ~100min the voltage limit of 1500V was attained and focusing was stopped. Isoelectric focusing experiments were performed at 15-18°C using n-decane as a heat exchanger, a FH15 Grant

heater/circulator, and a FC25 Grant cooler.

Agarose gels were stained for protein by the Coomassie Blue R250 method described in Section 3-6-1. After destaining, gels were transferred to water for ~15min and then dried at 37°C. Active arginase was localized by overlaying the gel with a strip of Whatman No. 1 filter paper moistened with 20mM L-arginine in 0.1M MOPS/NaOH buffer containing 5μM $MnSO_4$, pH9.0 (60°C). After 5-10 seconds the strip was removed and placed in a 65°C oven to dry. The ornithine formed while drying the paper overlay was detected by dipping the paper in a 0.2% solution of vanillin (AR, BDH) in acetone and heating for 10min at 100°C, followed by a dipping in 0.1% KOH in ethanol and repeating the heating step (Curzon and Giltrow, 1953).

2-8 Protein Determination and Enzyme Assays

A unit (U) of enzyme activity was defined as the amount of enzyme required to produce 1μmol of measured product/min under the specified assay conditions. As all the enzymes assayed in this project catalyse reactions with a 1:1 substrate to product ratio, the unit of activity also corresponds to the processing of 1μmol of substrate/min. Unless otherwise specified, assays were initiated by thorough vortex mixing of 25μl of enzyme solution (delivered from a glass syringe (25A-FN, SGE)) with 0.475ml of buffered substrate pre-equilibrated at the assay temperature in a 0.9x10cm borosilicate glass test-tube (Duran, Schott). The assay test-tubes were submerged in a water bath to between one third and one half of their length to ensure that the assay mixture was not cooled during mixing. Assays were for 6min unless otherwise specified.

2-8-1 Determination of protein concentration

In cell-free bacterial extracts and during enzyme purification protein concentration was measured by a Pierce Co. modification of the dye binding method of Bradford (1976). The dye reagent (made up freshly every 2 weeks) consisted of 100mg Coomassie Blue G-250 dye (Pierce product no. 20279) dissolved in 50ml ethanol to which 100ml 88% (w/w) orthophosphoric was added. The solution was made up to 1 litre with water and filtered through Whatman No. 1 paper. The assay procedure involved the thorough mixing of 5ml of dye reagent and 100μl sample containing 10-100μg of protein. The absorbance at 595nm was read after 15-20min. The protein concentration in appropriately diluted unknown solutions was determined from the non-linear standard curve for BSA (A 7638, Sigma, prepared and stored as described by Peterson (1983)). A microassay in which 0.95ml of reagent was added to 50μl of sample containing 2-25μg protein was used when limited sample was available. Determinations were carried out in duplicate.

An attempt to use the less sensitive but more accurate Biuret reagent of Gornall *et al.* (1949) to measure the high protein concentrations in the cell-free bacterial extracts prepared for analytical RP-LC was unsuccessful owing to strong interference by dithiothreitol present in the extraction buffer. Components of the extraction buffer did not interfere with the dye-binding assay.

The UV absorption method of Scopes (1974) was used to determine protein concentration in dialysed solutions of purified arginase. Measurements at 205nm and 280nm were conducted in 1cm pathlength cuvettes (cleaned with hot 10% (w/v) SDS) at 25°C using a UV-VIS 280 spectrophotometer (Shimadzu) with a slit width setting of 0.5nm. Protein solutions were mixed in the cuvette using pasteur pipettes that had been cleaned with chromic acid, as uncleaned pipettes contributed

significantly to A_{205}. Extinction coefficients (1mg/ml at 205nm) were calculated for each sample using the formula

$$E_{205}^{1mg/ml} = 27.0 + 120 \times (A_{280}/A_{205})$$

and protein concentrations then determined using the A_{205} value. The extinction coefficient for both *B. caldovelox* and *Thermus* 4-1A arginases was ~29.5. The dye-binding method typically gave protein concentrations that were 75-85% of those obtained by the UV method. The UV results were considered to be more reliable as the relative dye-binding capacities of arginase and the BSA standard are unknown.

2-8-2 Arginase assays

The most common method used to measure arginase activity involves the colorimetric estimation of the product urea. Determination of urea by the method of Archibald (1944) is based on Fearon's condensation reaction (Fearon, 1939) in which carbamido compounds such as urea and citrulline form coloured complexes with diacetylmonoxime when heated in acid solution. This method has been used more recently (Moore and Kauffman, 1970) but the sensitivity and linearity of the method and stability of the chromogen towards light were improved by adding thiosemicarbazide (Marsh *et al.*, 1965; Geyer and Dabich, 1971), 2,3-dimethyl-1-phenyl-3-pyrazoline-5-one (phenazone, antipyrine) (Ceriotti and Spandrio, 1963; Prescott and Jones, 1969), or *N*-phenyl-p-phenylenediamine•HCl (4-aminodiphenylamine, semidine) (Hunninghake and Grisolia, 1966) to the diacetylmonoxime reagent. The time required for maximum colour development at 100°C was decreased by including ferric ions in the reaction mixture (Ceriotti and Spandrio, 1965). Archibald (1945) also used α-isonitrosopropiophenone as a reagent for urea determination. The reagent was more specific for urea

(compared with other carbamido compounds) than diacetylmonoxime and the chromogen formed by heating at 100°C exhibited a high molar extinction coefficient but was light sensitive (Moore and Kauffman, 1970). Despite this drawback the method has been used in several studies of arginase (e.g. O'Malley and Terwilliger, 1974; Tarrab *et al.*, 1974; Carvajal *et al.*, 1987). Hagan and Dallam (1968) described an arginase assay in which urea was quantified as a coloured complex formed with 4-dimethylaminobenzaldehyde (Ehrlich's reagent) at room temperature and this method has been used by other workers (e.g. Kaysen and Strecker, 1973; Rossi *et al.*, 1983). Colorimetric detection of the product ornithine was shown to be feasible by Chinard (1952) who found that heating ornithine with ninhydrin in phosphoric-acetic acid solutions gave an intense orange-brown colour. Of the other compounds tested, only proline, citrulline, cysteine, lysine and hydroxylysine gave significant reactions. This finding was recently applied to the estimation of serum arginase activity in domestic animals (Mia and Koger, 1978).

A few workers have estimated the arginine remaining colorimetrically by the Sakaguchi reaction (Sakaguchi, 1925) or followed the disappearance of arginine by UV absorption. The latter method, initially presented by Ward and Srere (1967), was extended to steady-state kinetic studies of arginase by Pace *et al.* (1980) and is based on a decrease in the combined extinction coefficients of urea and ornithine compared to arginine at 205.7nm. However, the poor sensitivity of this assay method at saturating substrate concentrations makes it unsuitable for most characterization studies. Novel arginase assay methods include the use of an ammonium-selective electrode in the presence of excess urease (Booker and Haslam, 1974; Aguirre and Kasche, 1983) and a radiochemical assay with L-[guanidino-^{14}C] arginine as

substrate, in which unhydrolysed arginine was removed from the assay by batch adsorption to a sulfonate resin and liberated urea determined in the supernatant (Rüegg and Russell, 1980).

For this project a diacetylmonoxime reagent was selected for urea determination in assay mixtures and culture supernatants, because it could also be used to measure citrulline formed in arginine deiminase assays (Hunninghake and Grisolia, 1966; Moore and Kauffman, 1970; Boyde and Rahmatullah, 1980) as well as other carbamido compounds. The method used was modified from Geyer and Dabich (1971). 1ml of colour reagent (3.6mM thiosemicarbazide and 61.7mM diacetylmonoxime) and 1.5ml acid reagent (0.5ml 0.06M $Fe_2(SO_4)_3$ in 56.7% (w/w) phosphoric acid diluted to 1 litre with 10% (v/v) sulphuric acid) was added rapidly with mixing to 0.5ml of a solution (assay or diluted culture supernatant) with a urea concentration of 0.04-0.5mM and placed in a 95°C water bath for 20min. After cooling for 5-10min in 20°C water, the absorbance at 520nm was read with appropriate blanks and a 0.2mM urea standard. The acid reagent was stable indefinitely, but a precipitate developed in the colour reagent on standing so decreasing assay sensitivity. Provided a duplicate urea standard was included with each set of assays the latter reagent was usable for one month. Ornithine was measured in assays and culture supernatants by the method of Mia and Koger (1978). 2ml of cold acid ninhydrin reagent (6.24g ninhydrin•H_2O (AR, BDH) dissolved in 80ml water by heating at 60°C, added to 10.6ml 88% (w/w) phosphoric acid (AR) and made up to one litre with glacial acetic acid (AR)) was added rapidly with mixing to a 0.5ml solution containing 0.015-0.25mM ornithine and incubated at 95°C for 15min then cooled to 20°C as above. The cooled assay solution tended to develop crystalline precipitates on standing for more than ~20min at room temperature. As this problem was more common at substrate concentrations greater than 20mM arginine it

is possible that the precipitate is due to ninhydrin-oxidised arginine. The coloured product also precipitated from solution on standing but this was only rapid at very high A_{515} values. These difficulties were avoided by reading the tubes within 20min of removal from the 95°C waterbath. The A_{515} was read with appropriate blanks and a duplicate 0.1mM ornithine standard for each set of assays to compensate for variations in waterbath temperature, reagent strength and the effect of substrate concentration on colour yield. The acid ninhydrin reagent was stable for at least two months when stored at 5°C in an amber bottle.

The ornithine assay was selected for routine use during enzyme purification and characterization of arginases because of its greater sensitivity and lower background (at appropriate substrate concentrations) and because only a single reagent was required. The colour yield for ornithine varied markedly with arginine concentration (Fig. 2-1) and assays with arginine concentrations above 40mM were stopped with 0.2ml of 36% (w/v) acetic acid and diluted to give 20mM arginine in a 0.5ml sample before analysing for ornithine in the usual way. However at a given substrate concentration the linearity of response for ornithine was excellent (Fig. 2-1). The diacetylmonoxime assay of urea showed less variation in colour response with changes in arginine concentration but blanks gave very high readings. Substrate solutions were stored at 4°C and made up freshly each week because of slow non-enzymatic hydrolysis of arginine at alkaline pH. Assay solutions for the measurement of enzyme activities in the RP-LC studies have been described (Section 2-3). Appendix 2 contains titration curves of 0.21M solutions of L-arginine against buffers at 70°C and titration curves of 0.1M solutions of the free acid forms of buffers with NaOH. These graphs were used to make up buffered substrate solutions of a particular pH and arginine concentration between 40 and 80°C.

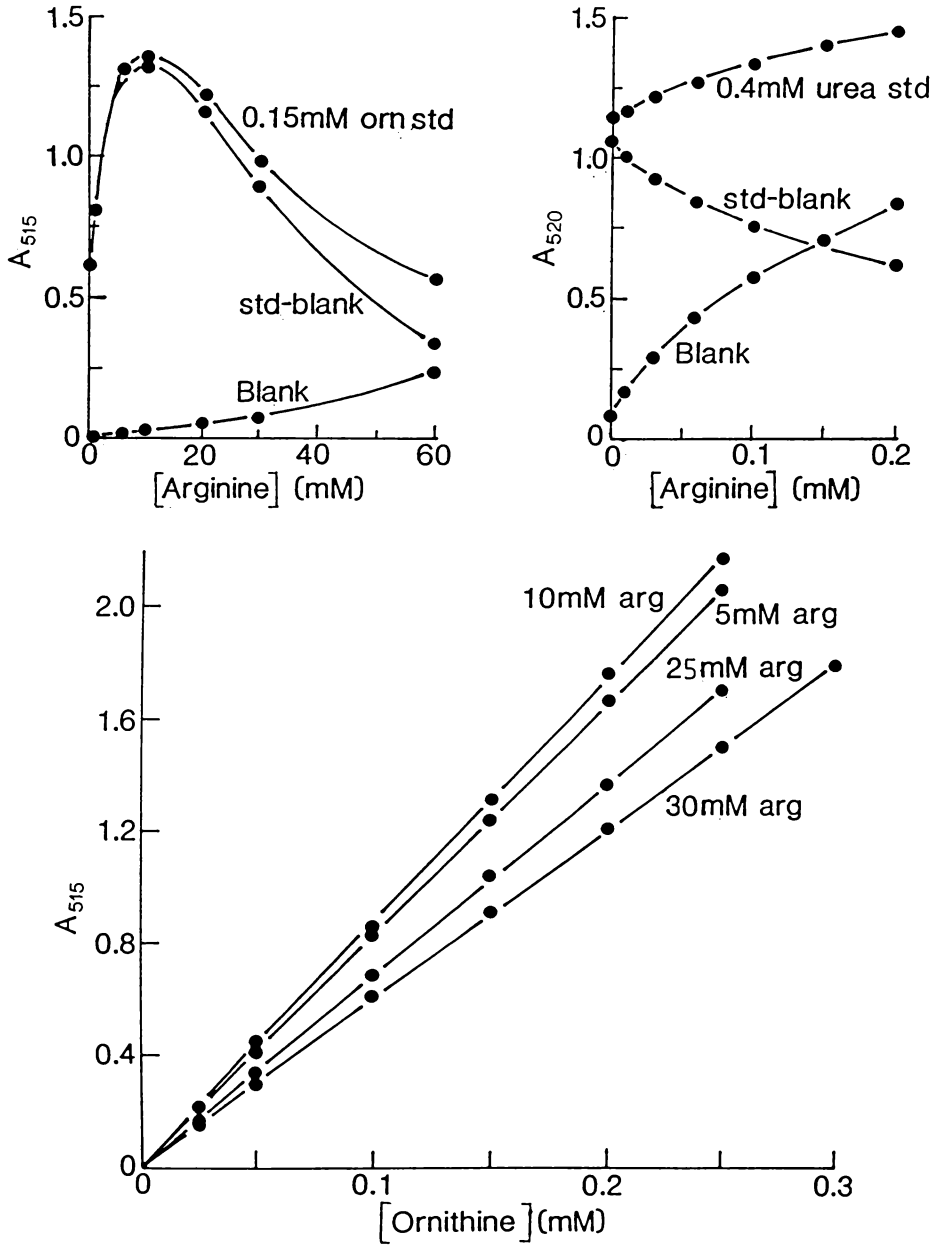

Figure 2-1 Effect of arginine concentration on colorimetric assays.

Top left: 0.5ml solutions containing 0.15mM ornithine and a range of arginine concentrations were assayed for ornithine using the acid-ninhydrin reagent. At high arginine concentrations the ninhydrin is no longer in large excess and the incomplete oxidation of arginine probably causes the higher blank absorbance.

Top right: 0.5ml solutions containing 0.4mM urea and a range of arginine concentrations were assayed for urea with the diacetylmonoxime reagent.

Bottom: Demonstrates the linearity of response for the determination ornithine with the acid-ninhydrin reagent. The spectrophotometer is apparently in its linear range up to an A_{515} of ~2. Millimolar extinction coefficients (l/mmol/cm) for ornithine were 42.5 (5mM arg), 44 (10mM arg), 42 (15mM arg, line not plotted), 38.5 (20mM arg, line not plotted)), 34.3 (25mM arg) and 30 (30mM arg).

Solutions of *B. caldovelox* "native metals" arginase lost activity rapidly at room temperature when diluted to concentrations appropriate for assaying, i.e. 0.13-0.2μg/ml. Enzyme activity was most stable when rapidly thawed concentrated arginase (≥0.33mg/ml) was diluted into cold alkaline buffer (CHES/NaOH or CAPS/NaOH, pH9.5-10) and stored at 4°C in a glass container. Under these conditions the loss of ~9% activity over 16h was tolerable for experimental purposes.

It was possible to measure arginase activity in small pellets of *Bacillus* and *Thermus* cells rendered permeable by treatment with an equal volume of a 1:1 toluene-acetone mix for 5min at 20°C but cell pellets were usually lysed by sonication and activity measured in cell-free extracts.

2-8-3 Miscellaneous colorimetric procedures

Parniak *et al.* (1983) reviewed four colorimetric methods for determination of arginine. From these the method of Van Pilsum *et al.* (1956) was selected for the quantification of arginine in some culture supernatants in this study. Ammonia was quantified by Nessler's reaction as described for the assay of L-asparaginase (Bergmeyer, 1974) using an ammonia colour reagent (14-2, Sigma). The deimination of agmatine could not be followed by this method due to the precipitation of Hg^{2+} in the colour reagent with SO_4^{2+} from the agmatine salt.

2-9 Miscellaneous Materials and Methods

Chromous acetate (chromium (II) acetate monohydrate dimer $[(CH_3CO_2)_2Cr{\cdot}H_2O]_2$) was prepared by the method of Balthis and Bailer (1939). The platinous salt $[Et_2NH_2]_2[PtCl_4]$ was kindly supplied by Dr Brian Nicholson. 0.2M solutions of the lanthanide ions (Gd, Ho, Yb, Lu) were prepared by heating the oxides in 1M HCl at 70°C except for La^{3+}

which was prepared from $La(NO_3)_3 \cdot 6H_2O$. 0.2M solutions of metal ions in water were prepared from the following AR grade salts: $MgSO_4 \cdot 7H_2O$, $AlCl_3 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, $MnSO_4 \cdot 4H_2O$, $CoSO_4 \cdot 7H_2O$, $NiSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$, $SrCl_2 \cdot 6H_2O$, $Na_2MoO_4 \cdot 2H_2O$, $AgNO_3$, $CdCl_2 \cdot 2.5H_2O$, $In_2(SO_4)_3$, $BaCl_2 \cdot 2H_2O$, $Na_2WO_4 \cdot H_2O$, $H_2PtCl_6 \cdot 6H_2O$, $HAuCl_4 \cdot 3H_2O$ and $Pb(CH_3COO)_2$. The laboratory grade salts $TiCl_3$, $VOSO_4 \cdot H_2O$, VCl_3 (in deaerated water), $Cr_2(SO_4)_3 \cdot 15H_2O$ and $[Et_2NH_2]_2[PtCl_4]$ (in deaerated water) were also prepared at a 0.2M concentration. 0.2M $FeSO_4 \cdot 7H_2O$ (Pronalys, M&B) was prepared in 50mM MOPS (free acid) and a saturated chromous acetate solution was made up in deaerated water. Soluble complexes of Pd(II) were formed by mixing $PdCl_2$ in 1M HCl at 70°C or 1M NH_3 at 25°C. When 0.2M solutions of the metal ions were diluted to 10mM they were not all stable at pH7.4 (i.e. dilution in 0.2M MOPS/NaOH buffer pH7.4) and deaerated buffers of lower pH (pH6.3 and pH5.2) were used for some metal ions.

2-Oxoarginine was synthesized enzymatically from arginine according to Meister (1954). 50mg crude *Crotalus altrox* venom (0.30U/mg, A 5147, Sigma) dissolved in 25ml 5mM potassium phosphate buffer, pH7 (20°C), was dialysed (3000MW cutoff tubing) with stirring against water for 16h at 5°C. The non-diffusible material was centrifuged for 15min at 31200g ($r_{av.}$ 6.98cm, SS-34 rotor, Sorvall) at 10°C and the supernatant (which served as a source of L-amino acid oxidase) was added to a 250ml Erlenmeyer flask containing 30ml 0.16M L-arginine (chromatographically homogeneous, BDH) and 3.3mg/ml dialysed catalase (1600U/mg, C-10, Sigma) adjusted to pH7.3 (20°C) and equilibrated at 37°C. The solution was stirred at 37°C with O_2 bubbling through until all the arginine had been oxidatively deaminated (20h) as indicated by no further increase in the $[NH_4^+]$. The reaction mixture was dialysed at 5°C against 2 500ml volumes of Milli Q water for 6h

each with stirring. The diffusates were concentrated to ~4ml at 42°C using a rotary evaporator and the solution cooled slowly to 0°C. The precipitated crystals were dissolved in a minimum volume of water at 50°C and recrystallized by cooling to 25°C. 2-Oxoarginine (α-keto-δ-guanidinovaleric acid) crystallized as the monohydrate, M_r ~191. An attempt to derivatize the product with OPA-ME and analysis by RP-LC confirmed the absence of primary amines.

The three standard buffers used to calibrate pH meters for the preparation of buffers used in this project were the NBS primary standards phthalate, phosphate D and borate, prepared according to Dawson *et al.* (1986) using $KHC_4H_4O_6$ (AR, Ajax), KH_2PO_4 (AR, Ajax), Na_2HPO_4 (AR, BDH) and $Na_2B_4O_7$ (AR, Ajax). Phosphate D and borate buffers were stable for 6 months at 4°C but the phthalate buffer was made up freshly each month. The need for accurate standards to calibrate Philips glass and reference electrodes (type G210 and R11 respectively, pH displayed on a Philips model PW9421 pH meter) was greatest when preparing the stock aqueous buffer for eluent A and the potassium borate derivatization buffer used in RP-LC work. The same standards were also used to calibrate combination pH electrodes over a range of temperatures when adjusting solution pH at elevated temperatures.

CHAPTER 3

ANALYTICAL REVERSE-PHASE LIQUID CHROMATOGRAPHY: METHOD AND APPLICATIONS

3-1 Introduction

It was hoped that the screening of thermophilic bacteria for enzymes suitable for characterization would also provide information about which pathways of arginine catabolism operated in a number of extreme thermophiles. Each of the three most thoroughly characterized catabolic pathways has a different enzyme that catalyses the first degradative reaction of arginine (see Figures 1-1, 1-2 and 1-3). Detection of the reaction products unique to each enzyme would therefore show that a particular pathway was present. However, the determination of these products in assay mixtures of bacterial extracts is complicated by the non-specific nature of some of the colorimetric methods normally used and the possibility that multiple pathways might occur in thermophilic bacteria as has been observed for a number of mesophiles (Abdelal, 1979; Cunin *et al.*, 1986; Table 1-1). Using the reverse-phase liquid chromatography (RP-LC) method developed it was possible to identify each product amine unambiguously so that the same method could be used for all assays. The development of the RP-LC method and the principles behind it are summarized in Section 3-2 below. A paper describing the complete method and some of its applications (Patchett *et al.*, 1988) is appended together with a few observations on its use (Appendix 1). The rest of the chapter presents the results obtained when this method was applied to bacterial extracts.

3-2 Analytical RP-LC: Method Selection and Optimization

Many of the diagnostically important intermediates of arginine catabolism are amino acids or primary amines. The available equipment allowed two options for the separation of these metabolites. The first was to use a Waters Associates automated ion exchange HPLC system normally employed for amino acid analysis with a fluoresence-based detection system involving post-column derivatization of primary amines using an *o*-phthalaldehyde-2-mercaptoethanol (OPA-ME) reagent. This system separated arginine, citrulline and ornithine using a conventional pH gradient elution method (Fig. 3-1) and detected ornithine in stopped-assay incubation mixtures (at pH7 at 70°C) of substrate arginine with cell-free extracts of *Thermus aquaticus* and the *Thermus* strain Tok1 A.1 (TRUCC 118). However, the ion-exchange system had a number of disadvantages when compared to the second option, pre-column derivatization of primary amines with OPA-ME followed by gradient elution on a RP-LC C_{18} 5μm Nova-Pak column (Waters Assoc.). A major consideration was the relative sensitivity of the two methods, the optimized RP-LC method being about 50 times more sensitive. In addition, the run-time between injections for the ion exchange method was more than twice the 60 minutes required in the RP-LC method. Sample preparation for RP-LC was straight forward, as diluted assay supernatants from which protein had been precipitated could be derivatized directly for injection (Rönnberg *et al.*, 1984; Qureshi *et al.*, 1984; Godel *et al.*, 1984). Finally, much smaller quantities of OPA-ME reagent are required for pre-column derivatization methods. The RP-LC method with pre-column derivatization was chosen for further study.

Chromatographic conditions required to separate the OPA-ME

derivatives of substrate arginine and the product amines agmatine, citrulline and ornithine on the Nova-Pak column were sought. The hydrophobic C_{18} groups of this column are supported by a silica-based matrix which is degraded outside the pH range of ~pH2-8. Buffer systems employed in other RP-LC methods typically contained phosphate (pH 6.5-7.5) or acetate (pH 4-6) salts. Lower pH values tend to improve column lifetimes and the apparent disadvantage of a more rapid rearrangement of fluorescent derivatives to non-fluorescent compounds at acidic pH values (Simons and Johnson, 1978) is unimportant in a chromatographic system because exposure to excess OPA is required to cause decay at a significant rate (Jacobs *et al.*, 1986). On the Nova-Pak column the initial composition of eluent A (0.1M phosphate buffer at pH 6.65) was successively modified over several runs by lowering the pH and salt concentration until a pH of 6 using a mixed phosphate-acetate buffer was reached (Fig 3-2). This modification caused a reversal in the elution order of arginine and citrulline. It is important to have a well buffered solvent as separation is dependent on ionic interactions (e.g. between protonated amine groups and ionised silanol groups on the silica matrix) as well as solvophobic effects. The greater effectiveness of K^+ compared to Na^+ in masking ionised silanol groups was a reason for selecting buffer salts containing the former cation (Papp and Vigh, 1983). The common practice of adding an organic modifier such as 1% (v/v) tetrahydrofuran (THF) (Fleury and Ashley, 1983; Buck and Krummen, 1984) or methanol (MeOH) and THF (Jones *et al.*, 1981; Winspear and Oaks, 1983; Venema *et al.*, 1983; Qureshi *et al.*, 1984; Méndez *et al.*, 1985) to eluent A was discontinued early in the development of a buffer system as it complicated buffer preparation and made no difference to the resolution of derivatives.

Baseline resolution of the amines of interest in this study

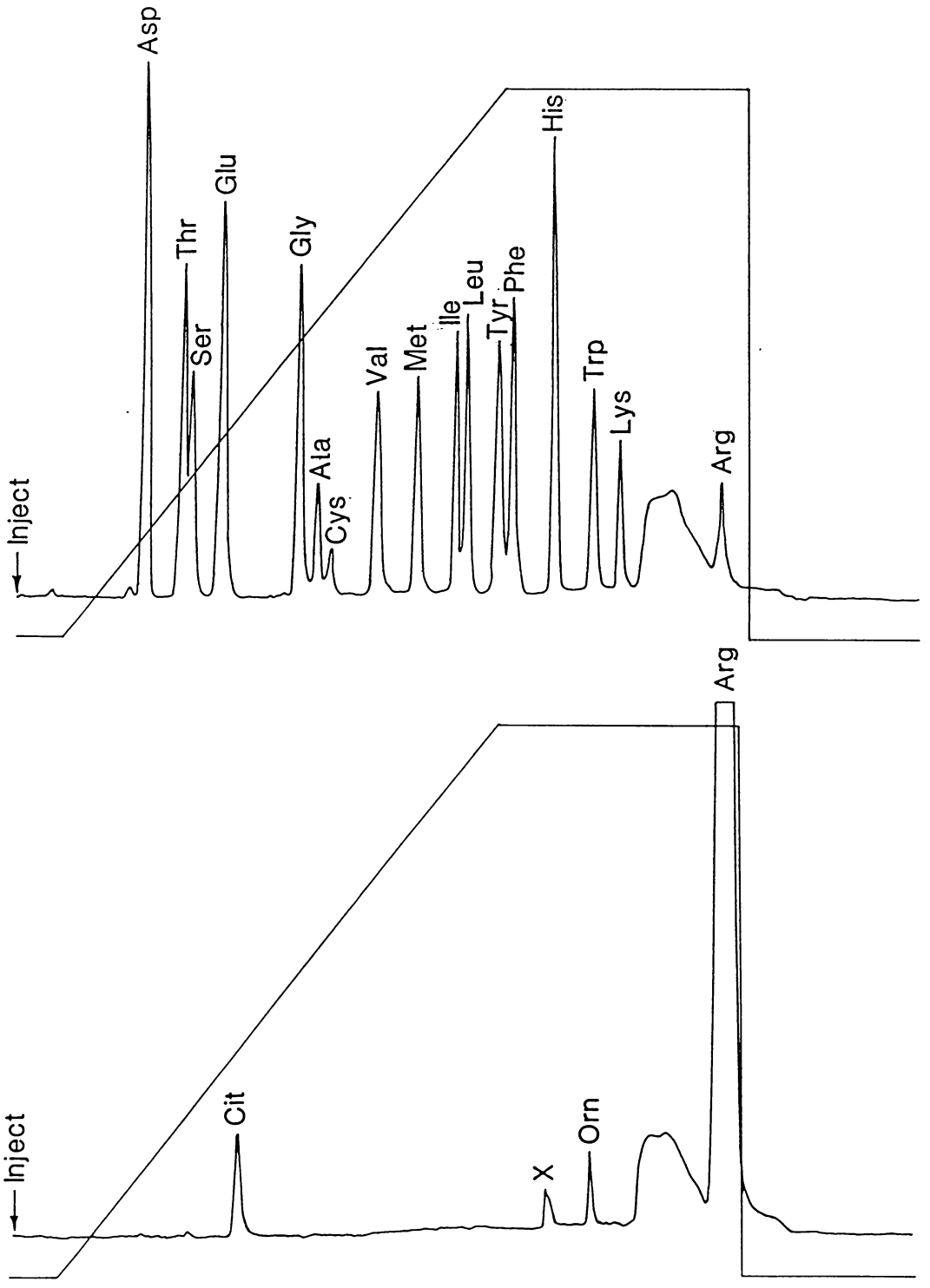

Figure 3-1 Ion exchange amino acid analysis.

Top: A Pierce H amino acid standard (product no. 20088, Pierce) sample supplemented with tryptophan and containing ~1nmole of each amino acid was separated on a Waters Assoc. amino acid analysis column using the gradient method and instrument settings recommended by Waters Assoc.

Bottom: Analysis of a sample containing 60nmole arginine and 0.6nmole of both citrulline and ornithine using the chromatographic system described above.

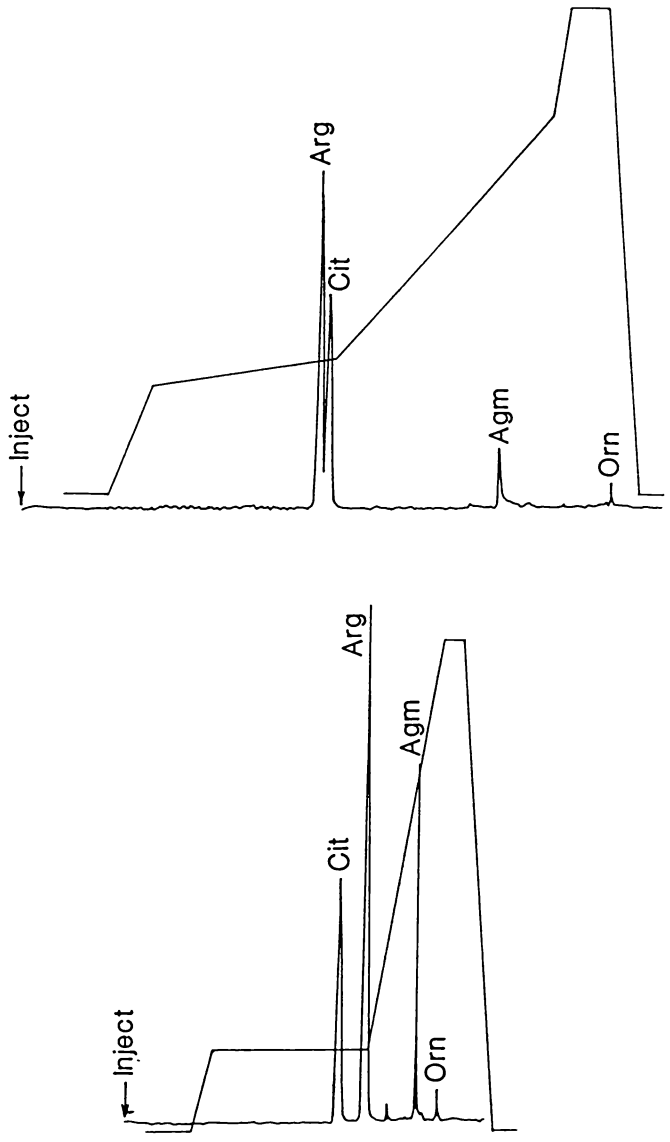

Figure 3-2 Resolution of OPA-ME-derivatized analytes by RP-LC.

Top: A sample containing 1nmole arginine, 0.75nmole citrulline, 0.3nmole agmatine and 0.1nmole ornithine was derivatized at pH7.5 with OPA-ME reagent. The gradient ran from 10% to 100% eluent B. A = 0.1M potassium phosphate buffer, pH6.65; B = 3:3:4 MeOH:CH_3CN:A. Citrulline and arginine derivatives eluted towards the end of a 10min linear segment of the gradient from 30% to 35% B. Chart speed 0.5cm/min, gain = 4.

Bottom: Sample and derivatization as above. A = 10mM KH_2PO_4 adjusted to pH6.05 with potassium acetate; B = 3:3:4 MeOH:CH_3CN:A. Arginine and citrulline derivatives eluted towards the end of a 17min isocratic segment of the gradient (25% B) in reversed order compared to the top figure. Chart speed 0.25cm/min, gain = 8.

The reversed elution order is beneficial as the large amount of arginine present in samples of stopped-assay mixtures produces a peak that tails and would interfere with the quantitation of any derivative eluting shortly after arginine.

required the use of a gradient method of derivative elution. Many workers separating OPA-ME-derivatized amines by RP-LC found an eluent B consisting of MeOH or acetonitrile (CH_3CN) (Hill *et al.*, 1979; Umagat and Kucera, 1982), a water-MeOH mix (Pfeifer *et al.*, 1983) or an aqueous buffer mixed with MeOH or CH_3CN (Jones *et al.*, 1981; Godel *et al.*, 1984; Qureshi *et al.*, 1984) provided satisfactory resolution. Pure organic solvents for eluent B were easy to prepare but on the Nova-Pak column use of 100% CH_3CN or 100% MeOH for eluent B produced broad peaks that were poorly resolved compared to peaks obtained when eluent B consisted of aqueous-organic mixtures. An eluent B composition of CH_3CN:MeOH:H_2O, 4:3:3 by volume (final volume less than added volumes) was arrived at after abandoning aqueous buffer-organic mixtures for eluent B because of difficulties experienced with precipitation of buffer salts during preparation. Eluent B was therefore unbuffered but the derivatives of interest were eluted before 100% B so eluent A still provided buffering capacity.

Two problems with early gradient systems were the poor resolution of arginine and citrulline derivatives (good resolution of product peaks from substrate arginine is important because of the large amount of arginine present in stopped-assay supernatants) and an irregular baseline in the region where the agmatine derivative eluted. Adequate resolution of citrulline from large amounts of substrate arginine was achieved by inserting an isocratic step in the gradient (see Fig. 3-2). The best %B of 27% for the isocratic step was identified by successive modifications to the gradient program. The noisy baseline in the region of agmatine elution was attributed to adsorption of trace NH_4^+ ions in eluent A on to ionised silanol groups of the column matrix. After accumulating during a chromatographic cycle, some of these ions were derivatized by excess OPA-ME reagent as it moved through the column.

However, the fluorescent response of the ammonia derivative is only ~2% of that seen for the analytes so no further steps were taken to improve the baseline in this region.

The effect of temperature on analyte response and peak resolution was assessed as some studies reported improved resolution at higher column temperatures (e.g. Méndez *et al.*, 1985). However at 40°C peak areas were half those seen at 20°C due to more rapid decay of fluorescent derivatives, while resolution was not affected. In terms of column lifetime low running temperatures are best but because elution times were temperature dependent a temperature of 30°C was chosen to avoid the effect of variations in ambient temperature.

3-2-1 Derivatization conditions and use of an internal standard

As previously mentioned, silica-based column matrices are damaged by exposure to alkaline pH. The direct injection of an alkaline derivatization solution (typically pH9.5-10.5 in most studies) onto the Nova-Pak column was therefore of some concern. A few researchers have neutralized the derivatized solutions prior to injection (Sista, 1986) but this limits the extent to which derivatization can be automated. The first studies on OPA-ME reactions with primary amines (Roth, 1971) suggested that a derivatization pH of ~7.5 would give only slightly reduced fluorecent response for the amines of interest especially if derivatization times could be increased. However, for the method used an increased derivatization time would have required the insertion of a pre-column mixing chamber as the maximum time was set by the minimum possible flow rate of 0.1ml/min. While partial derivatization was achieved at pH7.5 without a mixing chamber (Fig. 3-2) the sensitivity to all analytes, particularly ornithine, was poor. Further, the citrulline response was variable and decreased up to 5-fold in the

presence of excess arginine, a phenomenon not seen at higher derivatization pH's. It was decided that to maximize sensitivity a derivatizing pH of 9.4 would be a satisfactory compromise. By lowering the concentration of borate buffer to 0.2M (lower than the nearly saturated concentrations used in other studies but still enough to buffer any variations in sample pH) injection of the derivatized sample was unlikely to raise the column pH (buffered at pH 6) for any length of time. After adding OPA and ME to borate buffer and mixing with an equal volume of typical sample containing 20mM perchloric acid, the solution pH was 9.0±0.1 at 30°C.

Following the selection of a pH for derivatization, optimum OPA and ME concentrations were established. Because of the high concentrations of substrate arginine present in most samples (up to 1mM) an OPA concentration of ~40mM was used to ensure a large excess of OPA over total derivatizable amino groups in the sample. A 2:1 molar ratio of ME:OPA was used as this is the optimum for reaction rate (Wong *et al.*, 1985). This derivatization reagent gave a linear response for all analytes and the response was not decreased in the presence of excess sample arginine (Patchett *et al.*, 1988). The mixing of OPA-ME and sample takes place at a slow flow rate in the column frit and at the top of the column at a temperature of 30°C. The reaction time is less than 1 minute so a rapid derivatization reaction is important for maximum sensitivity. Faster flow rates during derivatization decreased fluorescent responses but also produced a more regular baseline in the region of elution of the agmatine derivative because of a less complete derivatization of adsorbed NH_4^+ ions. The instability of OPA-ME derivatives observed in studies on the chemistry of derivatization is now thought to be largely due to excess reagent (Cooper *et al.*, 1984) and in particular excess OPA (Jacobs *et al.*, 1986). The latter workers

indicated that using a minimum concentration of OPA during derivatization was the most obvious route to stabilizing derivatives but that the need for a rapid and quantitative derivatization reaction imposed limits in this regard. However, the automated pre-column derivatization used here is rapid and reproducible, and minimizes the time the derivatives are exposed to excess OPA before they are adsorbed to the column matrix. Other thiol reagents were reputed to yield more stable adducts or give a faster derivatization reaction (Simons and Johnson, 1977; Godel *et al.*, 1984). In this study the use of ethanethiol was rapidly abandoned because of its unacceptable odour and 3-mercaptopropionic acid offered no apparent improvement compared to ME despite a report by Godel *et al.* (1984) showing that derivatives of the former thiol reagent are more stable.

The importance of an internal standard in maximizing the accuracy of analyte quantification by correcting for variations in sample volume, reagent strength and mixing efficiency has been outlined by Patchett *et al.* (1988). Internal standards trialed and rejected included ϵ-aminocaproic acid, 2-aminoethanol (eluted too close to agmatine) and homoserine which converts to the γ-lactone at acid pH (pg. 18, Dawson *et al.*, 1986). Homocysteic acid was selected because it is stable across the pH range and eluted well clear of analyte peaks. Lindroth and Mopper (1979) reported that the related compound cysteic acid was relatively slow to derivatize. In this study the fluorescent response of homocysteic acid was usually at least as great as that of other analytes (Table 3-1). It was subsequently found that homocysteic acid eluted close to the derivative of glutamate which proved to be an important intermediate of arginine catabolism in some thermophilic bacteria. However the two derivatives were baseline resolved using the standard RP-LC method so the same internal standard was used for all

analyses.

3-2-3 Separation of amines: General applications of the RP-LC method

The elution times of derivatives of amino acids and other amines on this RP-LC system were studied to see if any had the potential to interfere with the determination of the intermediates under investigation. As shown in Table 3-1 threonine and phenylalanine could interfere with the determination of citrulline and agmatine respectively if present in bacterial extracts in large amounts but the 200-fold dilution of extract in the sample prevented interference by endogenous amines. The elution times indicated that the analysis of stopped-assay mixtures by this RP-LC method would permit the measurement of numerous enzyme activities that occur in amino acid metabolism. In this study ornithine aminotransferase and catabolic ornithine carbamyltransferase activities were detected in bacterial extracts without any modifications to the method. While simple colorimetric assay methods are available for most of the enzymes studied here not all of these have the specificity required for assays of crude extracts. The high assay temperatures used here might also preclude the use of conventional assay media when substrates or products are thermolabile. For example, the assay of catabolic ornithine carbamyltransferase in the reverse direction and detection of citrulline formed (Crow and Thomas, 1982) would not be feasible at 80°C because of the rapid breakdown of the substrate carbamyl phosphate (Jones and Lipmann, 1960; pg. 56, Dawson *et al.*, 1986). The method developed here was also used to determine the concentrations of catabolic intermediates present in culture supernatants of *B. caldovelox* and *Thermus* 4-1A (see Figures 3-3 and 3-7).

Table 3-1 Elution times and response factors of amino acids and amines

Amino acid/amine	Elution time (min)	Response factor*
Aspartic acid	4.44	0.66
Homocysteic acid	5.08	1.00
Glutamic acid	5.55	0.90
Asparagine	8.40	1.02
Serine	9.30	0.95
Histidine	9.75	0.76
Glutamine	10.22	0.94
Glycine	12.79	0.81
Citrulline	13.35	1.04
Threonine	13.39	0.88
Cadaverine	14.20	0.86
Arginine	15.22	1.15
Alanine	19.20	0.86
Taurine	20.75	1.43
α-amino-*N*-butyric acid	22.22	1.04
Tyrosine	22.30	0.89
Valine	24.88	1.02
Methionine	24.92	1.00
Tryptophan	25.46	0.89
Phenylalanine	25.80	0.94
Agmatine	25.98	1.16
Leucine	26.42	0.91
Ornithine	28.07	0.34
Lysine	28.50	0.57
Spermidine	32.79	0.20
Putrescine	33.06	0.41

*Response factor relative to the internal standard homocysteic acid.

Chromatographic conditions were as described in Patchett *et al.* (1988) (Appendix 1).

3-3 Distribution of Arginine-Catabolizing Enzymes in Thermophiles

3-3-1 Growth of bacteria and detection of enzymes

Studies with mesophilic bacteria have shown that the enzymes of arginine catabolism are often induced by arginine and repressed by carbon and nitrogen catabolites so growth media for thermophilic bacteria included 10-20mM arginine and an effort was made to decrease the concentration of other amino acids and ammonium ions where possible. The growth curves of some aerobic thermophilic bacteria screened for enzymes are shown in Figures 3-3 and 3-7. The supernatant pH typically increased during growth in arginine-supplemented medium. This was not necessarily related to arginine metabolism as the pH increase occurred for *Thermus* 4-1A which only degraded a small proportion of available arginine (Fig. 3-3) and even for *B. coagulans*, which possessed no detectable stationary phase arginase activity. From the few growth experiments performed it was apparent that enzyme specific activities varied with media composition (e.g. arginine concentration, amount of T.P. or Y.E. added), culture conditions (pH, aeration, temperature) and the time of harvesting. For example the specific activity (nmol ornithine formed/min/mg of cell-free extract protein) of arginase in the thermophilic *Bacillus* strain Ok3 A.1 was 150 (pH6.2), 1500 (pH6.8), 1200 (pH7.4) and 360 (pH8.0) at the initial culture pH values given. These factors influence enzyme levels in mesophilic bacteria (Cunin *et al.*, 1986).

As the enzymes assayed in this study differed in their requirements for optimal activity and stability, it was necessary to use sub-optimal conditions for the simultaneous determination of enzyme activities. Compared to assays of extracts under optimal conditions the RP-LC assays conducted at a lower (physiological) pH without an

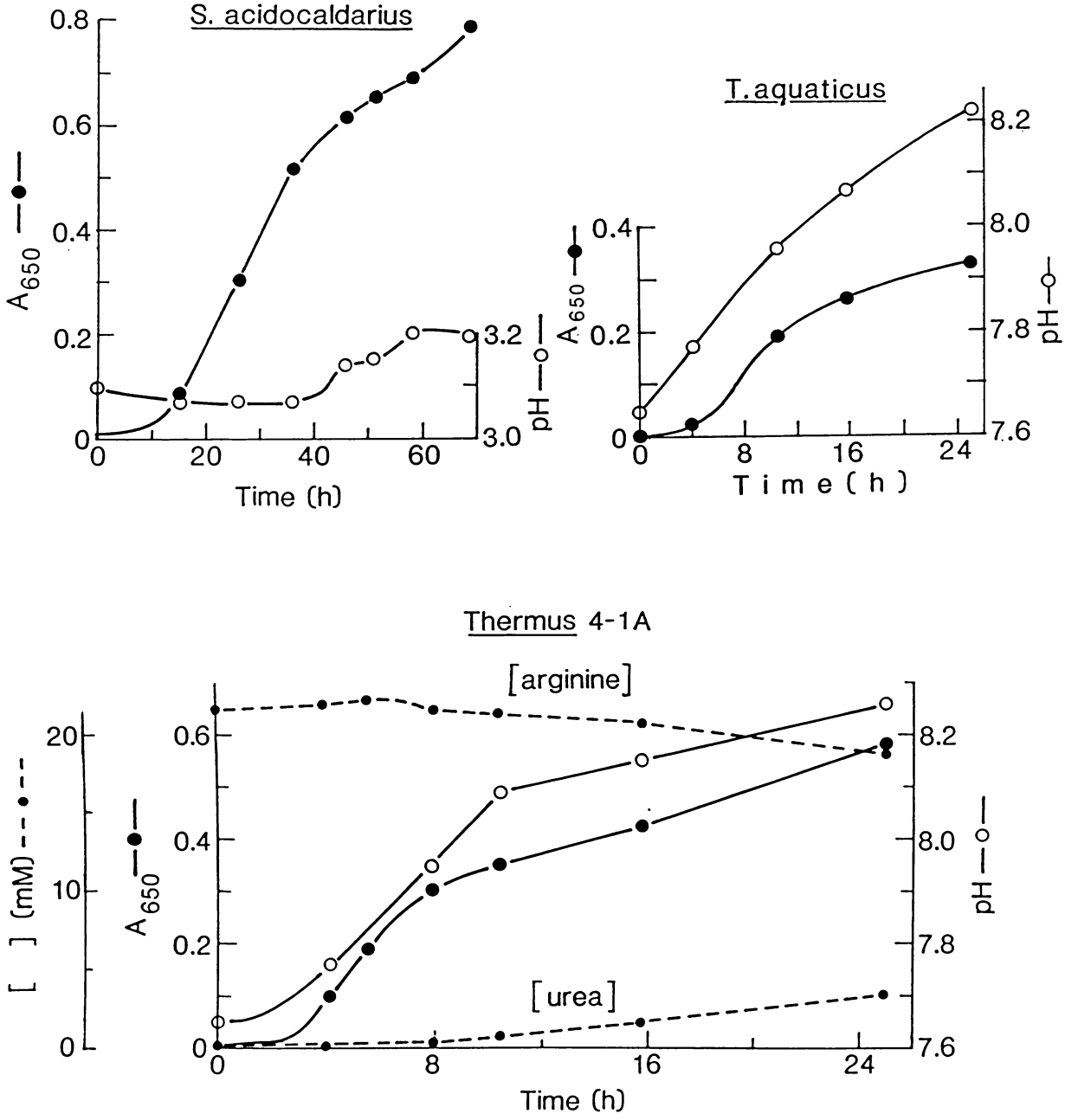

Figure 3-3 Growth curves on "inducing" media.

Enzyme activities at specified sampling times are listed in Tables 3-2 and 3-3. [Arginine] in the culture supernatant of *Thermus* 4-1A was determined by RP-LC and the [urea] was measured colorimetrically. No ornithine or glutamate was detected.

activating divalent transition metal cation (Mn^{2+}) gave decreased arginase activity but the activity measured is probably a more accurate indication of arginase activity *in vivo*. Potential cofactors and stabilizing chemicals (e.g. DTT and glycerol) were included in the extraction buffer when they were not strongly inhibitory to a particular activity and so were present in assays at a 10-fold dilution. Pyridoxal phosphate was included as a cofactor for aminotransferases and decarboxylases. Many arginine decarboxylases require Mg^{2+} for activity and stability (e.g. Rosenfeld and Roberts, 1976) and this cation may also be important for arginine deiminase activity (Broman *et al.*, 1975).

The RP-LC method was tested using *B. licheniformis* (DSM 13*) as a positive control for arginase and *P. aeruginosa* (DSM 1707) as a positive control for arginine deiminase and arginine decarboxylase activity. Under appropriate growth and assay conditions (those described by Mercenier *et al.* (1980) for arginine decarboxylase) the expected activities were detected in these test organisms by the RP-LC method.

Although each of the three first-step enzymes assayed is usually indicative of a particular pathway, a few organisms deviate from the expected pattern by using alternative enzymes. For example, some arginase-positive microorganisms degrade ornithine by cyclodeamination to proline rather than by the more usual aminotransferase reaction, and the detection of arginine deiminase activity does not guarantee that the other two enzymes of this pathway will occur in a bacterium (e.g. *Streptococcus faecium* (Pendey, 1980)). Further, Kamekura *et al.* (1987) was unable to detect agmatinase or agmatine deiminase in cell-free extracts of *Halobacterium halobium* despite the presence of arginine decarboxylase activity. The ability to detect the second enzyme of both

the arginase and arginine deiminase pathways by RP-LC was therefore useful in confirming the pathways operating in the thermophilic bacteria screened. Representative elution profiles for all the enzymes detected by RP-LC are shown in Figures 3-4, 3-5 and in Patchett *et al.* (1988) (Appendix 1).

The literature on microbial arginine catabolism is scattered with reports identifying enzyme activities that have subsequently been shown to be absent (e.g. arginine deiminase in *S. cerevisiae*, Roche and Lacombe, 1952; Middelhoven, 1964) or due to the non-specific activity of a different enzyme (e.g. ornithine aminotransferase in *P. aeruginosa*, Voellmy and Leisinger, 1975; Stalon *et al.*, 1987). Because of difficulties in distinguishing between the inability of a bacterium to express a particular activity and inappropriate growth or assay conditions for the enzyme, a negative result reported here does not necessarily indicate that a bacterium is incapable of expressing that activity. However, where a positive result was seen alternative explanations for the activity were eliminated by appropriate controls and whenever possible the activity was assayed colorimetrically for further confirmation.

3-3-2 Enzyme distribution

Tables 3-2 and 3-3 contain most of the results obtained from the RP-LC screening experiments. With the exception of *B. coagulans*, arginase activity was detected in all *Thermus* and thermophilic *Bacillus* strains. Although the range of activity seen for *Thermus* and *Bacillus* spp. overlapped, *Bacillus* strains typically showed higher activity and grew more rapidly and to higher A_{650} on the "inducing" medium than *Thermus* strains. The highest specific activity of arginase in both groups occurred in early stationary phase, after which activity

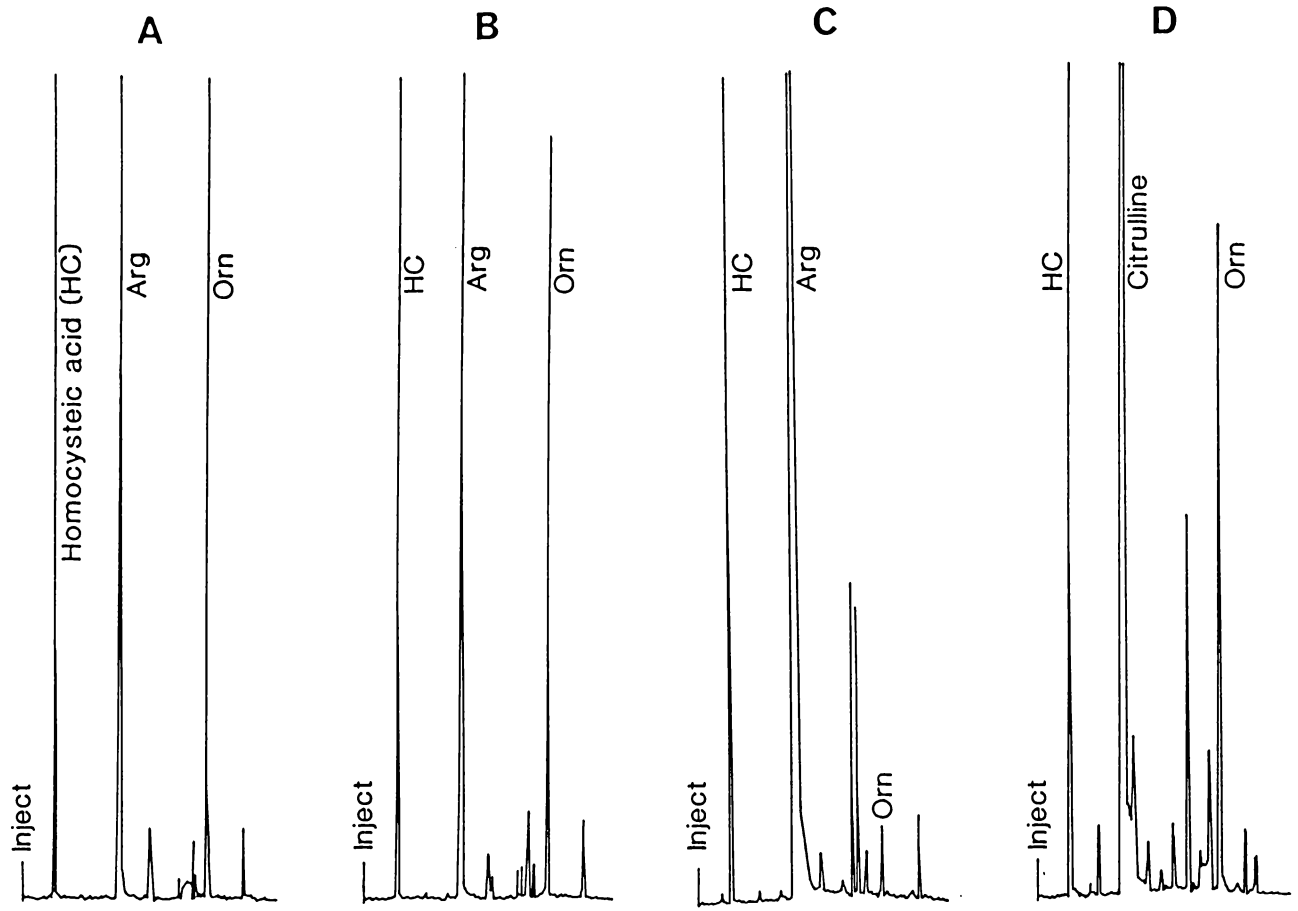

Figure 3-4 Representative RP-LC elution profiles.

Assays are described in Section 2-3.

A. Arginase: *B. caldovelox* 15.75h sample extract incubated with arginine. Stopped assay supernatant diluted 100×.

B. Arginase: *Thermus* 4-1A 25h sample extract incubated with arginine. Stopped assay supernatant diluted 100×.

C. Arginase: *S. acidocaldarius* 46h sample extract incubated with arginine. Stopped assay supernatant diluted 10×.

D. Ornithine carbamyltransferase: Tok12 S.1 (growth described in Section 2-2-4) extract incubated with citrulline. Stopped assay supernatant diluted 10×.

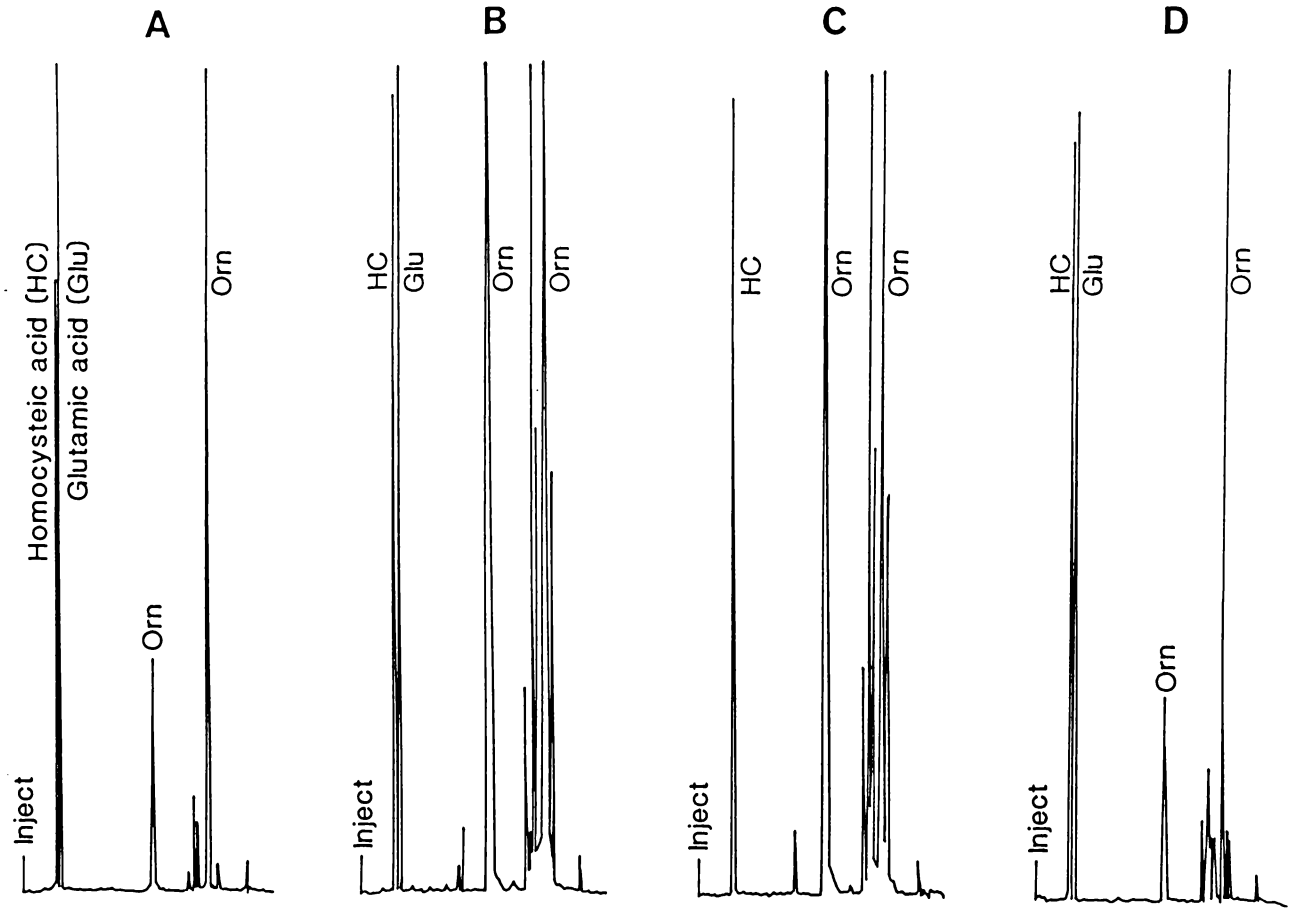

Figure 3-5 Representative RP-LC elution profiles.

Assays are described in Section 2-3.

A. Ornithine aminotransferase: *B. caldovelox* 15.75h sample extract incubated with ornithine and 2-oxoglutarate. Stopped assay supernatant diluted 100×.
B. Ornithine aminotransferase: *Thermus* 4-1A 25h sample extract diluted 5-fold and incubated with ornithine and 2-oxoglutarate. Stopped assay supernatant diluted 10×.
C. Ornithine aminotransferase substrate blank showing the multiple ornithine peaks that occur at high ornithine concentrations. Stopped assay diluted 10×.
D. Ornithine aminotransferase: Tok12 S.1 (grown on "inducing" medium described in Section 2-2-1) extract incubated with ornithine and 2-oxoglutarate. Stopped assay supernatant diluted 100×.

Table 3-2 Summary of arginine-catabolizing enzyme activities in thermophilic bacteria

Thermophilic group	Arginase	Ornithine-oxoacid aminotransferase	Arginine deiminase	Ornithine carbamyltransferase	Arginine decarboxylase
Bacillus	200-3000[a]	1520 (*B. caldovelox*, 15.75h)	not detected	not detected	not determined[b]
Thermus	40- 870	160 (*T. aquaticus* & 4-1A, both 25h)	not detected	not detected	not determined[b]
Anaerobic archaebacteria[c]	not detected	390 (Tok12 S.1)	5-15	80 (Tok12 S.1)	5-15
Sulfolobus[d]	6 - 11	not determined	not detected	not determined	not detected

[a]Values are the range seen for 5 *Bacillus* strains (see Table 3-3). *B. coagulans* had no detectable arginase activity.
[b]Unable to determine product agmatine because of high [ornithine] in stopped-assay supernatants (Patchett *et al.*, 1988).
[c]Tok12 S.1 (TRUCC 95) and AN1 (DSM 2770). Growth at 88°C, assays at 80°C.
[d]*S. acidocaldarius* and *S. solfataricus* (see Table 3-3).

Activity expressed as nmol product formed/min/mg of cell-free extract protein at pH7 and 70°C unless otherwise indicated (see also Table 3-3). All cell-free extracts were prepared from bacteria grown on "inducing" media.

Table 3-3 Arginase activity in thermophilic bacteria

Species/strain	Activity (nmol ornithine produced/min/mg cell-free extract protein)	Sample time[a] (h)
B. caldovelox (DSM 411, YT-F)	500	5.7
	1740	8
	3000	10.5
	2780	15.75
B. caldotenax (DSM 406)	2100	
B. caldolyticus (DSM 405)	200	
Bacillus Ok3 A.1 (TRUCC 253)	1210	
B. stearothermophilus (DSM 22*)[b]	920	
B. coagulans (DSM 459)[b]	0	
T. aquaticus (ATCC 25104*)	80	12.75
	160	25
T. `thermophilus' (ATCC 27634)	120	
T. filiformis (ATCC 43280^T)	270	
Thermus sp. strain T-351 (ATCC 31674)	320	
Thermus Rt350 A.12 (TRUCC 251)	180	
Thermus Rt8 A.1 (TRUCC 100)	200	
Thermus HS1 A.1 (TRUCC 271)	230	
Thermus 4-1A (TRUCC 52)	420	4.7
	870	25
T. ruber (DSM 1279)[b]	40	
S. acidocaldarius (type 7, TRUCC 305)[c]	10	46
	6	69
S. solfataricus (DSM 1616)[c]	11	

[a]If not specified sampling was in early-mid logarithmic phase with the exception of *B. coagulans* (stationary phase). Not all bacteria displayed classical growth curves in "inducing" media.
[b]Growth and assays at 60°C
[c]Growth at 75°C

Growth and assays at 70°C unless otherwise indicated.

gradually declined as stationary phase lengthened. The next step of arginine catabolism in these bacteria is apparently catalysed by ornithine aminotransferase. No urease activity could be detected in any extracts including those from archaebacteria.

Sharp *et al.* (1980) observed that several thermophilic *Bacillus* strains including *B. caldovelox* grew (albeit poorly) under anaerobic conditions. *B. caldovelox*, *B. caldotenax* and *Bacillus* Ok3 A.1 were grown under conditions of oxygen limitation in "inducing" medium to see if enzymes of the deiminase pathway would be induced as in *B. licheniformis* (Broman *et al.*, 1978). These bacteria all grew very poorly, however, and no arginine deiminase activity could be detected in extracts. Further, no catabolic ornithine carbamyltransferase activity could be detected in the same *B. caldovelox* extract. Arginase activity was still present in the three strains at ~5-10% of the specific activity seen in aerobic cultures. These findings are similar to those for mesophilic *Bacillus* spp. that are deiminase-negative (Cunin *et al.*, 1986).

The occurrence of arginine decarboxylase in *Thermus* and thermophilic *Bacillus* strains is unlikely considering the current information on this enzyme's distribution and the presence of an arginase pathway in these bacteria. Interference from ornithine (produced by arginase activity) in the detection of agmatine by RP-LC (Patchett *et al.*, 1988) meant that the absence of arginine decarboxylase activity could only be confirmed for *B. coagulans* and *T. ruber*. A radiochemical assay based on detection of $^{14}CO_2$ (e.g. Morris and Boeker, 1983) could be used for the other bacteria but only after checking extracts for ornithine decarboxylase activity. This assay could also be used to confirm the level of arginine decarboxylase activity in archaebacterial extracts and the further metabolism of

agmatine. The 25h extract of *T. aquaticus* did produce a carbamido compound (detected colorimetrically) when incubated with 20mM agmatine (pH7, 70°C) at a rate of ~5nmol/min/mg of cell-free extract protein. This may have been due to agmatinase activity possibly from a non-specific arginase. The agmatine deiminase alternative is unlikely for obligate aerobes.

The specific activity of arginase from *Sulfolobus* spp. was the same whether the strains were grown on "standard" or "inducing" media, but better growth was found on the former. As arginase is normally an inducible enzyme and the level of activity was very low, the possibility that ornithine detected in RP-LC assays was formed by arginine amidinotransferase activity was examined. Using cell-free extracts of *S. acidocaldarius* prepared as described in Fig. 3-6 the enzyme was assayed at pH9 at 60°C with 20mM arginine and 50μM Mn^{2+}. The product ratio of ornithine to urea (both measured colorimetrically) was 1.04 indicating that no endogenous guanidino acceptor was present. However, as some arginine amidinotransferases exhibit weak hydrolase activity (e.g. Srivenugopal and Adiga, 1980) the enzyme was also assayed with potential acceptors present. 10mM hydroxylamine failed to activate the enzyme and 10mM L-glycine caused ~50% inhibition of activity under the above assay conditions at pH8 and 9. This result and a pH optimum of ~9 both suggest an arginase is responsible for the activity, although inclusion of 50μM Mn^{2+}, Ni^{2+}, Co^{2+}, Cd^{2+}, Mg^{2+}, Ca^{2+} or 4mM CDTA in 20min assays had negligible effect on activity.

Fractionation of a *S. acidocaldarius* cell-free extract by GP-FPLC yielded two peaks of activity with M_rs of ~140000 and ~30000 (Fig. 3-6) which may correspond to the native oligomer and monomer of the arginase. The low specific activity of arginase in *Sulfolobus* strains might be related to inactivation/dissociation caused by equilibration

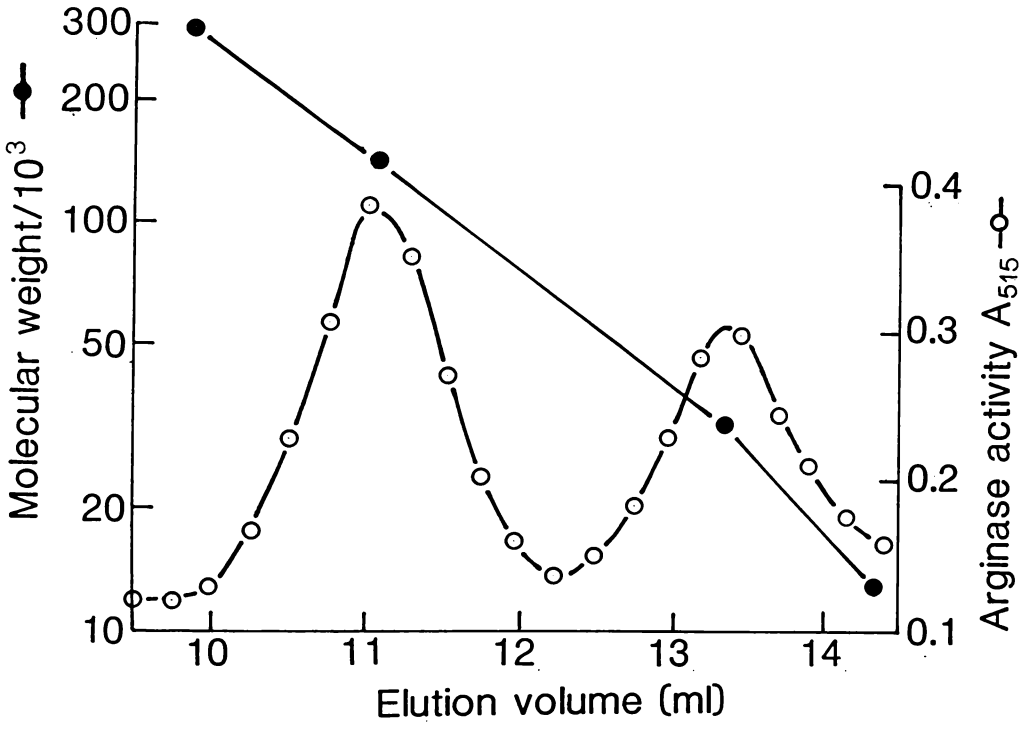

Figure 3-6 GP-FPLC of a *S. acidocaldarius* extract.

S. acidocaldarius cells grown on "inducing" medium and harvested at 46h were washed with 0.1M CHES/NaOH buffer pH9.6, resuspended in 0.6ml of the same buffer and sonicated on ice for 1.5min with a Dynatech Sonic Dismembranator (Series 300, Artek) using a 4mm micro-tip at a relative output of 0.4. The lysate was centrifuged in a TLA-100.3 fixed angle rotor (Beckman) for 20min at 100000rpm (max. 540000*g*) at 20°C and 100μl loaded onto a Superose 12 HR 10/30 FPLC column equilibrated at 0.7ml/min with 0.1M CAPS/NaOH buffer pH9.9 containing 50mM K_2SO_4. 0.25ml fractions were collected and 200μl of each fraction was assayed for 20min at pH9 at 60°C with 20mM arginine in the presence of 30μM Mn^{2+}. The column was calibrated with glutamate dehydrogenase, lactate dehydrogenase, adenylate kinase and cytochrome C (see Table 5-2-2).

of the cytoplasmic pH with the extracellular pH of 3-4 during cooling and harvesting of cells. Whether this occurs is uncertain as some acidophilic bacteria can maintain a near-neutral intracellular pH under non-metabolising conditions (e.g. *Thiobacillus acidophilus*, Matin (1984)). This is the first report of an archaebacterial arginase.

Of the four archaebacterial anaerobes studied, the rods Well44 S.2 (TRUCC 111) and *Thermoproteus tenax* (DSM 2078*) did not produce detectable levels of arginase, arginine deiminase or arginine decarboxylase activity. This was also the case for the extremely thermophilic anaerobic eubacterium FjSS3 B.1 (TRUCC 299). The two archaebacteria above did not grow well on "standard" or "inducing" media. AN1 produced both deiminase and decarboxylase activity but neither were induced by "inducing" media. However, Tok12 S.1, an anaerobic, extremely thermophilic, sulphur-metabolizing archaebacterium exhibiting characteristics typical of *Desulfurococcus* spp. (Patel *et al.*, 1986) elaborated an arginine deiminase that was induced 2-fold and an arginine decarboxylase activity. Extensive nutritional studies (Dr B. A. Huser, unpublished results) had suggested that Tok12 S.1 used small peptides or amino acids as a source of carbon and energy and this was confirmed by the disappearance of Ile, Leu, Phe, Met and Arg during growth on a trypticase peptone fraction containing only free amino acids (B.A. Huser and K. A. Hofman, unpublished results). As only the first two enzymes of the arginine deiminase pathway were assayed in this organism an energy generating role for this pathway in Tok12 S.1 remains to be proven. The carbamyl phosphate intermediate required for ATP synthesis is very labile (although it could be stabilized as an enzyme-bound intermediate) and this pathway may serve mainly as a means of utilizing arginine as a carbon and nitrogen source. The high level of ornithine aminotransferase activity in Tok12 S.1 tends to support

this proposal. When ornithine was omitted from the assay solution for ornithine aminotransferase no glutamate was formed, indicating that endogenous substrates were not present at the concentrations required for activity. This is the only report of ornithine aminotransferase activity from an archaebacterium.

The catabolic ornithine carbamyltransferase activity could be due to an ornithine carbamyltransferase capable of catalysing the reaction in both directions as high levels of (anabolic) ornithine carbamyltransferase have been detected in methanogenic bacteria (Meile and Leisinger, 1984). However, if Tok12 S.1 elaborates distinct catabolic and anabolic enzymes as is the case for mesophiles then the arginine present in the "inducing" medium should repress anabolic activity.

Tok12 S.1 arginine deiminase was apparently still active with D-arginine as a substrate while arginine decarboxylase activity was abolished. The former enzyme was stable to freezing and storage at 4°C in cell-free extracts but arginine decarboxylase was very unstable, losing ~60% activity after 24h at 4°C. Both enzymes were more active at pH6.2 than at pH7 and deiminase activity was stimulated ~50% by 1mM Mg^{2+}. This is the first report of the arginine deiminase pathway in an extreme thermophile, although the RP-LC assays also detected slight arginine deiminase activity (~4 nmol citrulline/min/mg at 60°C) in the extremely thermophilic eubacterium *Clostridium thermohydrosulfuricum* (ATCC 33223). No arginine decarboxylase activity was detected, whereas Paulin and Pösö (1983) found low levels of this activity. Differences in the growth media may be responsible for this discrepancy.

3-3-3 Utilization of arginine by *B. caldovelox*

B. caldovelox was one of the few bacteria studied that grew well on "inducing" medium, attaining A_{650} values similar to those found for the "standard" medium. This suggested that *B. caldovelox* was capable of efficient utilization of arginine as a carbon and nitrogen source and the extent of arginine utilization was examined by analysing culture supernatants for intermediates of arginine catabolism. The results (Fig. 3-7) show that all arginine is metabolized during growth, the stoichiometric formation of urea being consistent with the absence of urease activity in this bacterium and *Bacillus* spp. generally. The build-up of ornithine while arginine is still available and its removal once arginine is exhausted suggests that the ornithine aminotransferase reaction is the rate-limiting step for arginine utilization and that the increasing rate of arginine breakdown during growth is not matched by an increase in aminotransferase activity. This is reflected by the relative specific activities measured for this enzyme and arginase (Tables 3-2 and 3-3). Although growth ceases when all the arginine has been degraded, the high ornithine concentration and its continued catabolism to glutamate in stationary phase indicates that growth has not stopped because of carbon or nitrogen limitation. The quantitative formation of urea during growth on arginine indicates that breakdown of urea is not rapid under these growth conditions and the 1-2mM of toxic cyanate that may be formed would be unlikely to inhibit growth (Taussig, 1960). Similarly the increase in medium pH is insufficient to inhibit growth as the pH range for optimal growth is ~pH6.3-8.5 (Heinen and Heinen, 1972).

The pattern of intermediates seen here provides support for arginase and ornithine aminotransferase as the first two enzymes of arginine catabolism in *B. caldovelox*. The same system probably operates

in *Thermus* spp. but the pathway is less active in these organisms under the growth conditions used.

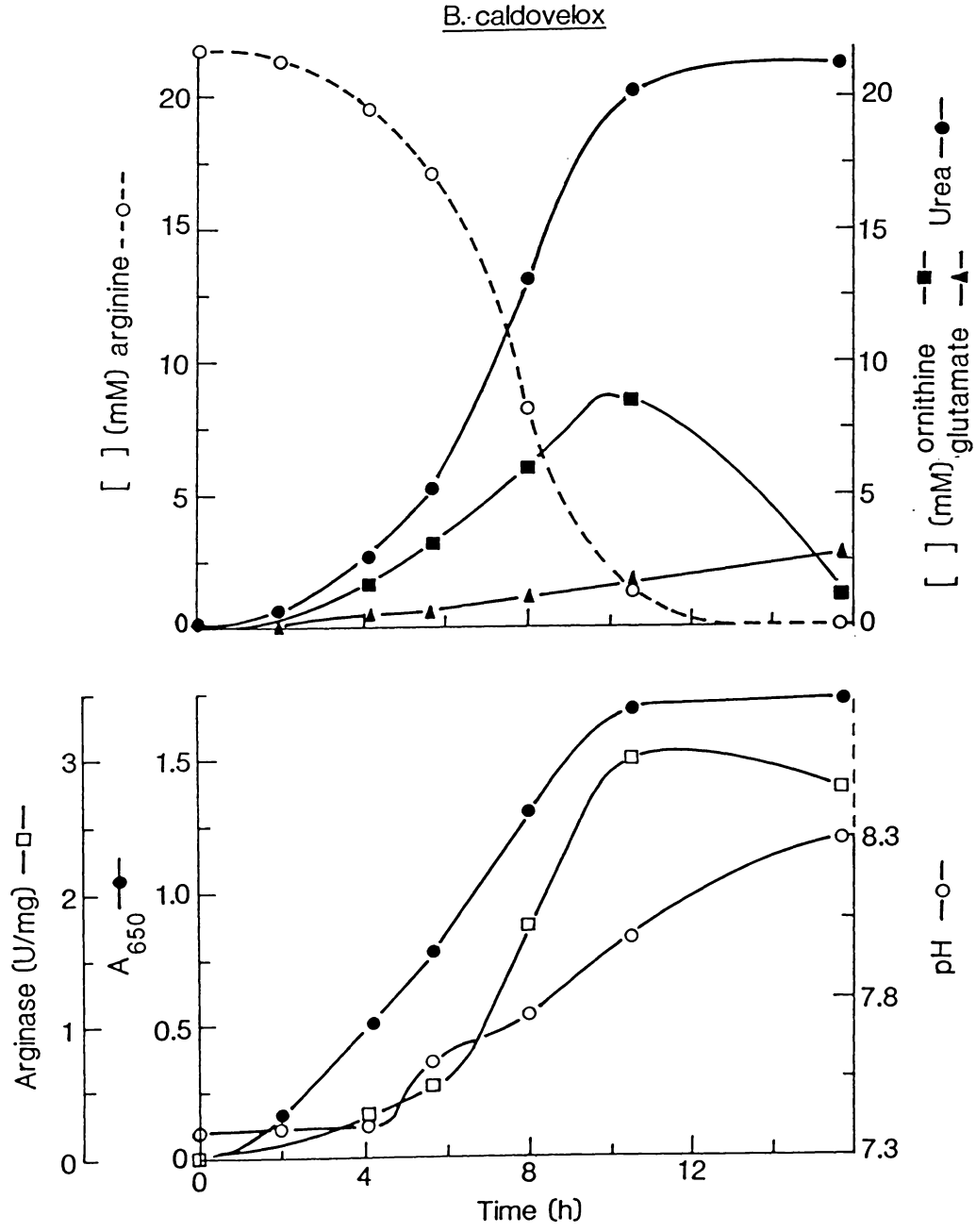

Figure 3-7 Growth of *B. caldovelox* on "inducing" medium.

Arginase activity was assayed by the RP-LC method. [Arginine], [ornithine] and [glutamate] in the culture supernatant was also determined by RP-LC and the [urea] was measured colorimetrically.

CHAPTER 4

PRODUCTION AND PURIFICATION OF ARGINASE

4-1 Introduction

Arginase has been purified from three bacteria to date, two of these being mesophilic *Bacillus* spp. (Simon and Stalon, 1976; Soru, 1983), and there are no reports of the purification of arginase from a thermophilic bacterium. After selecting *B. caldovelox* as a source of arginase attempts were made to optimize bacterial growth and the specific activity of arginase in this organism prior to large-scale cultivation. *B. caldovelox* is one of three strains of extremely thermophilic bacilli isolated from thermal springs in Yellowstone National Park by Heinen and Heinen (1972). Although the temperature range for its optimum growth is broad and somewhat lower than the other two *Bacillus* strains, *B. caldolyticus* and *B. caldotenax*, *B. caldovelox* does grow optimally at 70°C and therefore may be catagorized as an extreme thermophile. A phenotypic and genotypic characterization of these and other thermophilic bacilli has been published (Sharp *et al.*, 1980).

Initial purification schemes for *B. caldovelox* arginase employed a heat-treatment step in the presence of 50mM $MnCl_2$ which may have modified the enzyme in numerous ways, the alteration of metal ion composition being an obvious possibility. It is known that the properties of arginase can vary depending on the metal ion cofactor (e.g. Middelhoven, 1969). As there is no conclusive evidence that Mn^{2+} is the only *in vivo* cofactor in other arginases an attempt was made to

isolate the arginases from *B. caldovelox* and *Thermus* 4-1A using a modified purification scheme designed to maximize the retention of native metal ion cofactors. It was hoped that this would provide near-native arginase preparations with properties similar to those of the enzyme *in vivo* and that chemical analysis would reveal the *in vivo* cofactor(s).

4-2 Selection of a Thermophilic Source of Arginase

On the basis of the results of screening (Chapter 3) five thermophilic bacteria were chosen for further testing as sources of arginase. Rudimentary thermostability and kinetic studies were performed with cell-free extracts, and yield (U/ml) and specific activity experiments repeated using the colorimetric assay for ornithine. The results, presented in Table 4-1, confirmed the RP-LC screening result that the better thermophilic *Bacillus* spp. produced significantly higher levels of arginase activity compared to the *Thermus* strains. For thermostability comparisons, all enzyme solutions were preincubated at 95°C at pH7 with 2mM Mn^{2+} for 10min before taking a sample for zero time activity. Without this preincubation the loss of activity over 8min due to denaturation was offset by activation for some arginases. This effect was strongest for *Thermus* T-351 arginase which doubled its activity over the first 8min at 95°C. Degryse *et al.* (1976) reported that arginase in crude extracts of *T. aquaticus* was activated by Mn^{2+}. Plots of Michaelis-Menten data using linear transformations of the Michaelis-Menten equation often deviated from linearity so the K_m's listed are only approximate values. The value for crude *B. caldovelox* arginase was the same as that obtained in experiments with the purified enzyme at pH7 (see Section 5-3-3).

B. caldovelox arginase was selected primarily for its high specific activity and because it was apparently more thermostable than other *Bacillus* arginases. Discrepancies between the specific activities of arginase seen in this section of work and the RP-LC screening studies are due to the different growth conditions, assay conditions and harvesting times.

Table 4-1 Properties of arginase from five extreme thermophiles

Property	*Thermus* T-351	*Thermus* 4-1A	*Bacillus caldotenax*	*Bacillus caldovelox*	*Bacillus* Ok3 A.1
Harvest time (h)	13.5	11	13	11	11
A_{650}	1.06	1.24	0.96	1.2	1.04
Units/ml of culture	0.58	0.54	2.4	3.3	1.2
Units/mg of protein	3	2.5	19	22	11
K_m (mM)	12	9	30	25	20
Thermostability	53	61	14	24	15

35ml cultures were grown at 70°C in 250ml flasks at 120rpm on the "inducing" medium described in Section 2-2-1 supplemented with 1g/l TP and 1g/l YE (cf. 0.5g/l YE). Cell-free extracts were prepared by sonication of cells in 25mM MOPS/NaOH buffer pH7.5 (20°C) containing 80mM KCl and 2mM $MnCl_2$, followed by centrifugation. The extracts were diluted in sonication buffer, heated for 30min at 70°C and the assays performed at pH7 at 70°C with 20mM arginine in the presence of 0.1mM Mn^{2+}. Specific activities were calculated as units/mg of cell-free extract protein. K_m values were determined for cell-free extract arginase at pH7 using arginine concentrations between 2mM and 40mM. The thermostability figure is the percentage of t=0min arginase activity (measured after a 10min preincubation) remaining after an 8min incubation at 95°C at pH7 with 2mM Mn^{2+}. Protein concentrations during heating were ~0.2mg/ml for *Thermus* extracts and ~0.02mg/ml for *Bacillus* extracts.

4-3 Attempts to Optimize the Yield of *B. caldovelox* Arginase

Arginase activity in early stationary phase cultures of *B. caldovelox* was increased ~3-fold when 20mM L-arginine was added to a rich medium, i.e. basal salts + 3g/l yeast extract (YE) and 3g/l trypticase peptone (TP). A similar induction has been demonstrated for *T. aquaticus* arginase (Degryse *et al.*, 1976). *B. caldovelox* grew slowly on the basal medium described in Section 2-2-2 supplemented with 20mM arginine and 30mg/l YE and although a final A_{650} of ~2 was obtained the slow growth resulted in low arginase specific activity. The high cell density attained in this medium indicated that *B. caldovelox* utilizes arginine as a carbon and nitrogen source. Thorough aeration was necessary to achieve maximum A_{650} values as stationary phase optical densities in cultures shaken at 120rpm were ~60% of those seen at 200rpm.

The effects of arginine, YE and TP concentration on bacterial growth and enzyme levels are summarized in Fig. 4-1 and Table 4-2. Provided at least 20mM L-arginine and ~1g/l YE (required for rapid growth) was present in the medium and the culture was well aerated further additions of catabolites in the form of TP or YE did not significantly alter arginase specific activities in stationary phase. However, these additives did decrease the utilization of ornithine which built up to high concentrations in some cultures, e.g. 24mM after 10h growth in medium number 4 (Table 4-2).

As it was planned to use the optimized medium for a 400-600l fermenter batch culture practical considerations included the ease with which medium constituents would pass through the sterilising filters and the cost of the inducer used. L-arginine was the least expensive of the potential arginase inducers. The medium used contained the lowest

concentration of YE giving rapid growth (1g/l) and 25mM arginine. The final A_{650} in the fermenter batch culture was not as high as for small volume cultures (Fig. 4-2). This may have been due to impurities in the tap-water used to constitute to medium, different stirring and agitation methods, localized heating and the higher average hydrostatic pressure on the bacteria. The level of arginase activity approached that seen in small-scale cultures but decreased rapidly during harvesting. Small-volume cultures did not show this decrease but were well aerated in stationary phase whereas aeration was stopped when the

Table 4-2 Growth of *B. caldovelox* on different media

Culture conditions*	pH	mM arginine metabolized		mM ornithine metabolized		Arginase (U/ml)	(U/mg)
	10h	8h	10h	8h	10h	10h	
30mM arginine							
1. + 0.5g/l YE	7.5	ND		ND		5.8	18.5
2. + 1g/l YE	7.7	16	23	6.8	13	5.4	16.4
3. + 2g/l YE	7.9	ND		ND		7.5	18.4
4. + 1g/l YE, 3g/l TP	8.2	17	28	3.4	3.6	6.7	19.4
1g/l yeast extract							
5. + 20mM arginine	8.1	18	20	15	20	6.1	17.6
6. + 40mM arginine	7.8	22	33	11	12	7.9	17.3

*Basal medium is that described in Section 2-2-2, except that the sodium phosphate concentration was increased to 10mM in an attempt to control the shift in pH. Growth was at 70°C and was initiated by a 1% inoculum of late logarithmic culture grown in medium number 2. Cultures were shaken at 200rpm.

The pH (20°C) after autoclaving was ~pH7.0. Preparation of cell-free extracts and assays were performed as described in Table 4-1. Activity was not significantly altered when medium number 2 was supplemented with 1g/l TP or 0.135mM Mn^{2+}.

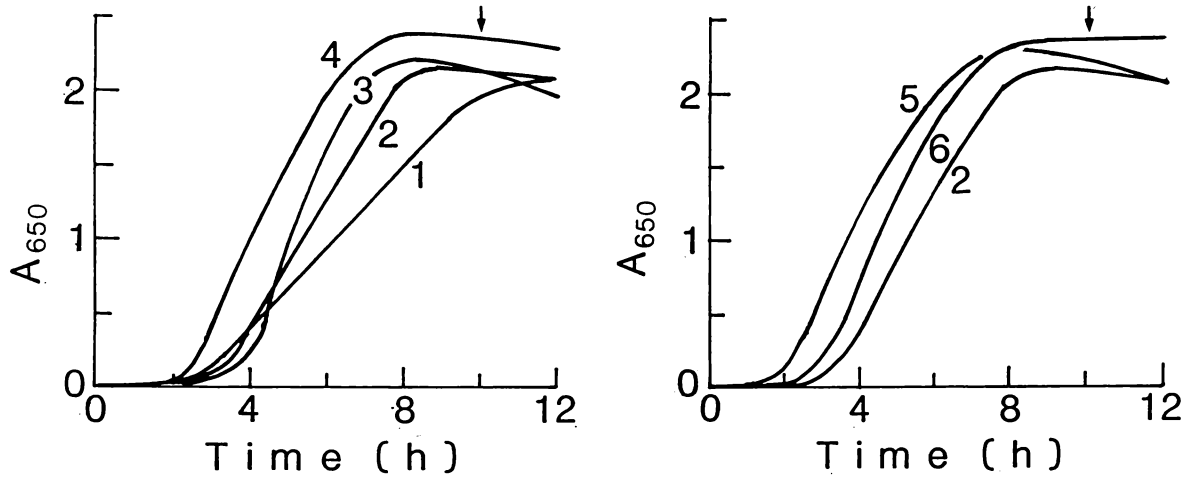

Figure 4-1 Growth curves for *B. caldovelox* on "inducing" media.

The numbering of the growth curves corresponds with that of the six media described in Table 4-2. Growth medium 2 is shown on both sets of curves for ease of comparison. The arrows indicate the time at which samples were taken for determination of arginase activity.

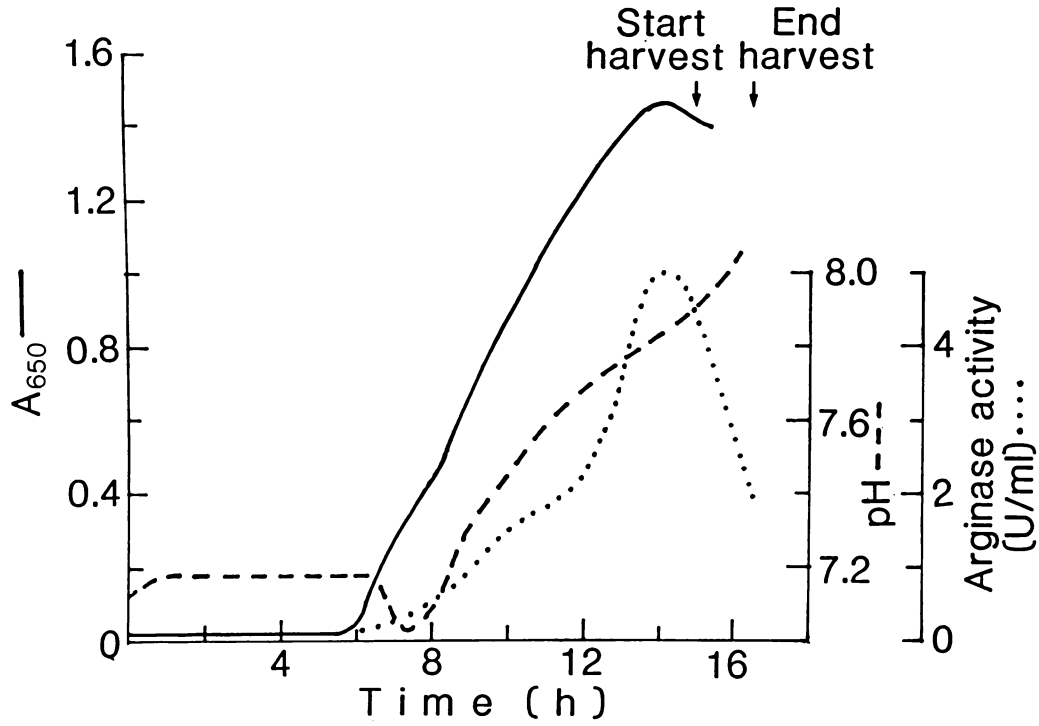

Figure 4-2 Growth of *B. caldovelox* in 450 litres.

Cell-free extracts were prepared and assayed as described in Table 4-1 except that the extracts were not preincubated at 70°C before the assay.

fermenter harvest was started. The resulting anaerobic conditions during harvesting might have caused the decrease in activity by hastening the shift to a stationary phase or sporulating metabolism with concomitant induction of proteases. Cell-free extracts of samples taken during the fermenter batch culture had protease activity (measured as clearing around wells in casein-agar plates incubated at 65°C) which began to appear at 12h, peaked at 14-15h and then declined. Thus it is conceivable that *B. caldovelox* arginase was subject to proteolytic attack *in vivo* during harvesting. It has been suggested that proteases produced in stationary phase are responsible for the instability of *Neurospora crassa* (Borkovich and Weiss, 1987a) and *Saccharomyces cerevisiae* (Penninckx *et al.*, 1974) arginases during heat treatment. These workers found it necessary to use logarithmic phase cells as a source of arginase.

The sensitivity of the *B. caldovelox* protease (15h extract) to three protease inhibitors was examined with a view to minimizing this activity during cell lysis. Addition of 5mM neutralized *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*',*N*'-tetraacetic acid (CDTA, D 1383, Sigma) caused complete inhibition but protease activity in cell-free extracts was not inhibited by treatment with 1mM phenylmethanesulphonyl fluoride (PMSF, P 7626, Sigma) or 0.15mM pepstatin A. CDTA was not added to the lysis buffer because of adverse effects this metal chelator might have had on arginase activity.

4-4 Purification of Arginase

Arginase from 1.5kg of *B. caldovelox* cells was purified 65-fold with a yield of 6% by purification scheme I (summarized in Table 4-3) and typical elution profiles for the last three steps of this sequence

are shown in Figures 4-3, 4-4 and 4-5. Cells from the fermenter batch culture were lysed chemically. A low initial pH of 5.5 resulted in more complete and reproducible lysis than was found at higher pH values and the final pH of the lysate supernatant was ~6.5. The inclusion of Triton X-100 in the lysis buffer decreased the volume of the pelleted cell debris and increased the total activity released but resulted in a slightly lower supernatant specific activity. Sonication of the pelleted cell debris in 50mM MOPS/NaOH buffer pH7 released ~15% more arginase activity but the specific activity was about half that seen for the initial supernatant and the two were not combined. Activity was lost in all purification steps except for heat treatment with Mn^{2+}. The recovery from heat treatment steps of arginase purification schemes is seldom less than 80% and if performed in the presence of Mn^{2+} apparent recoveries can exceed 100% (e.g. Sakai and Murachi, 1969; Tarrab *et al.*, 1974; Berüter *et al.*, 1978). Addition of 5mM 2-mercaptoethanol to the lysis buffer and dithiothreitol at other stages of the purification (e.g. at a concentration of 1mM in the GP-HPLC (TSK) running buffer) decreased the recovery of *B. caldovelox* arginase activity and these reagents were therefore omitted from all purification buffers. This loss of activity may be associated with the metal-chelating properties of thiol reagents (Miyano *et al.*, 1985). Other workers found that 2-mercaptoethanol minimized the appearance of multiple forms of arginase during purification, possibly by preventing aggregation of the enzyme (Sakai and Murachi, 1969; Vielle-Breitburd and Orth, 1972).

Some arginases are particularly susceptible to inactivation during purification unless the buffers used contain Mn^{2+} (e.g. Wright *et al.*, 1981). However, the inclusion of 1mM Mn^{2+} in purification buffers did not improve the recovery of *B. caldovelox* arginase activity and Mn^{2+} was therefore omitted. Vielle-Breitburd and Orth (1972) suggested that

Table 4-3 Purification of *B. caldovelox* arginase: Scheme I

Purification step	volume (ml)	protein concentration (mg/ml)	activity (kU/ml)	specific activity (kU/mg)	relative purification step	relative purification total	% recovery[a] step	% recovery[a] total
Cell-free extract[b]	6850	12.5	0.88	0.070	-	1.0	-	100
Dialysed $(NH_4)_2SO_4$ pellet	520	26.3	7.54	0.287	4.1	4.1	65	65
MnCl2 heat treatment	495	14.2	8.24	0.58	2.0	8.3	104	68
Dialysis, freeze-drying	1200	3.1	1.70	0.548	0.95	7.8	69.4	47
DEAE Sepharose CL-6B	18.5	24	52.8	2.20	4.0	31.4	48	22.6
Gel permeation HPLC (TSK)	25	5.2	24.6	4.73	2.15	67.6	63	14.2
Lysine-Sepharose CL-6B	3.7	15	67.2	4.48	0.95	64	50.6	7.2
IE-FPLC (Mono Q)	6	7.5	34.2	4.56	1.02	65	83	6.0

[a]28% of the dialysed and freeze-dried material and 21% of the post-Lysine-Sepharose arginase were used for other experiments or for testing possible purification methods. The % recoveries of these steps were corrected for the losses and the recoveries of other steps modified accordingly.
[b]Prepared from 1.5kg (wet weight) of cells from the fermenter batch culture described in Section 2-2-2.

Assays were carried out in 0.1M CHES/NaOH buffer pH8.5 at 70°C, containing 20mM arginine and 5μM Mn^{2+}.

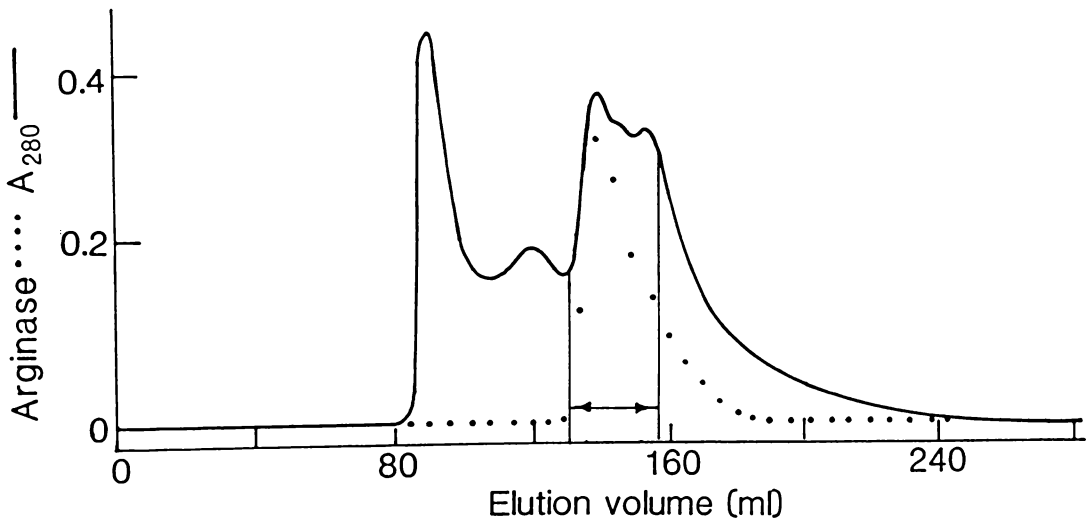

Figure 4-3 GP-HPLC of *B. caldovelox* arginase (purification scheme I).

Experimental details are described in Section 2-4-4. The first substance to elute was yellow in colour. The activity of each fraction is plotted in arbitrary units. The fractions between the vertical lines were taken into the Lysine-Sepharose step.

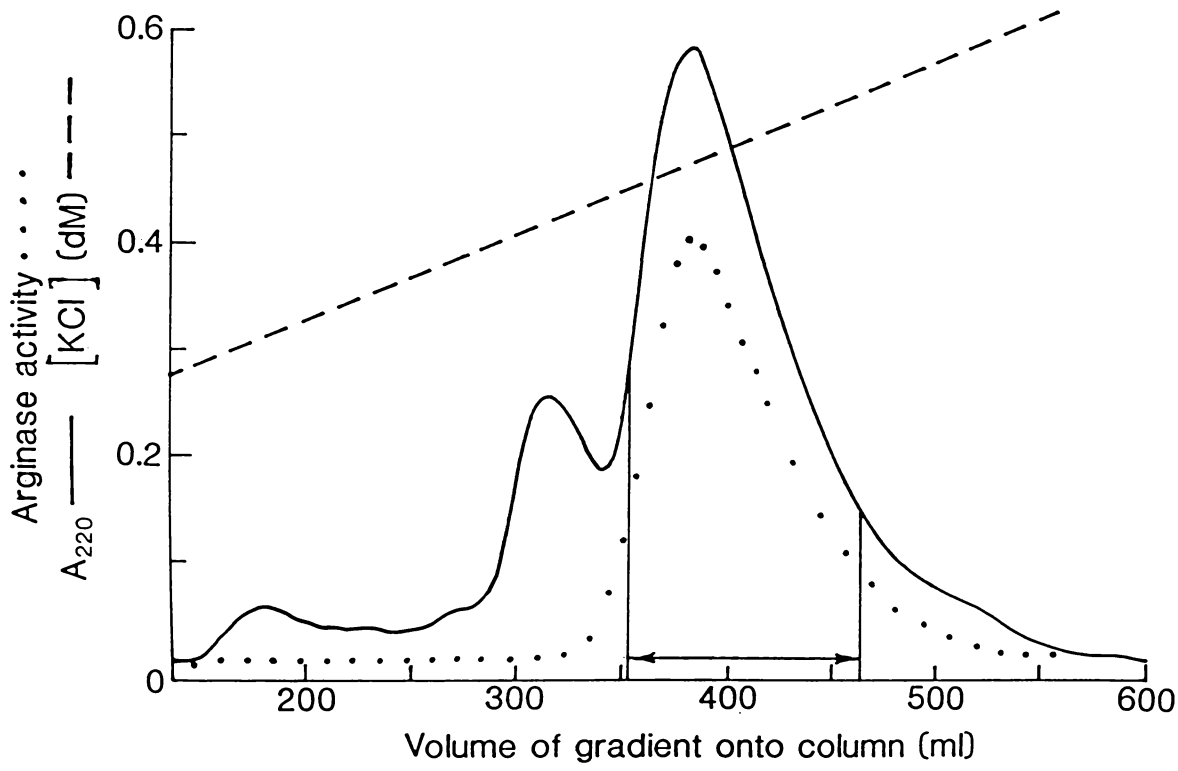

Figure 4-4 Lysine-Sepharose chromatography of *B. caldovelox* arginase (purification scheme I).

Experimental details as described in Section 2-4-5. Arginase activity is plotted in arbitrary units. The fractions bounded by the vertical lines were taken into the IE-FPLC step.

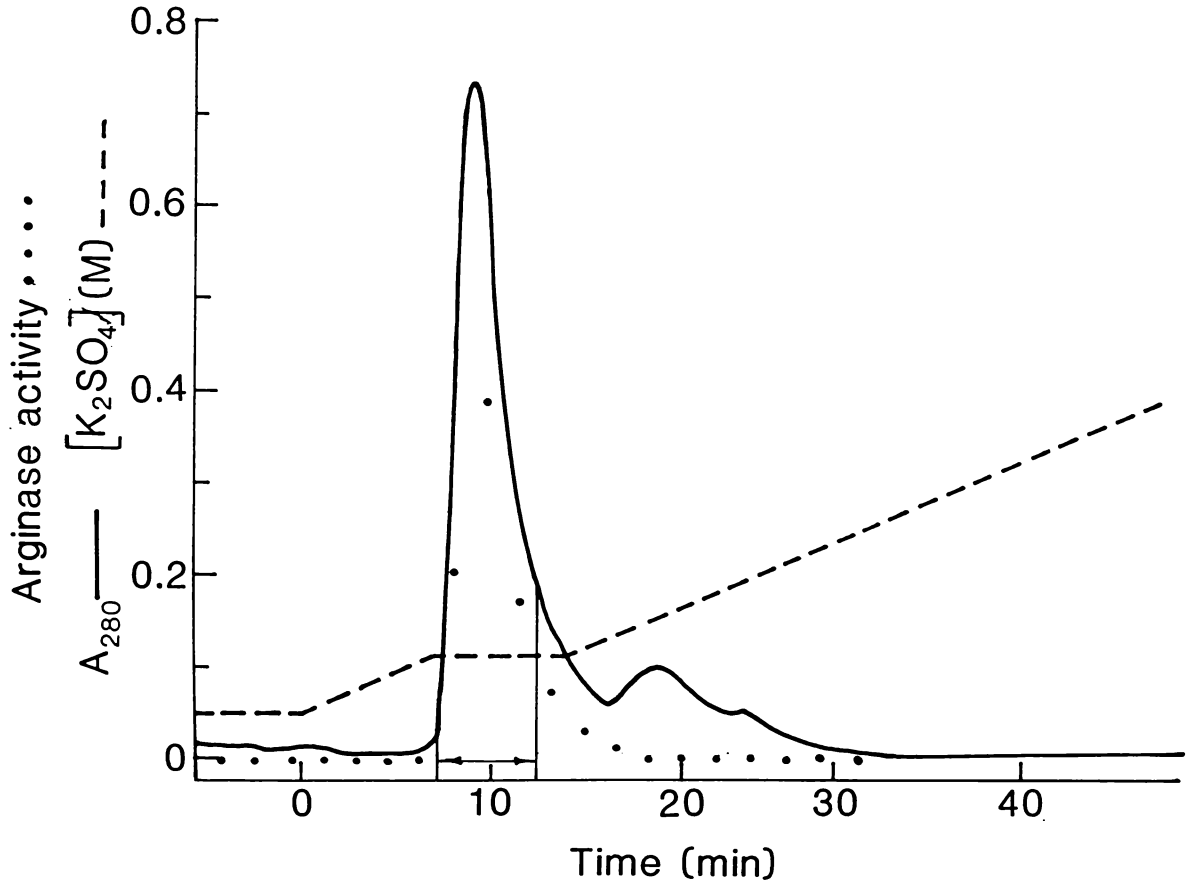

Figure 4-5 IE-FPLC of *B. caldovelox* arginase (purification scheme I).

Experimental details as described in Section 2-4-6. The most active fractions (bounded by the vertical lines) were concentrated and dialysed.

loss of metal ions from arginase holoenzymes might change the properties of the enzyme sufficiently for the species to be separable by ion exchange chromatography. With the exception of the lysine-Sepharose step all chromatographic separations of *B. caldovelox* arginase produced a single active peak. Approximately 7% of the activity loaded onto the lysine-Sepharose column was not eluted by the salt gradient but was removed by a buffer containing 1M KCl. When the tightly bound arginase fraction was desalted and rechromatographed on the column ~90% of the activity was eluted by the normal 20-80mM KCl salt gradient and 10% bound more strongly. It is possible that in each chromatographic run a small proportion of arginase molecules bind lysine as an affinity ligand whereas for most of the enzyme lysine acts as a weak anion exchanger.

4-4-1 Purification of arginases for native cofactor analysis

It was hoped that under appropriate conditions it would be possible to purify *B. caldovelox* and *Thermus* 4-1A arginases without loss or substitution of the *in vivo* metal ion cofactor(s). Where possible the purification was conducted above pH9 to maximize the stability of the metal ion-arginase complex and the use of metal chelating/precipitating buffers or reagents was avoided. The relatively successful $(NH_4)_2SO_4$ precipitation and DEAE-Sepharose steps of scheme I were retained in purification scheme II. Using DEAE-Sepharose at pH9.4 (in scheme II) decreased the binding capacity of the matrix as the DEAE (diethylaminoethyl) groups are only partially ionized at this pH. All activity loaded was, however, bound by this matrix. The FPLC columns became available during the purification of *B. caldovelox* arginase by scheme I. Unlike the silica-based HPLC columns (e.g. the GP-HPLC (TSK) column) the FPLC matrices are stable over a pH range of pH1-14

(Superose columns, crosslinked agarose beads) or pH2-12 (Mono Q column, hydrophilic polymer beads) and could therefore be used for the purification arginase at alkaline pH. When the arginases were chromatographed on a Superose 12 prep grade HR 16/50 column (bed volume ~100ml, void volume ~35ml) they eluted in the void volume of the column but some purification was achieved (Fig. 4-8). A yellow high M_r substance that eluted before *B. caldovelox* arginase on the preparative GP-HPLC (TSK) column used in scheme I (see Fig. 4-3) coeluted with this enzyme on the Superose 12 column and this is consistent with the higher M_r exclusion limit of the HPLC column. This yellow material separated from *B. caldovelox* arginase in the subsequent anion exchange FPLC step of scheme II. Sulphate rather than chloride was used as the counter-ion for the Mono Q IE-FPLC step as the buffer solutions for the FPLC columns were pumped through a metal HPLC solvent delivery system (Waters Assoc.) which may be subject to corrosion if exposed to high concentrations of halide ion for extended periods of time. K_2SO_4 was also suitable for Phenyl-Superose HI-FPLC as the buffering effect of ammonium ions above pH8 precluded the use of $(NH_4)_2SO_4$.

The purification of *B. caldovelox* and *Thermus* 4-1A arginases by scheme II is summarized in Tables 4-4 and 4-5 and elution profiles are shown in Figures 4-6 to 4-11. While the main aim of this purification was to retain the native metal cofactor(s) it was hoped that this might also improve recovery. However, as in purification scheme I, activity was lost in all steps. The arginases were not activated by Mn^{2+} in crude extracts. Assuming that some activity loss during purification is due to dissociation of the active metal ion-arginase complex, it seems permissible to correct the final yields (and purification factors) for the % activation of the purified enzymes by Mn^{2+}. Correcting for the 29% and 150% activations seen for purified *B. caldovelox* and *Thermus*

Table 4-4 Purification of *B. caldovelox* arginase: Scheme II

Purification step	volume (ml)	protein concentration (mg/ml)	activity (kU/ml)	specific activity (kU/mg)	relative purification step	relative purification total	% recovery step	% recovery total
Cell-free extract[a]	82.5	21.6	1.04	0.048	-	1.0	-	100
Protamine sulphate	118	11.1	0.76	0.068	1.42	1.42	104	104
Dialysed $(NH_4)_2SO_4$ pellet	33.6	19.5	2.43	0.125	1.84	2.6	92	95
DEAE Sepharose CL-6B	1.45	35.7	30.5	0.853	6.8	17.8	54	52
GP-FPLC (Superose)	8	2.67	3.9	1.46	1.71	30.4	73	36
IE-FPLC (Mono Q)	4	1.0	5.5	5.45	3.73	114	72	25
HI-FPLC (Phenyl-Superose) 1							57	14
HI-FPLC (Phenyl-Superose) 2	4.2	0.33	1.41	4.27	0.78	89	52	7

[a]Prepared from 21.3g (wet weight) of cells grown as described in Section 2-2-3.

Assays as described in Table 4-3.

Table 4-5 Purification of *Thermus* 4-1A arginase: Scheme II

Purification step	volume (ml)	protein concentration (mg/ml)	activity (kU/ml)	specific activity (kU/mg)	relative purification step	relative purification total	% recovery step	% recovery total
Cell-free extract[a]	116.5	17.2	0.136	0.008	-	1.0	-	100
Protamine sulphate	144	11.1	0.105	0.0095	1.2	1.2	95	95
Dialysed $(NH_4)_2SO_4$ pellet	29.9	21.1	0.237	0.0112	1.0	1.4	78	45
DEAE Sepharose CL-6B	3.5	9.5	0.857	0.0902	8.1	11.4	44	19.4
GP-FPLC (Superose)	8	2.03	0.216	0.107	1.18	13.5	60	11
IE-FPLC (Mono Q)	6.3	0.283	0.173	0.611	5.73	77	65	7
HI-FPLC (Phenyl-Superose) 1							33	2.2
HI-FPLC (Phenyl-Superose) 2	4.8	0.15	0.0563	0.375	0.61	47.5	80	1.7

[a]Prepared from ~50g (very wet weight) of cells grown as described in Section 2-2-3.

Assays as described in Table 4-3.

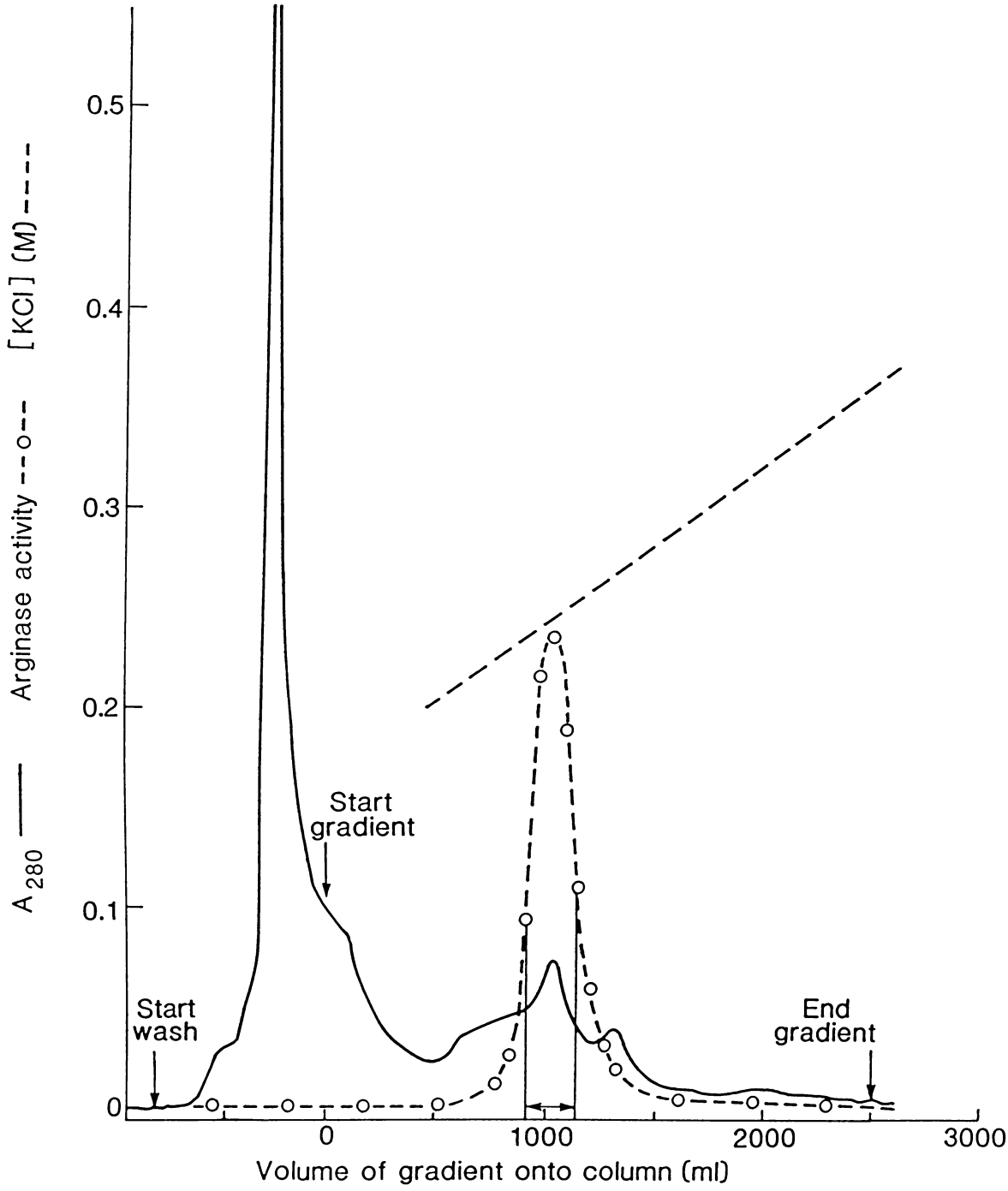

Figure 4-6 DEAE Sepharose chromatography of *B. caldovelox* arginase (purification scheme II).

Experimental details as described in Section 2-5-2. Arginase activity is plotted in arbitrary units and the most active fraction had an activity of 430U/ml when assayed as described in Table 4-3. The [KCl] plotted is that measured at the column outlet. The fractions bounded by the vertical lines were taken into the GP-FPLC step.

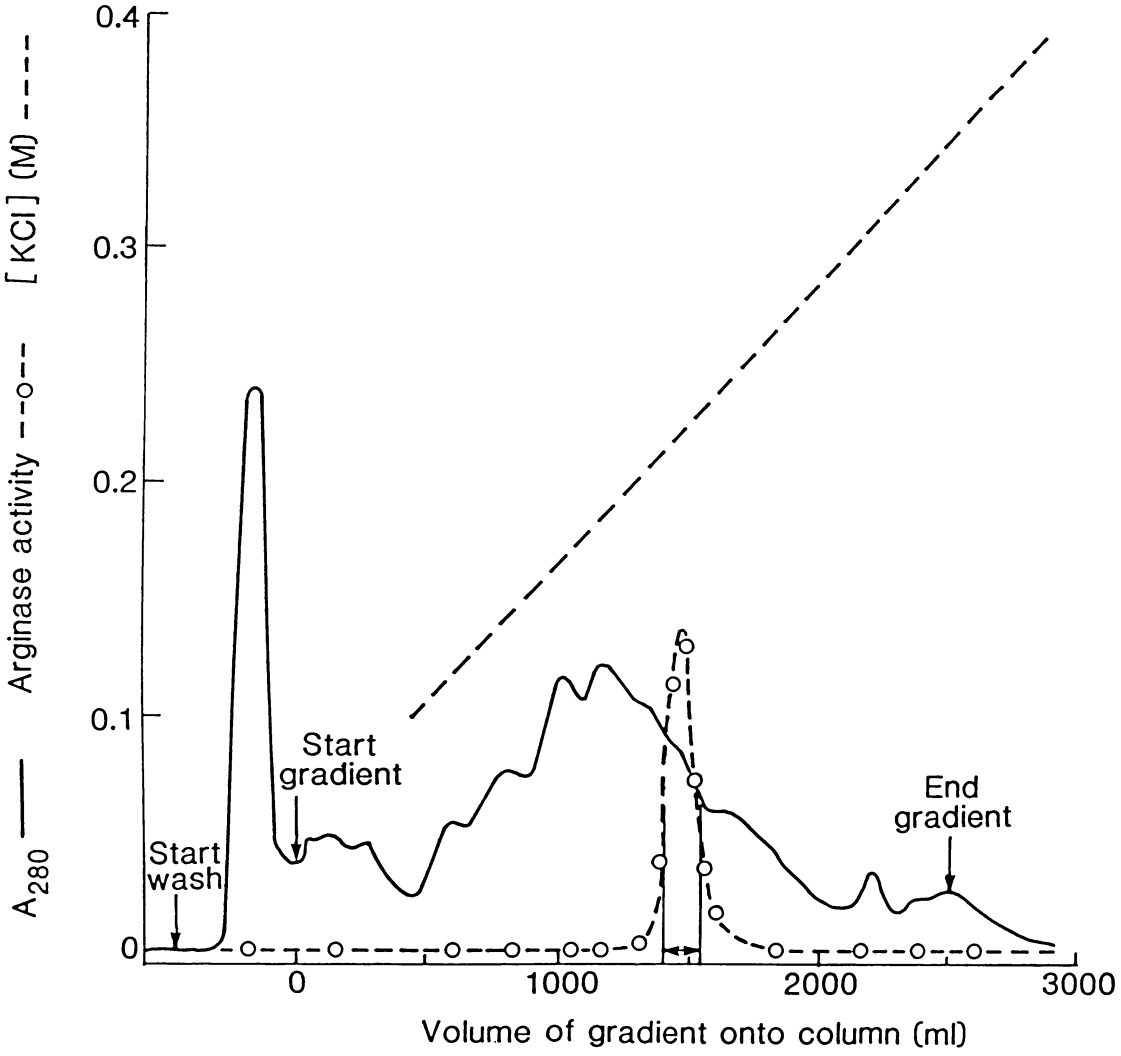

Figure 4-7 DEAE Sepharose chromatography of *Thermus* 4-1A arginase (purification scheme II).

Experimental details as described in Section 2-5-2. Arginase activity is plotted in arbitrary units and the most active fraction had an activity of 54U/ml when assayed as described in Table 4-3. The [KCl] plotted is that measured at the column outlet. The fractions bounded by the vertical lines were taken into the GP-FPLC step.

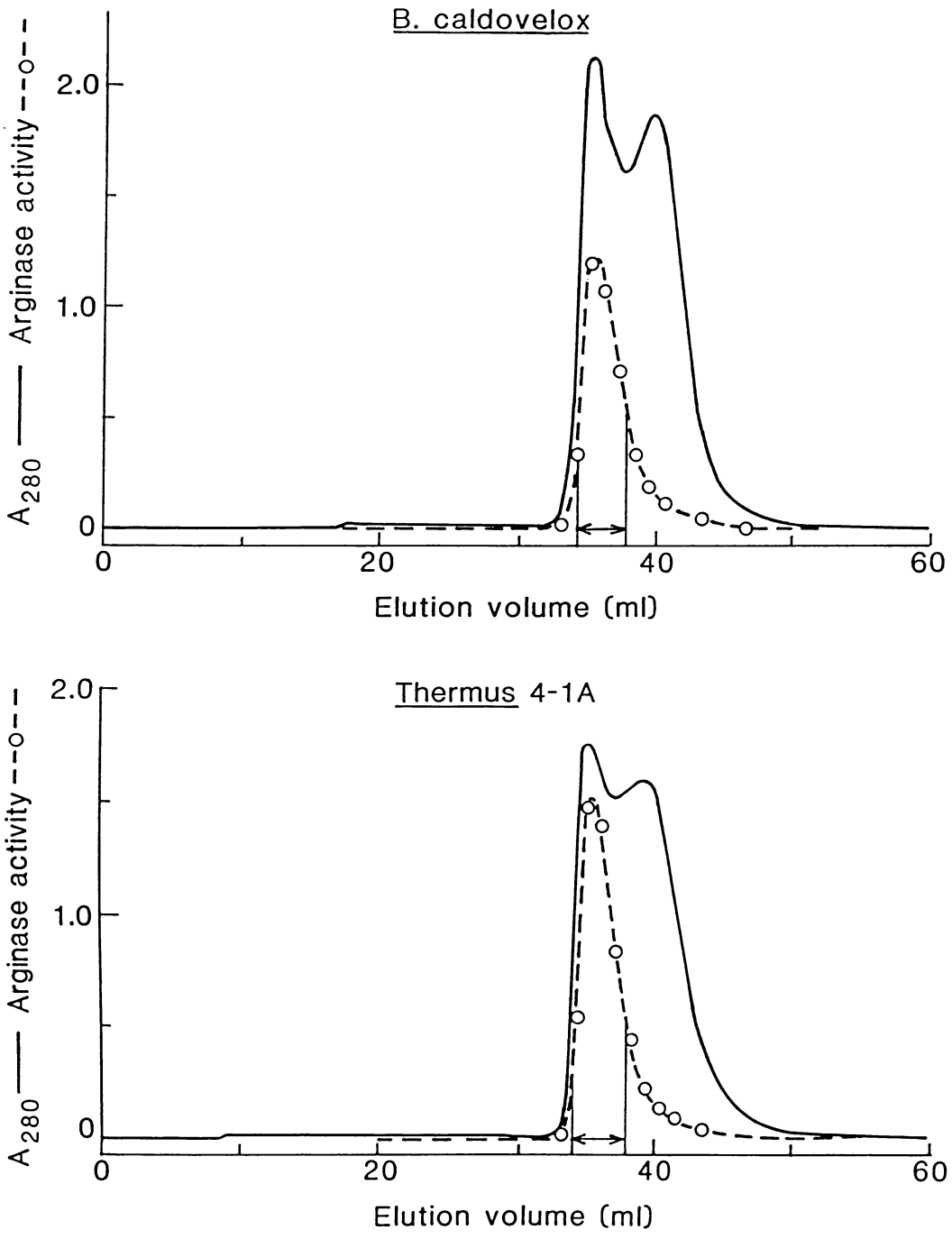

Figure 4-8 GP-FPLC of *B. caldovelox* and *Thermus* 4-1A arginases (purification scheme II).

Experimental details as described in Section 2-5-3. Arginase activity is plotted in arbitrary units and the activities of the most active fractions were 8kU/ml and 0.4kU/ml for *B. caldovelox* and *Thermus* 4-1A arginase respectively when assayed as described in Table 4-3. The fractions bounded by the vertical lines were taken into the IE-FPLC step. On this column *B. caldovelox* arginase coeluted with a yellow substance (compare Fig. 4-3).

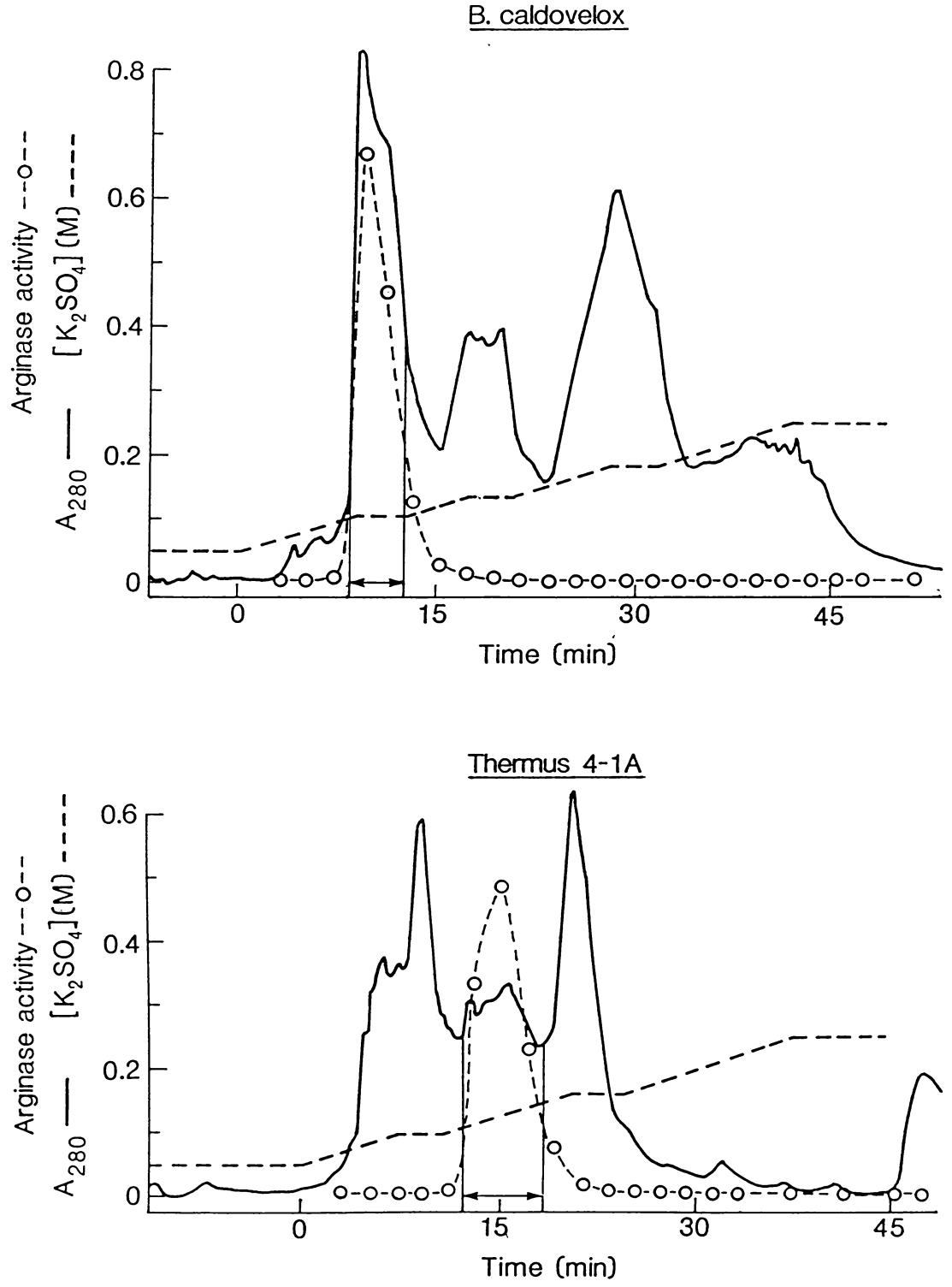

Figure 4-9 IE-FPLC of *B. caldovelox* and *Thermus* 4-1A arginases (purification scheme II).

Experimental details as described in Section 2-5-3. Arginase activity is plotted in arbitrary units and the activities of the most active fractions were 7.5kU/ml and 0.25kU/ml for *B. caldovelox* and *Thermus* 4-1A arginase respectively when assayed as described in Table 4-3. . The most active fractions (bounded by the vertical lines) were taken into the HI-FPLC step.

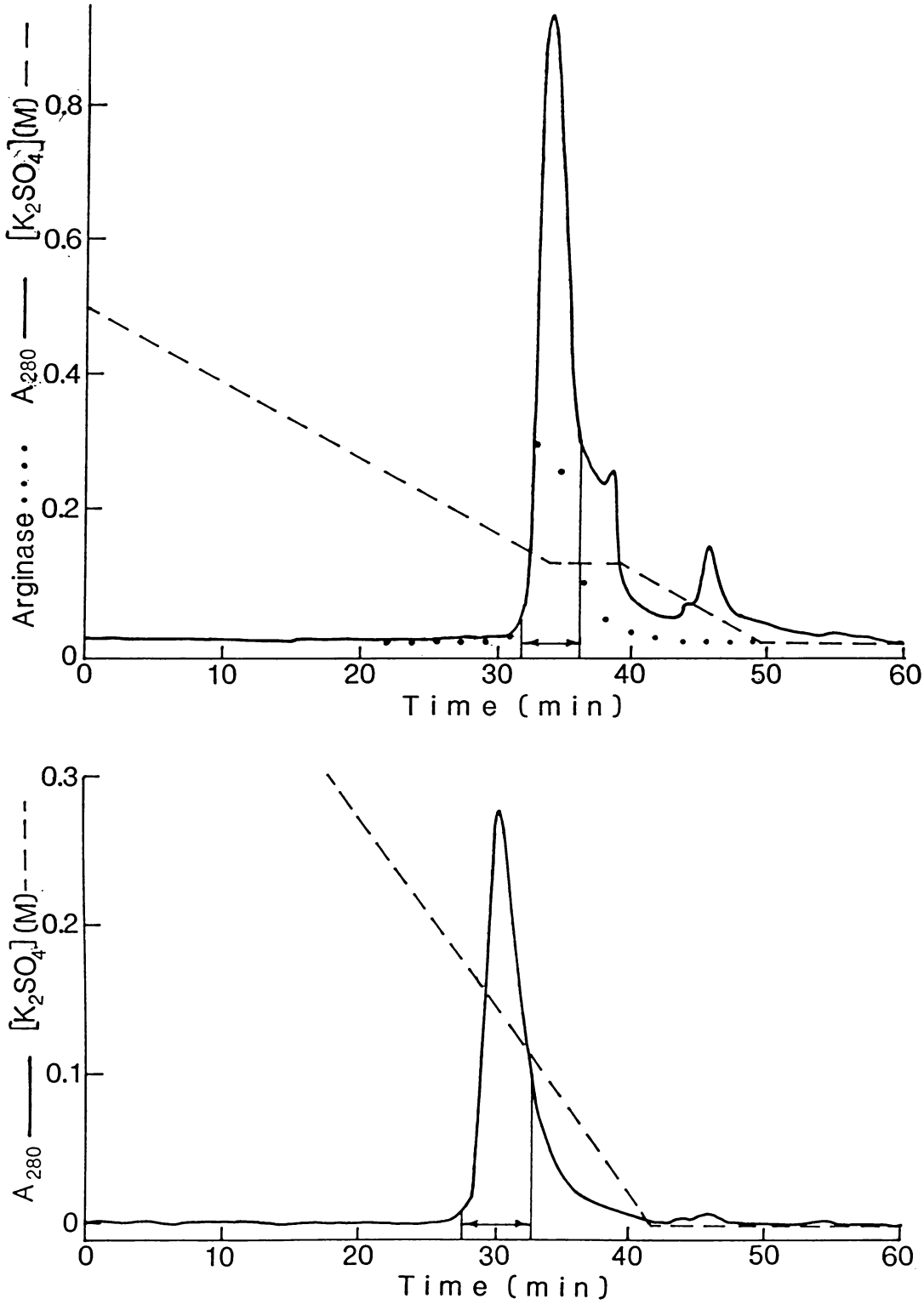

Figure 4-10 HI-FPLC of *B. caldovelox* arginase (purification scheme II).

Experimental details as described in Section 2-5-4. Arginase activity is plotted in arbitrary units. The most active fractions (bounded by the vertical lines) in the top diagram were rechromatographed as shown in the lower diagram and the fraction indicated was dialysed.

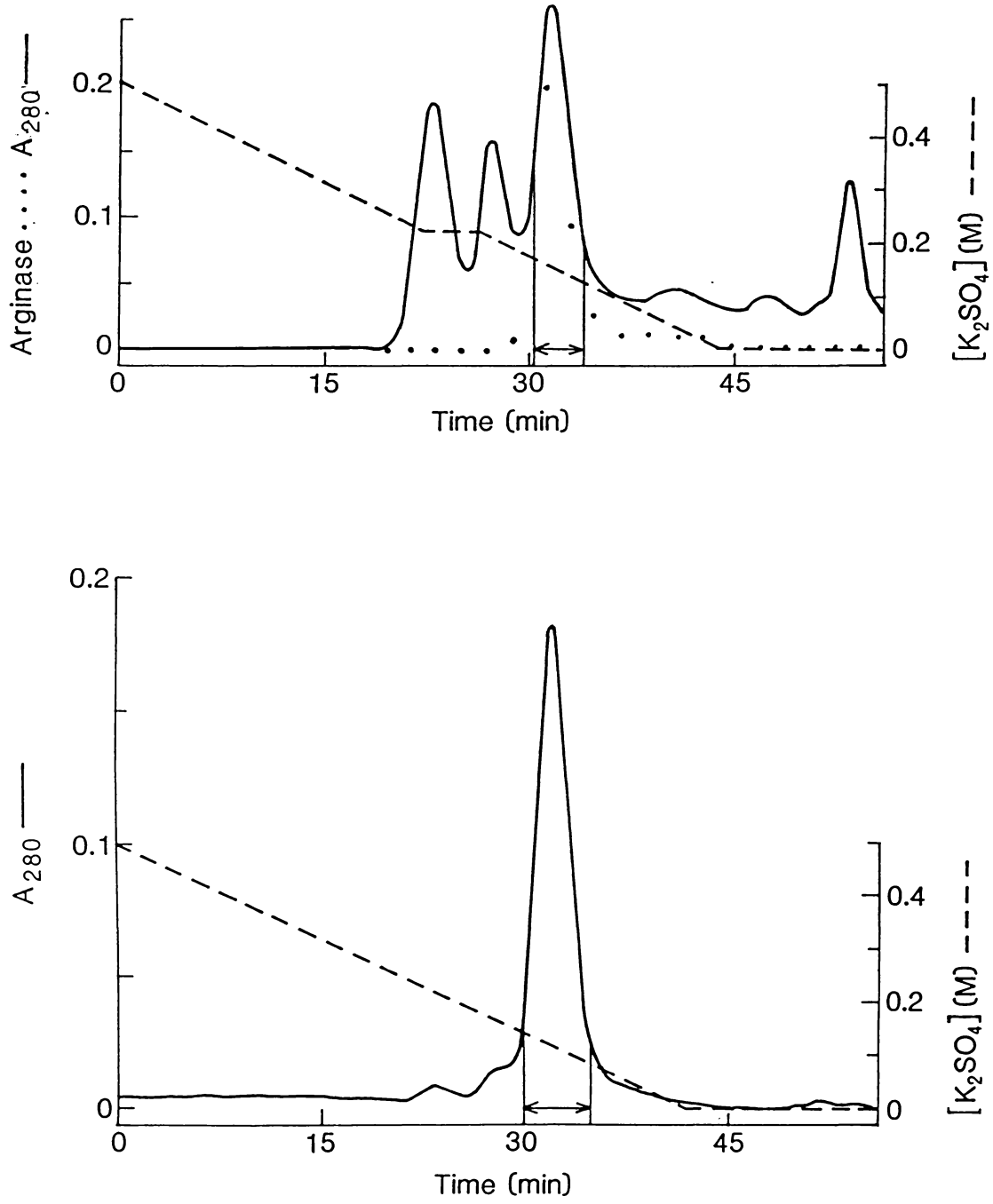

Figure 4-11 HI-FPLC of *Thermus* 4-1A arginase (purification scheme II).

Experimental details as described in Section 2-5-4. The most active fractions (bounded by the vertical lines) in the top diagram were rechromatographed as shown in the lower diagram and the fraction indicated was dialysed.

4-1A arginases after incubating with 0.5mM Mn^{2+} and 1mg/ml bovine serum albumin at pH7 at 75°C for 120min and 45min respectively (see Figures 5-4-1 and 5-4-2), the yields are 9% and 4.3%. Considering that there are 5 chromatographic steps in purification scheme II these low yields are not unexpected as chromatographic steps appear to cause substantial losses in most arginase purifications. Purification protocols involving one or two chromatographic procedures often achieve only 40-50% recovery (Vielle-Breitburd and Orth, 1972; Simon and Stalon, 1976; Soru and Zaharia, 1976; Soru, 1983) and those requiring three or more chromatographic steps for homogeneity typically yield 2-11% (Harell and Sokolovsky, 1972; Penninckx *et al.*, 1974; Fujimoto *et al.*, 1976; Wright *et al.*, 1981; Boutin, 1982; Iino and Shimadate, 1986; Borkovich and Weiss, 1987a).

Using purification scheme II *B. caldovelox* and *Thermus* 4-1A arginases were purified 89-fold and 48-fold respectively. Correcting for the activation seen for the purified enzymes the purification factors increase to 116 and 120 respectively. The purification factors for other bacterial arginases range from 56 (Simon and Stalon, 1976) to 148 (Soru, 1983). The greater fold purification for *B. caldovelox* arginase in scheme II compared with scheme I is largely due to the lower initial specific activity of the extract as the final preparations are of similar purity.

4-5 Assessment of Arginase Purity

The purified arginases were subjected to non-denaturing discontinuous PAGE (Fig. 4-12). *B. caldovelox* "native metals" arginase (3μg) in lanes 1 and 5 showed a single broad band when silver stained, while for the *Thermus* 4-1A "native metals" arginase in lane 4 two minor

bands were evident. *B. caldovelox* arginase purified by scheme I (lane 10) showed the same electrophoretic mobility as that prepared by scheme II and the final IE-FPLC step eliminated the diffuse band close to the migration front in lane 10. Many purified arginases yield a single band when subjected to non-denaturing PAGE (Harell and Sokolovsky, 1972; Vielle-Breitburd and Orth, 1972; Penninckx *et al.*, 1974; Berüter *et al.*, 1978; Iino and Shimadate, 1986) and microheterogeneity arising from the removal of Mn^{2+} either prior to or during electrophoresis may be responsible for the broad or diffuse nature of the band (Campbell, 1966; Rossi *et al.*, 1983). Some arginases tend to dissociate during non-denaturing electrophoresis unless Mn^{2+} is included in the electrophoresis buffer (e.g. Boutin, 1982) but the oligomeric structures of *B. caldovelox* and *Thermus* 4-1A arginases were stable under the electrophoretic conditions used.

Manual N-terminal sequencing of the *B. caldovelox* arginase prepared by purification scheme I was possible to nine residues (Section 5-2-1) suggesting a high degree of purity, although the possibility of N-terminal-blocked contaminants are cannot be ruled out.

B. caldovelox and *Thermus* 4-1A arginase samples from various stages of purification scheme II were subjected to discontinuous SDS-PAGE (Fig. 4-13). The most highly purified sample of both enzymes shows a major band and several minor bands. Densitometry of photographic negatives indicated that the contaminants represented less than 5% of the total protein in each case. *B. caldovelox* arginase purified by scheme I also showed several minor bands when analysed by this method but these again represented less than 5% of total protein. From the SDS-PAGE experiments it is apparent that *B. caldovelox* arginase has not been purified to homogeneity. The pattern of contaminant bands was not completely reproducible and in some gels

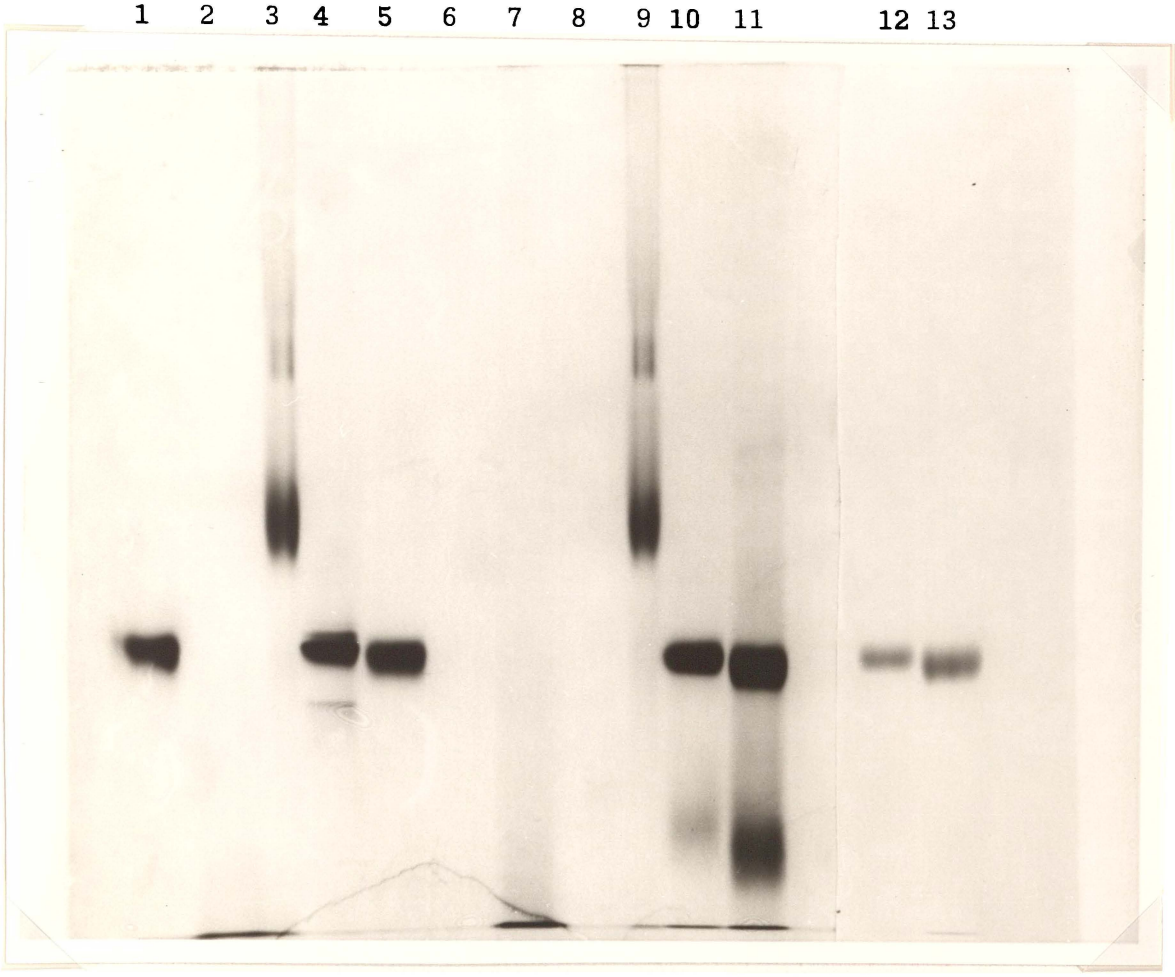

Figure 4-12 Non-denaturing PAGE of *B. caldovelox* and *Thermus* 4-1A arginases purified by purification schemes I and II.

Samples from left are: 1, *B. caldovelox* "native metals" arginase (3μg); 2, not applicable (NA); 3, NA; **4**, *Thermus* 4-1A "native metals" arginase (~3μg); **5**, as for sample 1; 6, Blank; 7, NA; 8, Blank; 9, NA; **10**, Post Lysine-Sepharose *B. caldovelox* arginase (purification scheme I) (~5μg); 11, NA; **12**, as for sample 10; 13, NA. Samples 12 and 13 were stained with Coomassie Blue R250 and the remaining samples were silver stained. The resolving gel was 5%T and the stacking gel was 3%T.

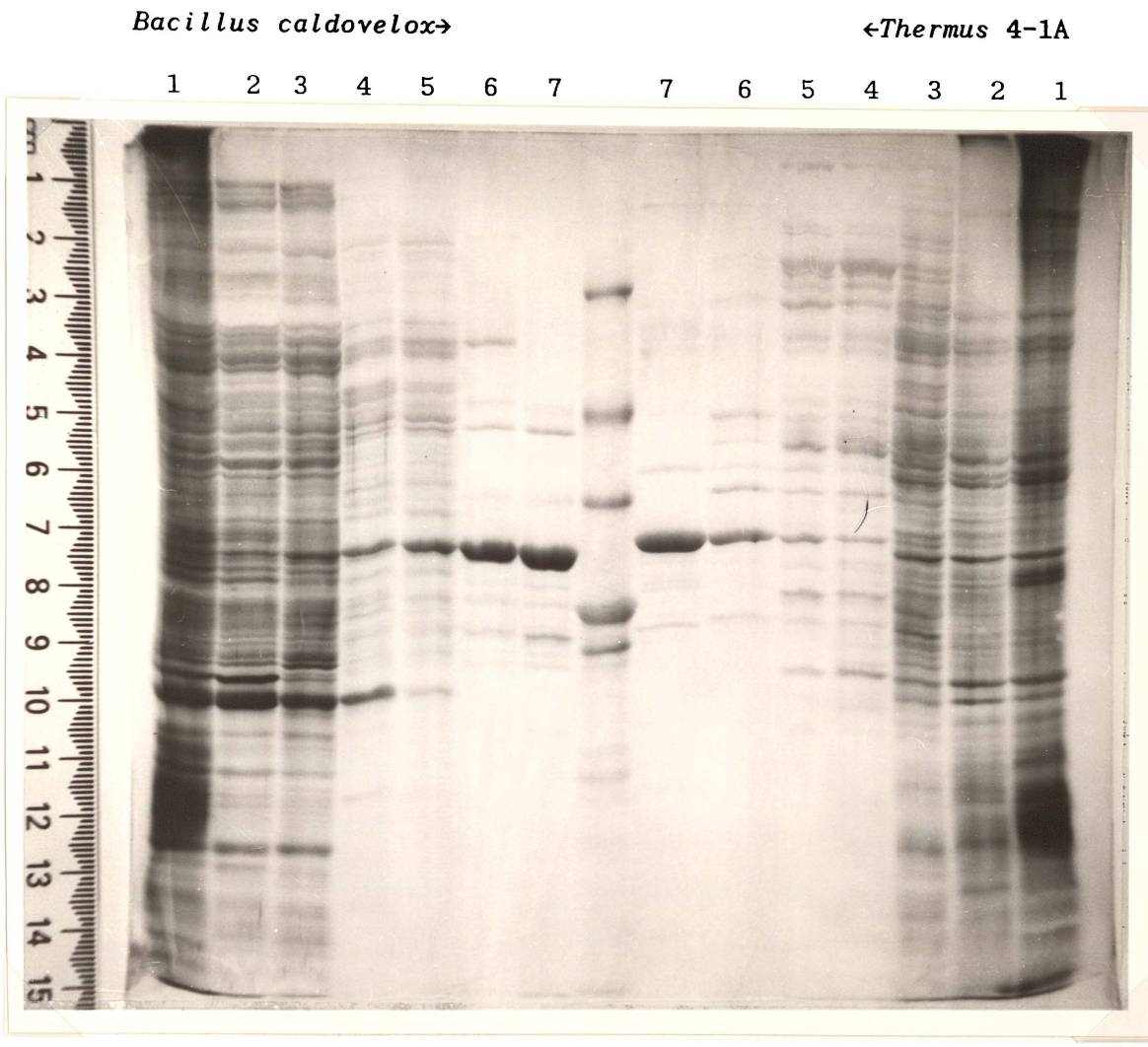

Figure 4-13 SDS-PAGE of *B. caldovelox* and *Thermus* 4-1A arginases at various steps in purification scheme II.

The 7 lanes on the left are *B. caldovelox* samples and the 7 lanes on the right are *Thermus* 4-1A samples. The centre sample contains the standard proteins bovine serum albumin, ovalbumin, glyceraldehyde-3-P DeH, carbonic anhydrase and trypsin inhibitor. Going inwards from the outermost lane on either side of the gel samples are: **1**, cell-free extract; **2**, protamine sulphate supernatant; **3**, dialysed $(NH_4)_2SO_4$ pellet; **4**, post DEAE-Sepharose; **5**, post GP-FPLC; **6**, post IE-FPLC; **7**, dialysed post HI-FPLC ("native metals" arginases). A 15μg loading was used for the first three samples of each purification sequence and ~3μg was used for the remaining four samples in each sequence. The resolving gel was 10%T and the stacking gel was 4%T. The gel was silver stained.

proteins with M_r's higher than the monomer were absent. The possibility that the low M_r contaminants were due to partial degradation of the arginase subunits during sample pretreatment was investigated but these bands were still present when sample was pretreated at 37°C. However, given the protease activity in cell-free extracts of *B. caldovelox* these low M_r bands could result from proteolytic degradation of the arginase *in vivo* or in the early stages of purification. Borkovich and Weiss (1987a) found that low M_r contaminant bands present in SDS gels of purified *N. crassa* arginase were almost completely abolished if the protease inhibitor PMSF was included in purification buffers and they showed that a major contaminant band and the arginase monomer had identical peptide maps. As intracellular protease activity could not be detected in early-mid logarithmic phase cultures of *B. caldovelox*, purification of arginase from these cells would show the extent to which proteolytic activity is responsible for the heterogeneity of arginase prepared by scheme II.

Attempts remove the minor contaminants of *B. caldovelox* "native metals" arginase by IEF or non-denaturing PAGE using various alkaline buffer systems were unsuccessful as recovery of activity was very poor and IEF yielded multiple active bands. Further anion exchange and hydrophobic interaction FPLC did not eliminate the minor contaminants seen on SDS gels.

CHAPTER 5

CHARACTERIZATION OF ARGINASE

5-1 Introduction

This chapter is divided into three sections that describe and discuss several properties of the purified "native metals" arginases from *B. caldovelox* and *Thermus* 4-1A. Section 5-2 covers physicochemical properties and focuses on the determination of native cofactors, molecular weights (M_r's) and a possible subunit structure for both enzymes. Enzymatic properties are discussed in Section 5-3 which includes the development of appropriate assay conditions for *B. caldovelox* arginase. Section 5-4 outlines properties which relate to both structure and activity, including enzyme stability and the substitution of metal ion cofactors. In the latter two sections much of the experimental work was performed using *B. caldovelox* arginase alone. Additional characterization of *Thermus* 4-1A arginase was hindered because its activation and assay conditions differed from those used for the *B. caldovelox* enzyme. This was due in part to the more extensive depletion of metal ion cofactors from *Thermus* 4-1A arginase during purification.

5-2 Physicochemical Properties of Arginases

5-2-1 N-terminal sequencing of arginases

N-terminal analysis of *B. caldovelox* and *Thermus* 4-1A "native metals" arginases was performed using a manual Edman degradation method (Tarr, 1986) with RP-LC analysis of the product phenylthiohydantoin

amino acids. For both enzymes the N-terminal residue was methionine and the second amino acid was tentatively identified as lysine. Further work on the *B. caldovelox* arginase (this time prepared by purification scheme I) showed the N-terminal sequence to be

Met-Lys-Lys-Ile-Ile-(Ser or Cys)-Ile-Gly(+Ile?)-Val-subunit.
1 2 3 4 5 6 7 8 9

The sixth residue is probably serine but the poor response of this amino acid and cysteine prevented definite identification. The eighth degradation cycle produced glycine and isoleucine derivative peaks and the latter was so strong that it was difficult to attribute it entirely to incomplete degradation in previous cycles. A possible interpretation is that the enzyme subunits are not identical and differ at the eighth residue but quantitative work would be needed to confirm this. The N-terminal amino acid of *Staphylococcus aureus* arginase is alanine (Soru and Zaharia, 1976) and that of *Bacillus anthracis* may be proline (Soru, 1983). The N-terminal sequence of mouse liver arginase (35000 M_r subunit) is Glu-Ser-Met- (Spolarics and Bond, 1988).

5-2-2 Analysis of the metal ion cofactors of arginases

Electron-probe analysis of arginase solutions evaporated onto sample stubs was attempted using a scanning electron microscope. It was hoped that this method would identify the metal ion component in the enzyme samples. Owing to the low sensitivity of the available detection system and the high background signals for several elements of interest including Fe, the analysis was of limited use. A weak Mn signal was, however, obtained for *B. caldovelox* arginase samples prepared by purification schemes I and II, and the "native metals" sample also gave a weak Ca signal. These elements were not detected in the *Thermus* 4-1A

sample.

Freeze-dried purified *B. caldovelox* and *Thermus* 4-1A "native metals" arginases were reconstituted in Milli Q water to protein concentrations of 0.7mg/ml and 0.5mg/ml respectively. These solutions were analysed for Mn, Fe, Co, Ni, Cu, Zn and Cd by carbon-rod atomic absorption spectrophotometry. For determination of Mn and Fe dilution of protein solutions were necessary. Analysis of Ca and Mg was also attempted by this method but background levels were too high and insufficient sample was available for a reliable measurement by flame photometry. The concentration of arginase subunits in each solution was calculated assuming subunit M_r's of 32000±2000 and 33000±2000 for *B. caldovelox* and *Thermus* 4-1A arginases respectively. Dividing the metal ion concentration by subunit concentration gave the number of metal ions per subunit as follows (the number of metal ions per hexamer (see Section 5-2-4) is given in parentheses): 1.2±0.15 (7) Mn^{2+} and 0.17±0.03 (1) Fe^{2+} for *B. caldovelox* arginase and 0.33±0.07 (2) Mn^{2+} and 0.1±0.03 (0.6) Fe^{2+} for *Thermus* 4-1A arginase. The maximum error in each result was calculated from the maximum percentage errors for protein concentration (including an allowance for contaminant proteins), metal ion concentration and subunit M_r. There were ~0.01-0.02 Ni^{2+} ions per subunit for both enzymes and the remaining elements were close to or at blank levels, i.e. ~0.001-0.003 ions per subunit.

Mn^{2+} is the predominant metal ion cofactor *in vivo* for *B. caldovelox* arginase and this is probably also true of the *Thermus* 4-1A enzyme. The presence of Fe^{2+} and measurable levels of Ni^{2+} in the enzymes suggests that formation of the M^{2+}-arginase complex *in vivo* is not absolutely specific for Mn^{2+}. Although a Fe^{2+}-specific binding site cannot be ruled out, the iron is most likely present as a result of

non-specific binding of intracellular Fe^{2+} at the same sites that bind Mn^{2+}. Studies on activation of arginases *in vitro* have shown that the active site will accept a number of activating cofactors, including Fe^{2+} (Middelhoven, 1969; Palacios *et al.*, 1969; Tarrab *et al.*, 1974), although this activation is usually achieved under non-physiological conditions. The amount of each metal ion bound by the arginases *in vivo* should depend not only on the stability of the various M^{2+}-arginase complexes but also on the intracellular concentrations of metal ions and the stability of the complexes these form with other ligands available *in vivo*. However, the apparent *in vivo* cofactor of *Saccharomyces cerevisiae* arginase (Fe^{2+}) could not be altered by the manipulation of exogenous metal ion concentrations (Middelhoven *et al.*, 1969). Bovine liver arginase purified 100-fold without exposure to activating cations contained Mn^{2+} and Fe^{2+} when analysed by flame photometry (Richards and Hellerman, 1940), while Mn^{2+} (but no Fe^{2+}) was detected in purified *Neurospora crassa* arginase (Davis, 1986).

There were fewer divalent metal ions per arginase subunit in the purified *Thermus* 4-1A enzyme than in *B. caldovelox* arginase and this is consistent with the relative activations of the enzymes by Mn^{2+} and the size of the decrease in specific activity towards the end of each purification. Both observations suggest that some metal ions are lost from the active sites during purification. It was not possible to activate these arginases with Mn^{2+} (activation conditions described in Fig. 5-3-8) in cell-free extracts prepared as described for purification scheme II. While this result may be complicated by the presence of proteases and inappropriate activation conditions, it would suggest that the enzymes are saturated with Mn^{2+} or a cofactor of similar catalytic effectiveness *in vivo*. The same result was obtained for *Bacillus licheniformis* arginase (Ramaley and Bernlohr, 1966).

Arginase in cell-free extracts prepared for purification scheme I was activated up to 50% by Mn^{2+} and this may be due to the lower pH of the extraction buffer used. The slight activation (~30%) of the "native metals" *B. caldovelox* arginase with Mn^{2+} (see Section 5-4-2) is open to several interpretations. Assuming Fe^{2+} is less effective catalytically, activation could be due to exchange of Fe^{2+} and Mn^{2+} in the active site. Alternatively some active sites that have lost metal ions may bind Mn^{2+} during activation. The latter proposal is consistent with the decrease in specific activity during the final purification step. Eight metal ions were detected per *B. caldovelox* oligomeric arginase. Unless there is more than one active site per subunit then some of the metal ions are presumably bound at other sites. A similar discrepancy occurs for the apparently trimeric arginases from rat liver and bovine liver which bind 4 Mn^{2+} ions, each Mn^{2+} ion being associated with an increase in activity (Hirsch-Kolb *et al.*, 1971; Harell and Sokolovsky, 1972).

5-2-3 Isoelectric points of arginases

Due to the large size of the arginase enzymes, agarose was selected as a stabilizing matrix in preference to polyacrylamide. Figure 5-2-1 shows the result of a broad-range electrofocusing experiment. A cluster of 3 main bands of equal intensity and several minor bands were observed for *B. caldovelox* arginase samples prepared by purification schemes I and II. *Thermus* 4-1A arginase gave a continuous smear of protein. For all samples protein was spread over ~0.2-0.3 pH units and no activity (detected by the paper overlay method) or protein was found outside this pH range. An agarose overlay for activity by the method of Farron (1973) on a narrow-range gel indicated that all the major bands were active. The isoelectric points of *B. caldovelox* and *Thermus* 4-1A arginase were estimated from the

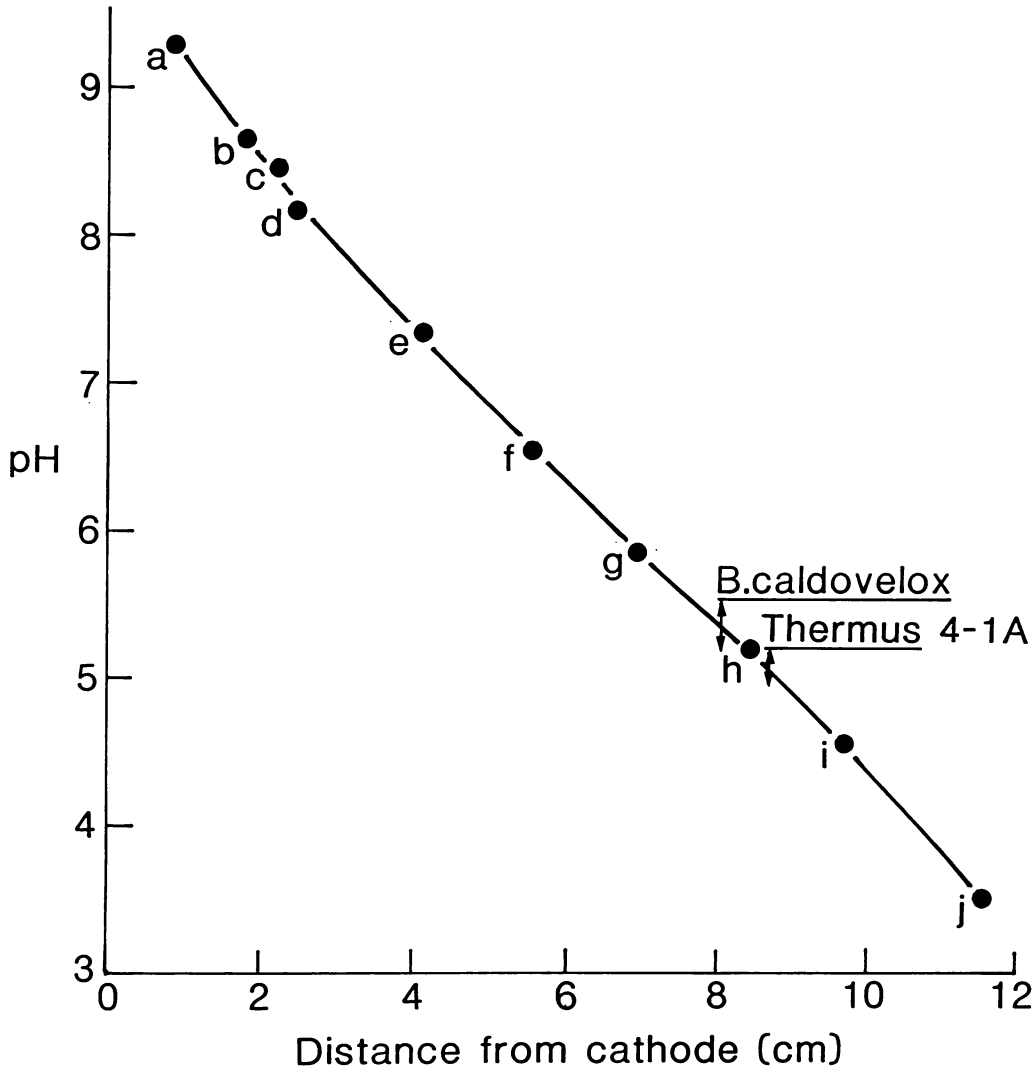

Figure 5-2-1 Isoelectric points of arginases.

Broad-range isoelectric focusing was conducted as described in Section 2-7. The standard proteins (from Pharmacia's broad pI calibration kit) were: a, trypsinogen (pI9.3); b, lentil lectin-basic band (pI8.65); c, lentil lectin-middle band (pI8.45); d, lentil lectin-acidic band; e, myoglobin-basic band (pI7.35); f, human carbonic anhydrase (pI6.55); g, bovine carbonic anhydrase (pI5.85); h, β-lactalbumin (pI5.2); i, soybean trypsin inhibitor (pI4.55); j, amyloglucosidase (pI3.5).

centre of the stained protein and are given in Table 5-2-1 with values for other arginases. These are the only bacterial arginases for which a pI has been determined. Despite the acidic pI and metal chelating properties of ampholytes (Righetti and Drysdale, 1976) the pH-dependence of the dissociation of *B. caldovelox* apoarginase at room temperature suggests that this enzyme retains its oligomeric structure during electrofocusing, i.e. the acidic pI is a property of the oligomeric enzyme. Rossi *et al.* (1983) observed a banding pattern similar to that obtained for *B. caldovelox* and attributed this microheterogeneity to loss of metal ions during electrofocusing. While this is likely to occur for the bacterial enzymes some heterogeneity may be caused by proteolytic degradation.

The proposal of Hirsch-Kolb *et al.* (1970) that neutral or slightly acidic arginases might be less stable that basic arginases because Mn^{2+} would dissociate more readily from acidic proteins is not tenable as the pI of a protein places minimal constraints on the residues present in metal ion-binding sites.

Table 5-2-1 Isoelectric points of arginases

Source	pI	Reference
Thermus 4-1A	5.1	
Bacillus caldovelox (5.2)	5.4 (5.5)	
Artichoke tubers	5.3	Wright *et al.*, 1981
Iris bulbs	5.6	Boutin, 1982
Bovine liver (5.7)	5.9 (6.0)	Rossi *et al.*, 1983
	5.9	Harell & Sokolovsky, 1972
Porcine liver	6.2	Sakai & Murachi, 1969
Human liver subunit	6.6	Carvajal *et al.*, 1971
oligomer	9.2	
Rat kidney	7.0	Reddi *et al.*, 1975
Rabbit liver	7.2	Vielle-Breitburd & Orth, 1972
Rat liver	9.3	Hirsch-Kolb *et al.*, 1970

5-2-4 Molecular weight and subunit structure of arginases

As standard proteins were used in all methods of M_r determination employed in this study it was important to have accurate M_r values for the standards. Several discrepancies exist between reference lists and the M_r values provided by manufacturers of standard protein kits. In some cases these were due to the use of old values, e.g. Sigma still gives a value of 150000 for yeast alcohol dehydrogenase (Hayes and Velick, 1954) for kits MW-GF-200 and MW-GF-1000 despite a more recent study showing the M_r to be 141000 (Bühner and Sund, 1969). In other cases the values given were simply inaccurate, e.g. yeast enolase, one of five standard proteins in a HPLC M_r marker kit (product no. 30180, USB) was assigned a M_r of 67000 by the manufacturer, considerably less than the value of 88000 reported for the dimer (Mann *et al.*, 1970). Because of the uncertainty surrounding the exact M_r of this protein it was not used to calibrate gel permeation columns, the remaining four standards proving adequate for calibration. Information on the standard proteins used in this study is given in Table 5-2-2.

The subunit M_r of *B. caldovelox* arginase was determined by SDS-PAGE (Figures 5-2-2 and 5-2-3) to be 31700±500 while the *Thermus* 4-1A arginase subunit was slightly larger at 32800±500. Both values were the mean of four independent experiments and the error is the standard deviation of these results. Although not significantly different at the 5% level, a direct comparison of band mobility on gels always showed the *Thermus* 4-1A arginase subunit to be slightly larger. The electrophoretic mobility of the *B. caldovelox* subunit did not vary with sample treatments of 1min to 15min in SDS sample buffer at 100°C. A treatment of 4h at 37°C gave a subunit M_r of ~32300 and a band representing ~5% of total protein with a M_r of ~101000 appeared. This band may be a trimeric derivative of arginase formed by incomplete

Table 5-2-2 Data on proteins used as molecular weight standards

Protein	Supplier	M_r (thousands)	Subunit structure	Sedimentation coefficient	References
Cytochrome C (horse heart)	Sigma C2506/USB	12.4(S)*	monomer	2.1	1, C-13, C-228
Lysozyme (hen egg white)	BDH 39024 2K	14.3(S)*	monomer	2.0	1, C-10; 2, 7:309; 6
Trypsin inhibitor (soyabean)	Sigma T9003	20.1	monomer	2.3	1, C-14; 3; 4
Trypsinogen	Sigma T9011	23.6	monomer	2.48	1, C-10
Carbonic anhydrase (SDS)	Sigma C2273				
(GP)	Sigma C7025	28.8	-		3
Adenylate kinase (yeast)	USB 30180	32	-		Determined by USB Corp.
Glyceraldehyde-3-P DeH (rabbit)	Sigma G5262	35.7	-		3
Ovalbumin (SDS)	Sigma A7642	42.7(S)*			Nisbet *et al.*, 1981
(SG)	Sigma A5503	43.5	monomer	3.66	1, C-15
Enolase (yeast)	USB 30180	88	dimer		2, 2:332
Serum albumin (bovine) (SDS)	Sigma A7517	66.3(S)*	133kDa dimer	4.41	1, C-16; 2, 7:497
(PA)	Sigma A8654				
Phosphorylase b (rabbit muscle)	Sigma P4649	97.4	-		4
β-Galactosidase (*E. coli*)	Sigma G8511	130	515kDa tetramer		1, C-11; 3; 5
Lactate DeH (porcine heart) (GP)	USB 30180	140	tetramer		2, 7:337
(PA)	Serva 27411				
Alcohol DeH (yeast)	Sigma A8656	141	tetramer	7.61	1, C-19; 2, 2:337; 6
Myosin (rabbit muscle)	Sigma M3889	212	468kDa dimer		2, 2:348; 5
Catalase (bovine liver) (SG)	Sigma C10	232(S)*	tetramer	11.3	1, C-11; 2, 2:343, 7:308
(PA)	Serva 26900				
Glutamate DeH (yeast)	USB 30180	290	hexamer		Determined by USB Corp.

*(S) indicates M_r calculated from amino acid sequence.

References: 1, CRC Handbook of Biochemistry 2nd edn; 2, CRC Handbook of Biochemistry and Molecular Biology 3rd edn; 3, Tabulation on p. 122 *In* Andrews (1986); 4, p. 1639-1642 Sigma catalogue 1988 edn; 5, Tabulation from Hames on p. 251 *In* Blackshear (1984); 6, Martin and Ames (1961).

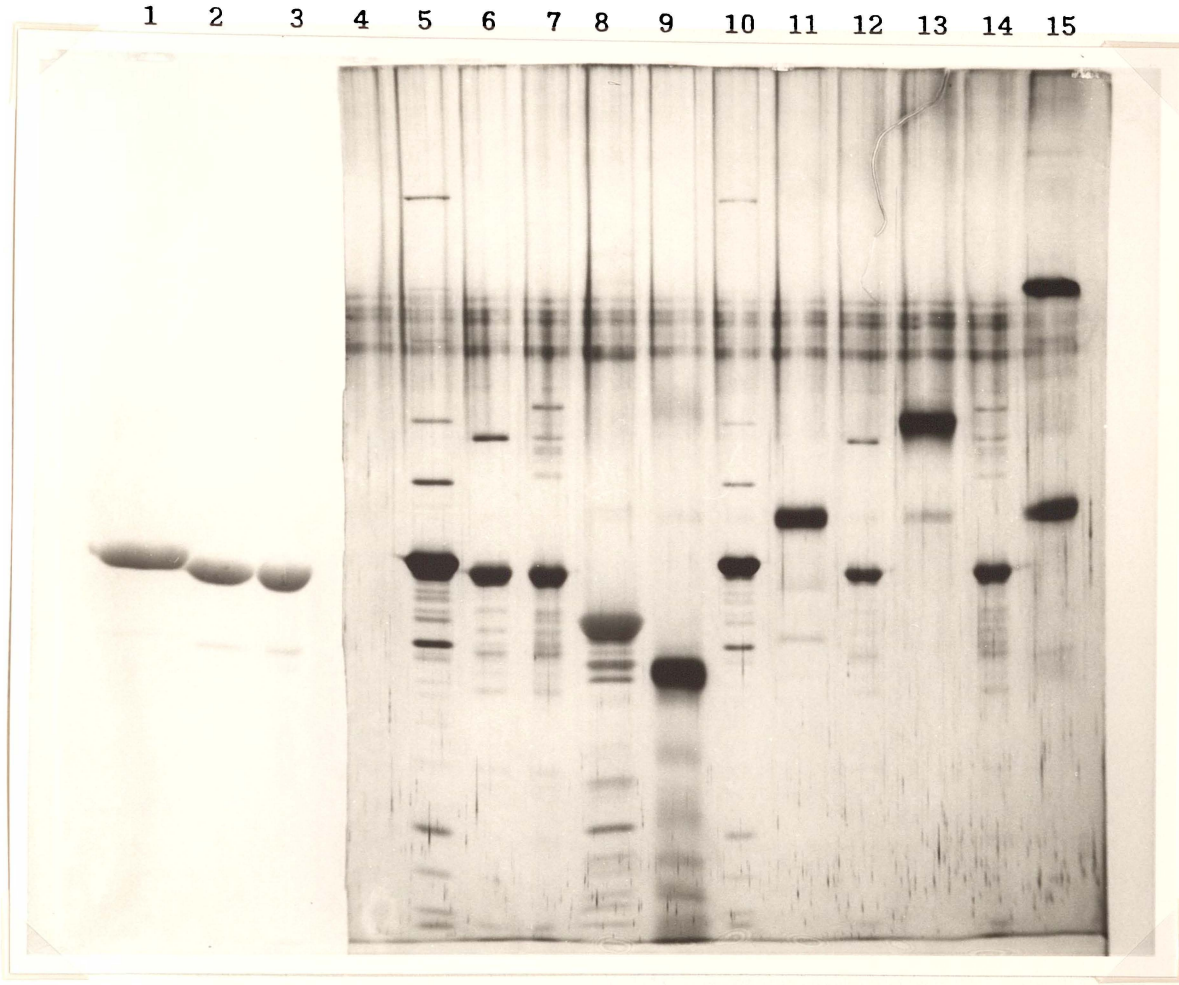

Figure 5-2-2 Molecular weights by discontinuous SDS-PAGE.

Samples from the left are

1. 9μg *Thermus* 4-1A "native metals" arginase
2. 7μg *B. caldovelox* "native metals" arginase
3. 7.5μg *B. caldovelox* arginase, purification scheme I
4. Sample buffer blank
5. 1.8μg of sample 1
6. 1.4μg of sample 2
7. 1.5μg of sample 3
8. 2μg trypsinogen
9. 5μg carbonic anhydrase
10. 0.9μg of sample 1
11. 0.5μg glyceraldehyde-3-phosphate dehydrogenase
12. 0.7μg of sample 2
13. 0.5μg ovalbumin
14. 0.75μg of sample 3
15. 0.5μg of BSA and ovalbumin

Lanes 1-3 were stained with Coomassie Blue R250 and the remaining lanes were silver stained. Samples were prepared by heating at 100°C for 10min. The resolving gel was 10%T and the stacking gel 4%T. Electrophoresis at 16°C with 13mA in the stacking gel, 18mA in the resolving gel and a voltage limit of 200V was complete in 5h. Sample buffer contamination and the use of BDH especially pure SDS rather than electrophoresis grade SDS account for some of the bands and streaks in the gel.

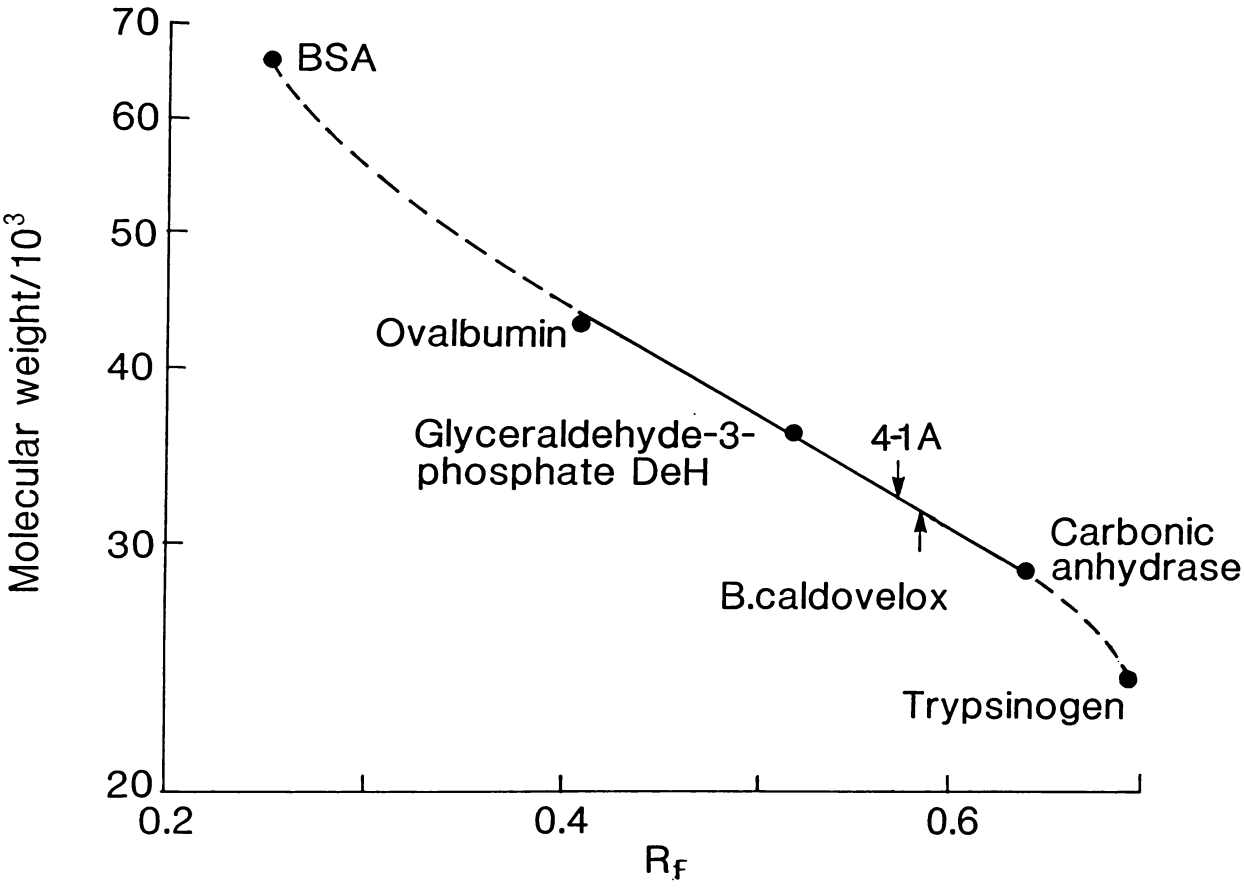

Figure 5-2-3 Molecular weights by discontinuous SDS-PAGE.

A plot of log molecular weight against the R_F values obtained from the SDS gel in Figure 5-2-2.

dissociation of the native oligomer under the milder sample treatment conditions. Omission of 2-mercaptoethanol from the SDS sample buffer made no difference to the R_F values of *B. caldovelox* and *Thermus* 4-1A arginase subunits prepared by heating for 10min at 100°C. Discontinuous non-denaturing PAGE with the Laemmli (1970) buffer system was used to determine the M_r's of the native arginases according to the method of Hedrick and Smith (1968). The standards (see Table 5-2-2 for details) BSA (monomer), lactate dehydrogenase and catalase were run with *B. caldovelox* and *Thermus* 4-1A arginase samples on 4%, 5%, 6%, 7% and 8% total acrylamide gels (see Section 2-6-1 for experimental details) and respective slopes (of $\log R_F$ vs %T plots) of 0.074, 0.096, 0.126, 0.106 and 0.108 were obtained. A plot of slope vs M_r yielded M_r estimates of 167000 and 172000 for *B. caldovelox* and *Thermus* 4-1A arginases respectively.

Sucrose density gradient centrifugation was used to determine the sedimentation coefficients ($s_{20,w}$) and M_r's of arginase subunits and native oligomers (Table 5-2-3). In a typical experiment arginase was centrifuged with two standard proteins that bracketed the expected M_r of arginase. The ratios of sedimentation distances of the standards and arginase were used to estimate $s_{20,w}$ and M_r values for the arginases according to the equations set out by Martin and Ames (1961). The assumptions of similar density (partial specific volume, $\bar{v}$) and molecular shape (frictional coefficient) for unknowns and standards made in the derivation of these equations were also made when applying them to the arginases. Good internal consistency for the high M_r pair of standard proteins catalase and alcohol dehydrogenase was seen under the experimental conditions, the ratio of measured migration distances (0.70) being similar to the ratio of the known sedimentation coefficients (0.67) and the $(M_r \text{ ratio})^{2/3}$ of 0.72. Thus the equations

derived by Martin and Ames (1961) for an isokinetic gradient also appear valid for the experimental conditions used in this study. A potential difficulty in the accurate estimation of $s_{20,w}$ values by this method is the sample convection that may occur in a preparative centrifuge but not in the sector-shaped cells used in analytical ultracentrifugation (Freifelder, 1982). However, as similar sedimentation coefficients have been obtained for rat liver arginase by analytical ultracentrifugation and sucrose density gradient centrifugation (see Table 5-2-4) this was not regarded as a problem. The M_r of the oligomeric arginases determined from a plot of log M_r vs

Table 5-2-3 *B. caldovelox* and *Thermus* 4-1A arginase sedimentation coefficients and M_r's by sucrose gradient centrifugation

Arginase & standard	Distance ratio (R)*	$s_{20,w}$	M_r (thousands)
B. caldovelox arginase &			
Alcohol DeH	1.20	9.1	187
Catalase	0.85	9.6	183
Cytochrome C	1.26	2.7	18[a]
Lysozyme	1.10	2.2	17[a]
Trypsin inhibitor	1.06	2.4	22[a]
Ovalbumin	0.76	2.8	29[a]
Thermus 4-1A arginase &			
Alcohol DeH	1.11	8.5	167
Catalase	0.78	8.8	161
Cytochrome C	1.28	2.7	18[a]
Ovalbumin	0.77	2.8	29[a]

*Distance travelled by arginase divided by distance travelled by standard.
[a]Acid-dissociated subunits

Arginase sedimentation coefficients ($s_{20,w}$) and M_r were calculated assuming the standard proteins and arginases have the same partial specific volume ($\bar{v}$) and shape.

migration distance gave values of 185000 and 164000 for the *B. caldovelox* and *Thermus* 4-1A enzymes respectively and these 'average' M_r's appear in Table 5-2-5. Attempts to find a suitable low M_r standard to use with ovalbumin for an estimation of the acid dissociated subunit M_r at pH2.7 were unsuccessful. Of cytochrome C, lysozyme and trypsin inhibitor the last gave the best overall internal consistency in combination with ovalbumin with an experimental distance ratio of 0.73 compared with the $s_{20,w}$ ratio of 0.63 and the $(M_r \text{ ratio})^{2/3}$ of 0.60. This deviation from ideal behaviour may be because some standard proteins do not conform to the two assumptions made in relating migration distance to M_r, resulting in different estimations of subunit M_r for each standard. Only the subunit M_r estimates obtained from ovalbumin approached the values obtained by other methods. Table 5-2-4 lists the sedimentation coefficients obtained for a number of native and acid-dissociated arginases. Comparison with the other values suggests that the arginases from chicken liver might be related as trimeric and hexameric forms of the enzyme.

Representative sedimentation profiles are shown in Figures 5-2-4, 5-2-5 and 5-2-6. The profile of yeast alcohol dehydrogenase at pH9.5 consisted of two protein peaks of which only the more rapidly sedimenting smaller peak showed alcohol dehydrogenase activity as determined by the assay method of Martin and Ames (1961). The larger inactive peak may have been due to a partial dissociation of alcohol dehydrogenase into subunits at pH9.5. As the location of the active alcohol dehydrogenase peak could be accurately established from A_{205} measurements no further alcohol dehydrogenase assays were conducted. Partially purified bovine liver catalase was used, necessitating the assay of this enzyme to locate it in sedimentation experiments at pH9.5.

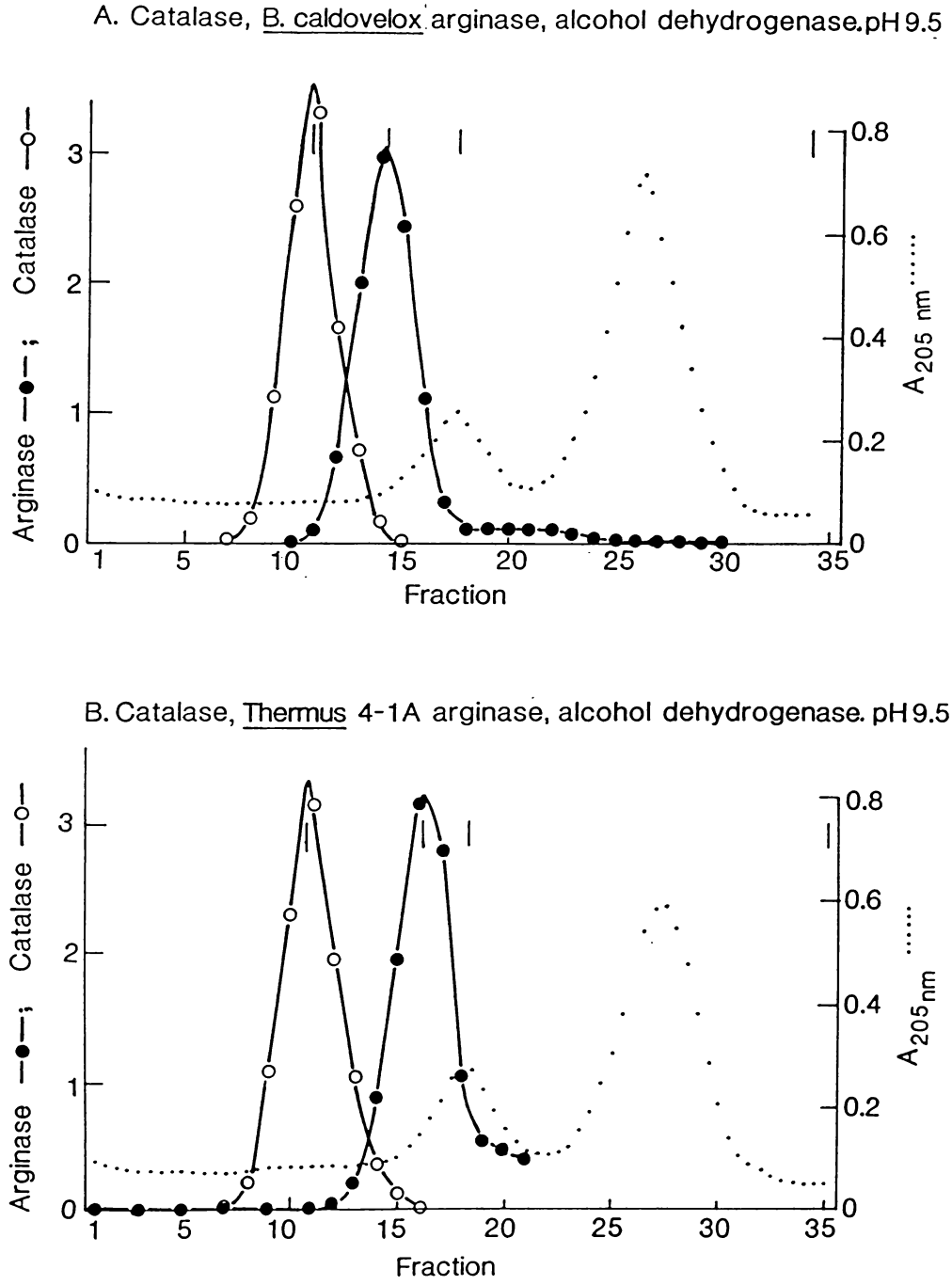

Figure 5-2-4 Sedimentation profiles of oligomeric arginases.

A 50μl sample containing 0.4mg/ml catalase, 2mg/ml alcohol dehydrogenase and either 0.015mg/ml *B. caldovelox* arginase (A) or 0.03mg/ml *Thermus* 4-1A arginase (B) in gradient buffer pH9.5 was layered onto a 5-20% sucrose density gradient. Centrifugation was for 5.5h and the experimental conditions are described in Section 2-6-3. Arginase activities are expressed in arbitrary units. Migration distances were determined from the vertical lines indicating the position of each peak and the meniscus.

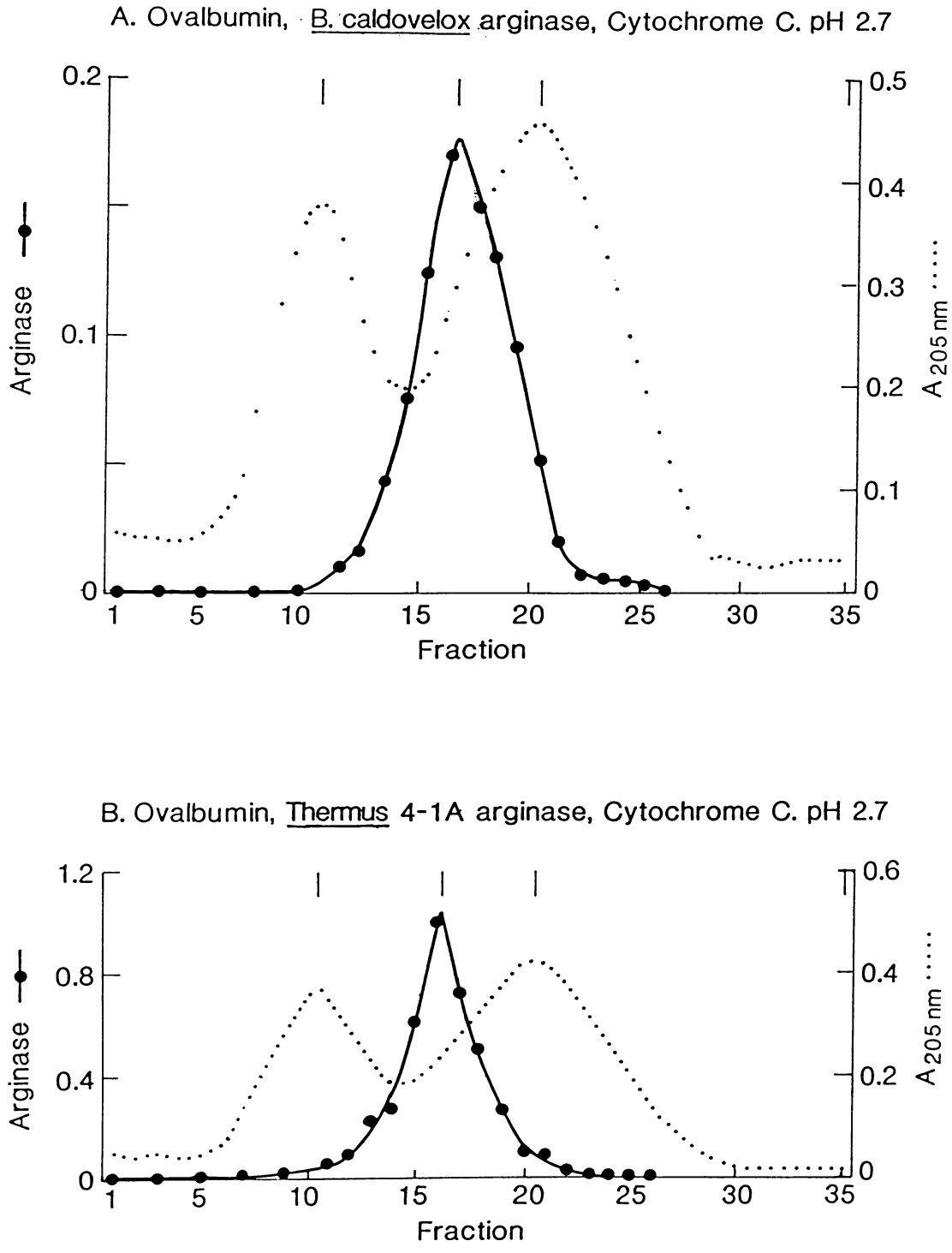

Figure 5-2-5 Sedimentation profiles of arginase subunits.

A 55μl sample containing either 1.3mg/ml ovalbumin, 1.3mg/ml cytochrome C and 0.11mg/ml *B. caldovelox* arginase (A) or 0.08mg/ml *Thermus* 4-1A arginase (B) in gradient buffer pH2.7 was layered onto a 5-20% sucrose density gradient. Centrifugation was for 19h and the experimental conditions are described in Section 2-6-3. See Fig. 5-2-4 for other details.

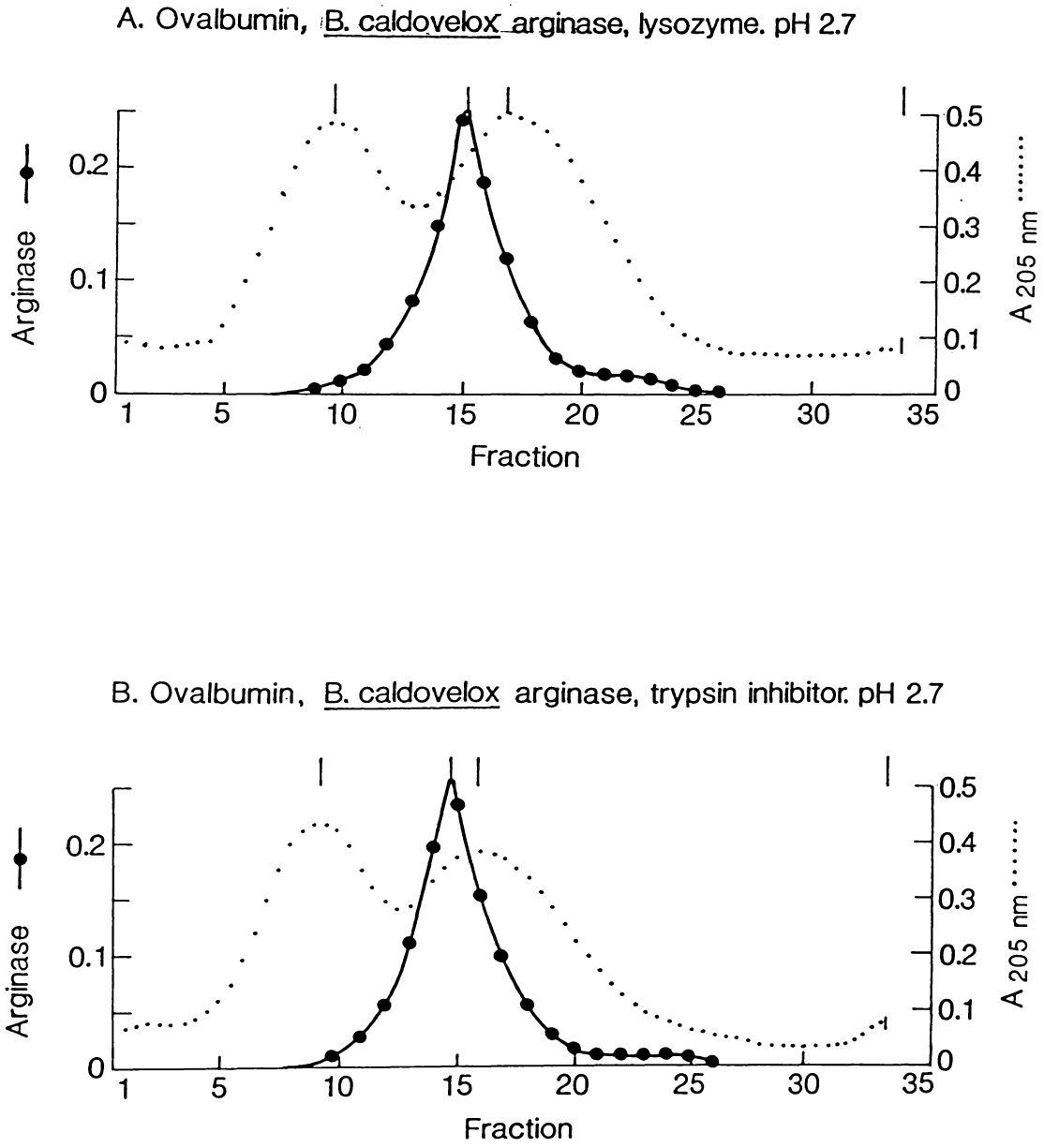

Figure 5-2-6 Sedimentation profiles of *B. caldovelox* arginase subunit.

A 55μl sample containing either 1.3mg/ml ovalbumin, 0.23mg/ml *B. caldovelox* arginase and 1.3mg/ml lysozyme (A) or 1.3mg/ml trypsin inhibitor (B) in gradient buffer pH2.7 was layered onto a 5-20% sucrose density gradient. Centrifugation was for 19h and the experimental conditions are described in Section 2-6-3. See Fig. 5-2-4 for other details.

Table 5-2-4 Sedimentation coefficients of arginases

Source	$s_{20,w}$		References
Rat liver (UC)	5.6		Schimke, 1964
(UC)	6.1	1.5[a]	Hirsch-Kolb & Greenberg, 1968
	6.2		Reddy & Campbell, 1970
Porcine liver (UC)	5.7	2.5*	Sakai & Murachi, 1969
Chicken liver (high act.)	5.7		Rossi & Grazi, 1969
(norm. act.)	9.0		
Rabbit liver	5.9	2.4*	Vielle-Breitburd & Orth, 1972
Human liver (UC)	5.9		Berüter *et al.*, 1978
Equine liver (UC)	5.95		Greenberg *et al.*, 1956
Bovine liver (UC)	6.0		Harell & Sokolovsky, 1972
Gull liver	6.75		Reddy & Campbell, 1970
Thermus 4-1A	8.7	2.8*	
Bacillus caldovelox	9.4	2.8*	
Land snail	10.1		Reddy & Campbell, 1970
Land planarian	10.1		Reddy & Campbell, 1970

*Sedimentation coefficient of arginase subunit formed by acid dissociation.
[a]Sedimentation coefficient in 8M urea.

Unless indicated by UC (analytical ultracentrifugation) the sedimentation coefficients were determined by centrifugation in sucrose density gradients.

Table 5-2-5 Oligomeric and subunit M_r's of *B. caldovelox* and *Thermus* 4-1A arginases

Method	*Bacillus caldovelox*		*Thermus* 4-1A	
	Oligomeric M_r (thousands)	Subunit M_r	Oligomeric M_r (thousands)	Subunit M_r
SDS-PAGE		31.7		32.8
Sucrose density gradient ultracentrifugation pH2.7		29		29
Sucrose density gradient ultracentrifugation pH9.5	185		164	
GP-FPLC pH2.7		30		31
GP-FPLC pH9.5 Dissociated enzyme		34		34
GP-FPLC pH9.5 Oligomeric enzyme	172		165	
Non-denaturing PAGE	167		172	

M_r determinations using an analytical TSK-Gel G3000SW HPLC column were abandoned when it was found that the apparent M_r's of *B. caldovelox* and *Thermus* 4-1A arginases were strongly pH-dependent between pH7.4 and pH3. Enzyme samples diluted in running buffer gave a single peak at pH7.4 (M_r ~160000), pH7.0 (M_r ~140000), pH6.5 (M_r ~110000), pH5.5 (M_r ~70000), pH4.5 (M_r ~50000) and pH3.5 (M_r ~30000). These M_r values did not correspond to integral multiples of the subunit M_r. Below pH6.5 the protein peak was not active when assayed with 20mM arginine at 60°C at pH9 in the presence of 5μM Mn^{2+}. The results suggest a pH dependent interaction of the oligomeric arginases with the column despite an ionic strength of at least 0.2M at all pH's in these HPLC experiments. At pH3.5 the enzymes will be dissociated and the M_r of 30000 for the subunit is similar to values obtained by GP-FPLC.

GP-FPLC on a Superose 12 HR 10/30 FPLC column permitted experiments at pH9.5 where arginase activity is most stable in the absence of Mn^{2+}, and at pH2.7 (see Section 2-6-2). Incubation of arginase samples at pH4 before GP-FPLC caused partial dissociation allowing oligomer and monomer M_r's to be estimated simultaneously using a pH9.5 buffer as eluent (Fig. 5-2-7). The subunit M_r's were also estimated in a pH2.7 running buffer after diluting samples in this eluent. Andrews (1970) found that many standard proteins used in M_r determinations were suitable for calibrating Sephadex columns at pH's as low as pH1.3. At pH2.7 glutamate dehydrogenase, lactate dehydrogenase, cytochrome C and yeast alcohol dehydrogenase were suitable standards but BSA and sweet potato β-amylase from Sigma's MW-GF-200 kit were not, the latter standard dissociating at the low pH. The apparently anomalous elution volume of adenylate kinase in this GP-FPLC system was also seen for carbonic anhydrase and these two standards provided the points on the calibration curve necessary for an

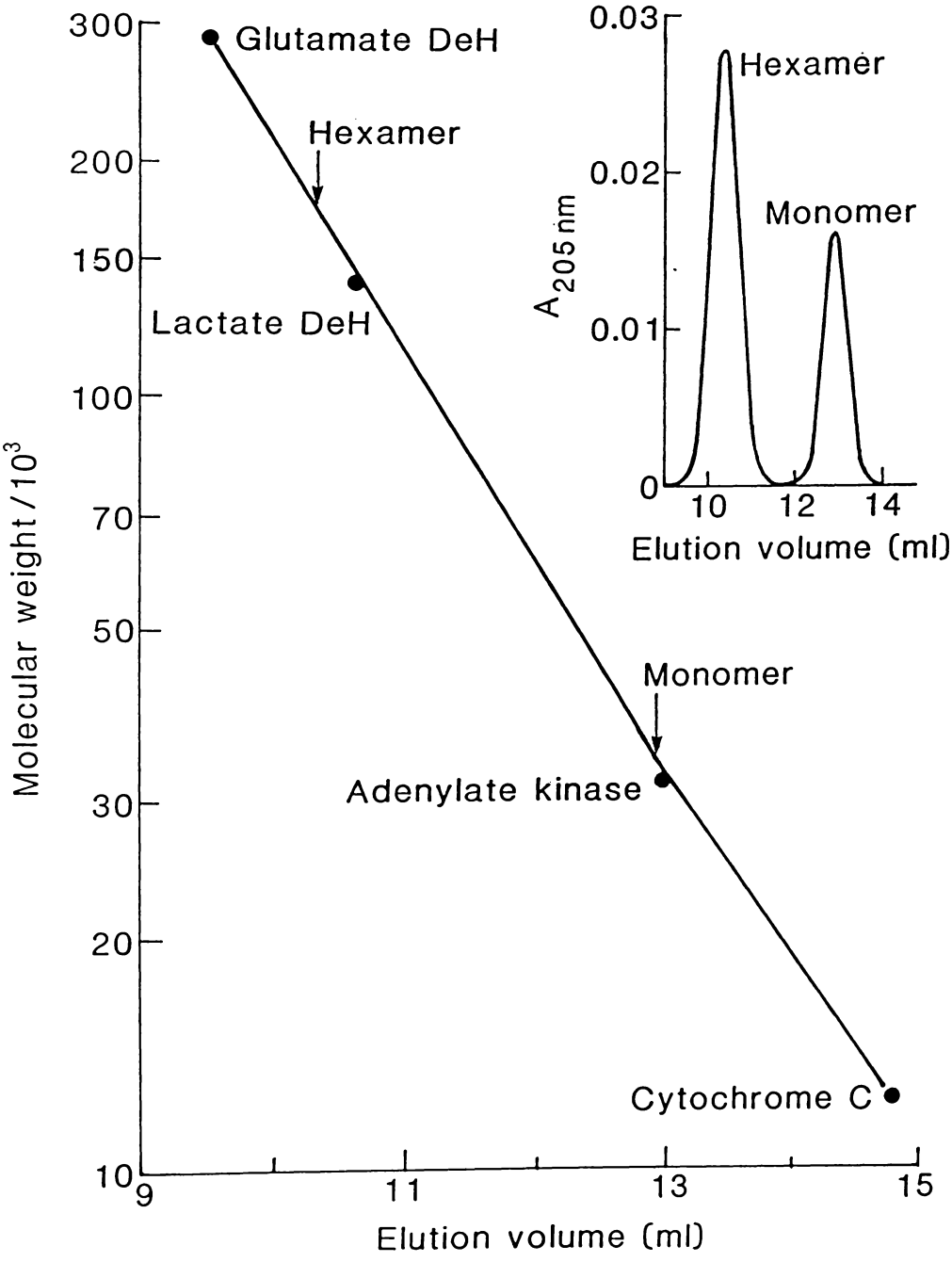

Figure 5-2-7 Molecular weights by GP-FPLC.

Plot of molecular weight (log scale) against elution volume on a Superose 12 HR 10/30 FPLC column.

Elution profile: FPLC column equilibrated at 0.7ml/min with 10mM sodium tetraborate buffer containing 7mM NaOH and 50mM K_2SO_4 pH9.5 (20°C). *B. caldovelox* "native metals" arginase (~0.15mg/ml) was equilibrated in 50mM citric acid/NaOH buffer pH4.0 for 28min and 20µl injected. The detector was set at 205nm and 0.05 AUFS. See Section 2-6-2 for other experimental details.

estimation of arginase subunit M_r's under acidic conditions. Results are summarized in Table 5-2-5.

The apparent M_r of some oligomeric enzymes determined by gel permeation chromatography can depend on enzyme concentration (Andrews, 1965; Mantle, 1984). Sakai and Murachi (1969) found that porcine liver arginase formed high M_r aggregates if concentrated prior to gel permeation chromatography. To preclude the possibility of concentration-dependent effects at pH9.5, 40-50μl samples of *B. caldovelox* arginase varying in concentration from 1.2mg/ml (detected at 280nm, 0.1 AUFS) to 12μg/ml (detected at 205nm, 0.05 AUFS) were chromatographed at pH9.5. No variation in elution volume or peak shape was seen and only a single protein peak was evident. A 1.2μg/ml sample gave a single peak of activity at the same elution volume and no activity (low M_r fractions were activated at 60°C, pH7 with 0.5mM Mn^{2+} and 1mg/ml BSA for 1h) or protein was detected in low M_r fractions which suggests that extreme dilution does not cause dissociation at pH9.5.

The results of the M_r determination methods applied to *B. caldovelox* and *Thermus* 4-1A arginases (summarized in Table 5-2-5) suggest that both enzymes are homomeric oligomers with either a pentameric or hexameric structure. Very few oligomeric proteins are homomeric pentamers (Darnall and Klotz, 1976). A number of arginases appear to be hexamers (entries 22, 23, 27 and 29 in Table 5-2-6 and the arginase from *Aspergillus nidulans* (Borkovich and Weiss, 1987a)), although the ratios of oligomeric to subunit M_r's do not always predict this structure exactly. For example, a hexameric structure has been demonstrated for *B. licheniformis* arginase by glutaraldehyde crosslinking of subunits despite an oligomer-to-subunit M_r ratio of 7.7. Hexameric structures have also been proposed for *Neurospora*

Table 5-2-6 Molecular weights and subunit structures of arginases

Source	Molecular weight (thousands)	Subunits number	Subunits M_r	References
1 Earthworm gut	25 (GP)	1		Iino & Shimadate, 1986
2 Earthworm gut	27 (GP)	1		Reddy & Campbell, 1968
3 Concholepas concholepas	27.5 (GP)	1		Carvajal *et al.*, 1984
4 Human liver	98 (GP)		39.2(SP)	Brusdeilins *et al.*, 1985
	107 (GP)		35 (SP)	Berüter *et al.*, 1978
	118 (GP)	4	30 (GP)	Carvajal *et al.*, 1971
			35 (SQ)	Spolarics & Bond, 1988
5 Rat liver	105±10(SPC)	3(SPC)	35 (SP)	Penninckx *et al.*, 1974
	150±15(GP)			
	110 (GP)			Tarrab *et al.*, 1974
	120 (GP)		30 (GP)	Barańczyk-Kuźma *et al.*, 1976
	120 (UC)	4	31 (UC)	Hirsch-Kolb & Greenberg, 1968
	121 (GP,SG)			Reddy & Campbell, 1970
	138 (SG)			Schimke, 1962
			32 (PA)	Hosoyama, 1972
			35 (SQ)	Spolarics & Bond, 1988
			39.2(SP)	Brusdeilins *et al.*, 1985
			39.3(SP)	Borkovich and Weiss, 1987a
6 Rabbit liver	110 (GP)		36.5(SP)	Vielle-Breitburd & Orth, 1972
			38.5(SP)	Brusdeilins *et al.*, 1985
7 Staphylococcus aureus	110±5 (GP)	4	30 (SP)	Soru & Zaharia, 1976
8 Saccharomyces cerevisiae	114±7 (GP,SPC)	3	39 (SP)	Penninckx *et al.*, 1974
			35.6(SQ)	Sumrada & Cooper, 1984
9 Bovine liver	114±3 (UC)			Kuchel *et al.*, 1975
	115±5 (UC)			Harell & Sokolovsky, 1972
			39.7(SP)	Brusdeilins *et al.*, 1985
10 Rat small intestine	120 (GP)			Fujimoto *et al.*, 1976
11 Pig liver	120 (GP)			Sakai & Murachi, 1969
			38.7(SP)	Brusdeilins *et al.*, 1985
12 Ox liver	120 (GP)			Sakai & Murachi, 1969
13 Human heart	120 (GP)	4	30 (GP)	Barańczyk-Kuźma *et al.*, 1980
14 Gull liver	134 (GP,SG)			Reddy & Campbell, 1970
15 Mouse liver	137 (GP)			Hirsch-Kolb *et al.*, 1970
			35,38(SP)	Spolarics & Bond, 1988
			39.5(SP)	Brusdeilins at al., 1985
16 Horse liver	138 (UC)			Greenberg *et al.*, 1956
			39.6(SP)	Brusdeilins *et al.*, 1985
17 Bullfrog kidney	140 (SG)			Carlisky *et al.*, 1972
18 Bacillus anthracis	160±2 (PA)			Soru, 1983
19 Thermus 4-1A	167±5	6	33±2	
20 Bacillus caldovelox	176±10	6	32±2	
21 Evernia prunastri	180 (GP)			Legaz & Vicente, 1980
22 Iris hollandica (bulb)	191 (GP,PA)	6	36.5(SP)	Boutin, 1982
23 Pista pacifica	205 (GP)	6	35±1(GP,SP)	O'Malley & Terwilliger, 1974
24 Land snail	232 (GP,SG)			Reddy & Campbell, 1970
25 Land planarian	238 (GP,SG)			Reddy & Campbell, 1970
26 Silkworm moth (fat body)	240 (GP)			Reddy & Campbell, 1969
27 Bacillus licheniformis	255 (GP)	6(SPC)	33 (SP)	Simon & Stalon, 1976
28 Chicken liver	276 (SG)			Mora *et al.*, 1966
29 Neurospora crassa	266 (GP)	6	38.3(SP)	Borkovich & Weiss, 1987a
			36.1,41.7	Borkovich & Weiss, 1987b
	278 (SG)			Mora *et al.*, 1966
30 Lizard liver	280 (SG)			Mora *et al.*, 1965
31 Bacillus subtilis	296±20(GP)			Issaly & Issaly, 1974
32 Evernia prunastri	330 (GP)			Legaz & Vicente, 1982

Method abbreviations: GP, Gel permeation chromatography; PA, Non-denaturing PAGE; SG, Sucrose gradient centrifugation; SP, SDS-PAGE; SPC, SDS-PAGE of crosslinked arginase; SQ, Sequence data; UC, Ultracentrifugation.

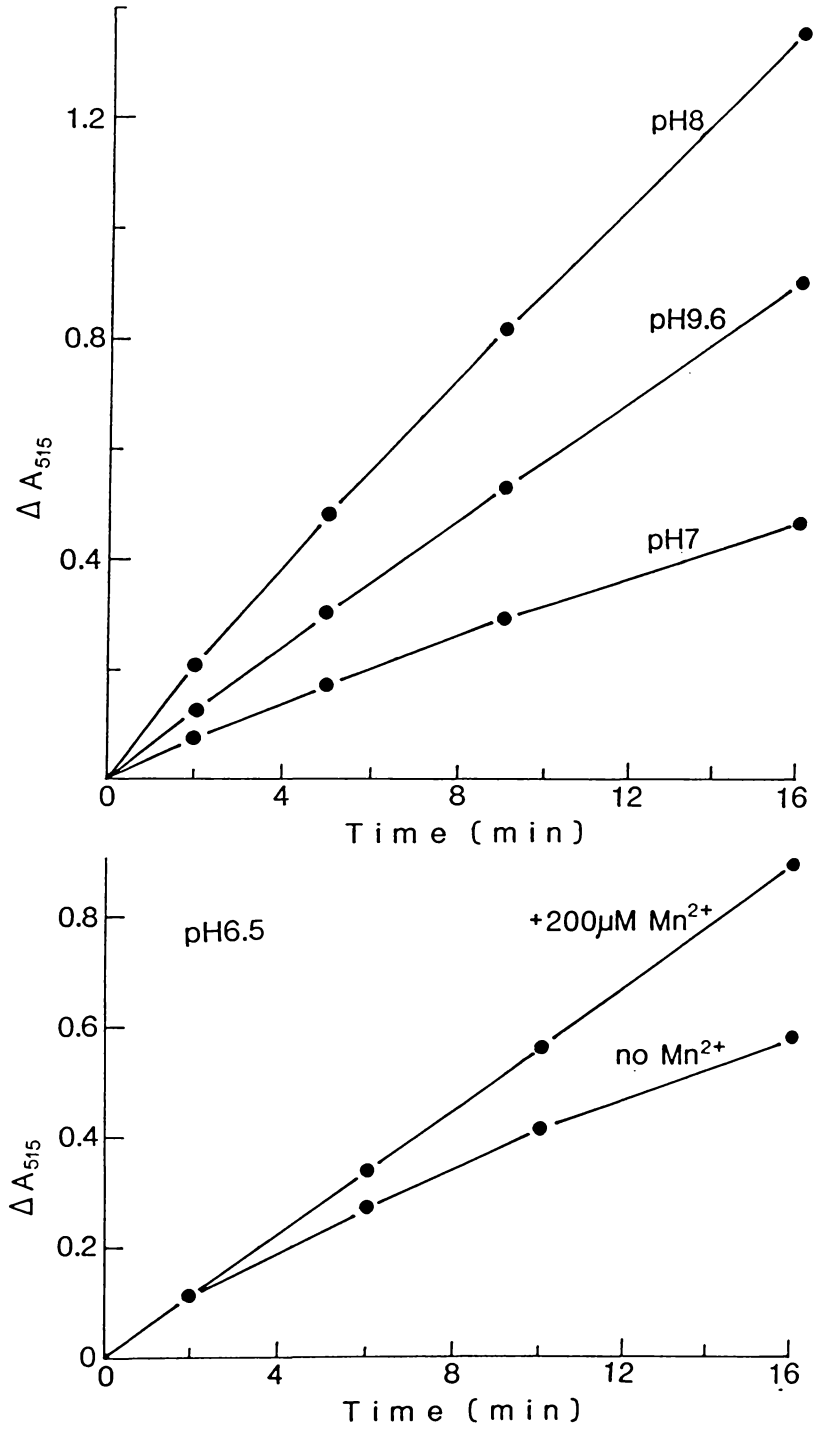

Figure 5-3-1 Linearity of the *B. caldovelox* arginase assay with time.

Top: *B. caldovelox* arginase ("native metals", 0.33mg/ml) was diluted 3000× (pH7 and 8) or 6000× (pH9.6) in 50mM CHES/NaOH buffer pH9.5 (20°C) and assayed at 60°C with 20mM arginine at the pH indicated.

Bottom: The lower graph shows how addition of 200μM Mn^{2+} to the assay of a 700-fold dilution of the same enzyme linearized the assay at 60°C with 20mM arginine at pH6.5.

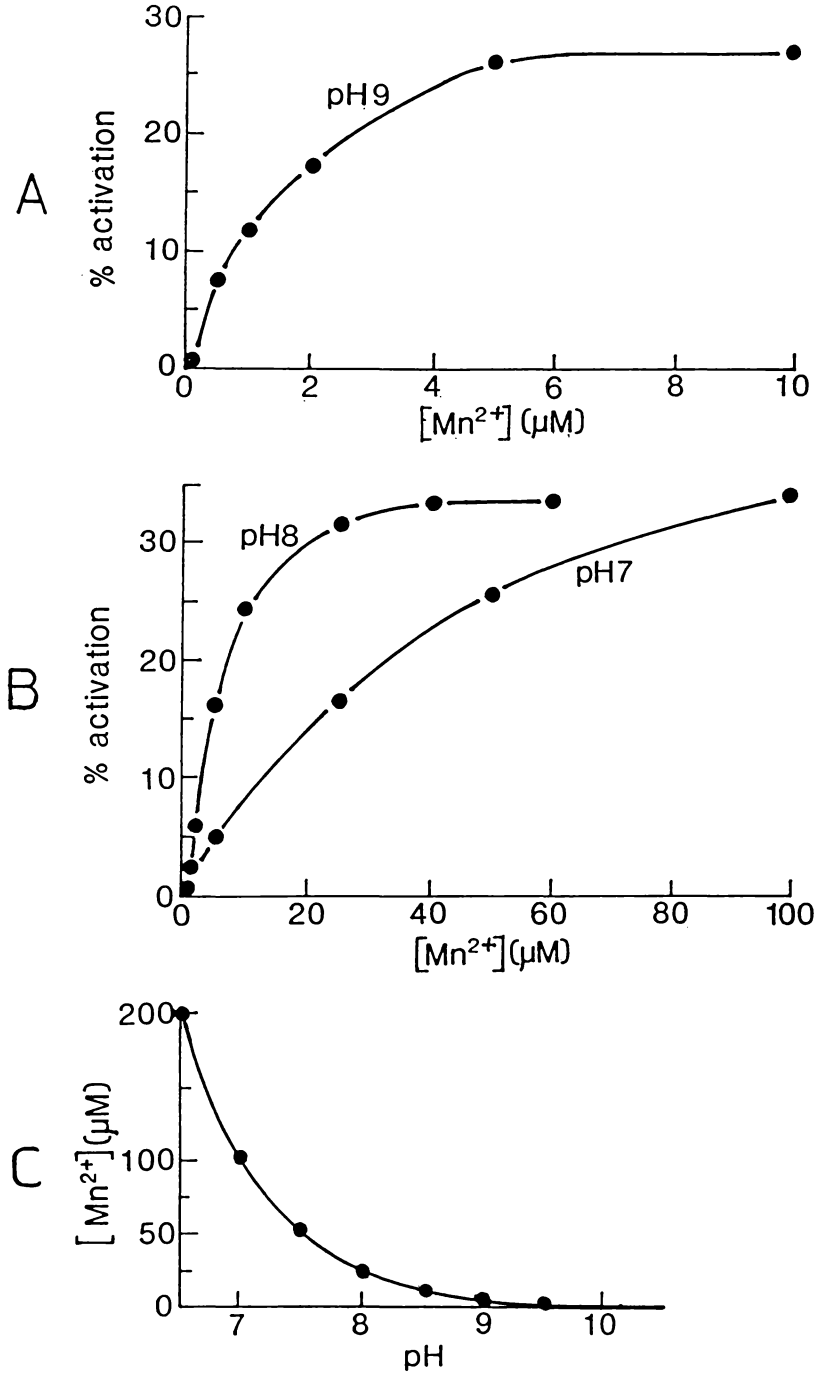

Figure 5-3-2 The effect of $[Mn^{2+}]$ on the activity of *B. caldovelox* arginase.

A and B: *B. caldovelox* arginase ("native metals", 0.33mg/ml) was diluted 2000× (pH7), 4000× (pH8) and 6000× (pH9) in 50mM CHES/NaOH buffer pH9.5 (20°C) and assayed for 16min at 60°C with 20mM arginine at the pH and in the presence of the $[Mn^{2+}]$ indicated.

C: Plot of the $[Mn^{2+}]$ required at various assay pH values for optimal or near-optimal activity of unactivated arginase when assayed at 60°C with 20mM arginine. The data points are (6.5, 200μM), (7, 100μM), (7.5, 50μM), (8, 25μM), (8.5, 10μM), (9, 5μM), (9.5, 1.5μM).

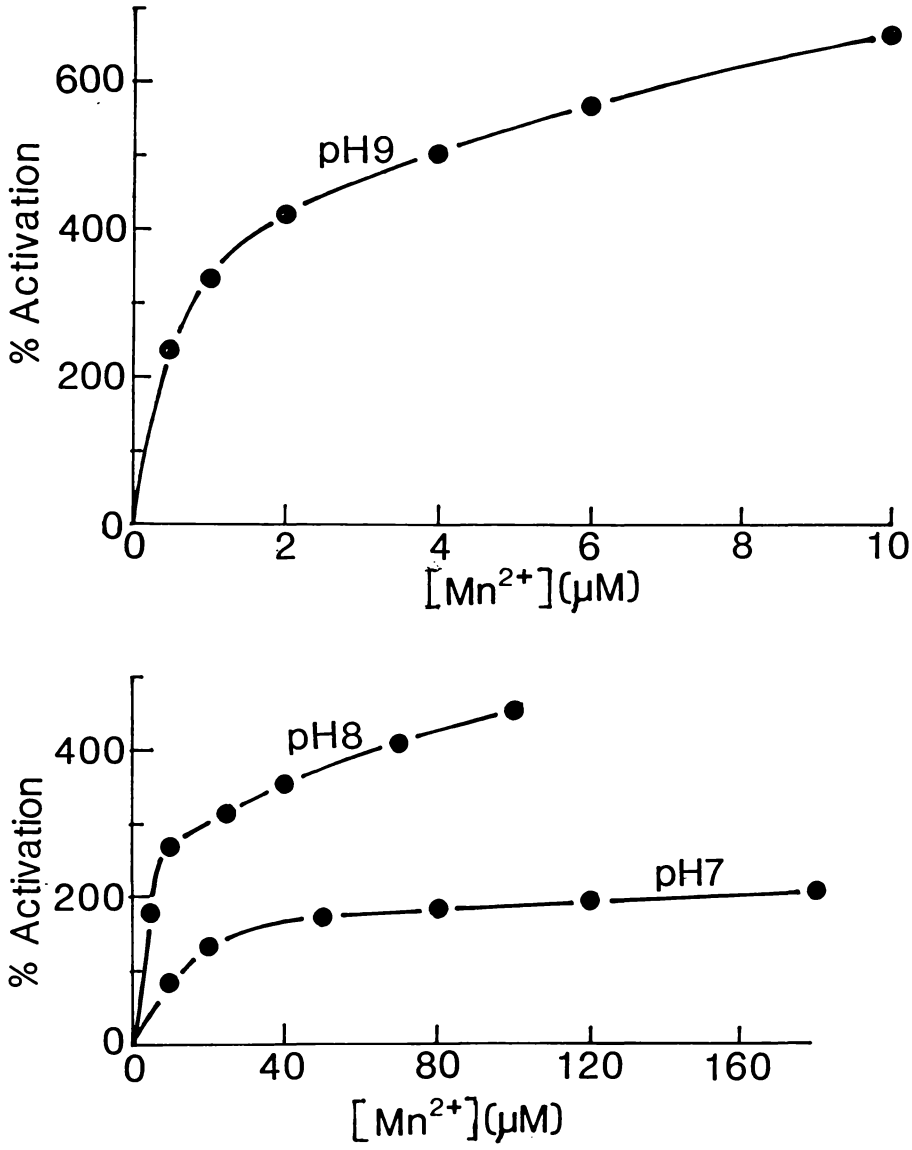

Figure 5-3-3 The effect of $[Mn^{2+}]$ on the activity of *Thermus* 4-1A arginase.

Thermus 4-1A arginase ("native metals", 0.15mg/ml) was diluted 50× (pH7), 100× (pH8) and 200× (pH9) in 50mM CHES/NaOH buffer pH9.5 (20°C) and assayed for 16min at 60°C with 20mM arginine at the pH and in the presence of the $[Mn^{2+}]$ indicated.

crassa, *Pista pacifica* and *Iris hollandica* arginases which have ratios of 6.9, 5.9 and 5.2 respectively. These compare with ratios of 5.5 for *B. caldovelox* arginase and 5.1 for *Thermus* 4-1A arginase. A number of factors could be causing these ratios to deviate from the expected value of 6. Significant differences in the density ($\bar{v}$) or shape (frictional coefficient) between one of or both oligomeric and monomeric arginase and standard proteins would result in inaccurate M_r estimations as the methods used assume these properties to be the same for all proteins. Andrews (1965) found the M_r of the tetrameric metalloenzyme catalase to be ≤200000 by gel filtration and suggested that the metal ion cofactors in this protein might enforce a closer packing within and between subunits. This might result in a more compact/dense oligomeric structure in solution compared with most proteins including arginase subunits which lack a cofactor. Removal of metal ion cofactors from oligomeric *B. caldovelox* arginase by chelation at 60°C or acid treatment did not significantly alter M_r, but the apoarginase might also have a relatively compact oligomeric structure at 20°C. Non-spherical oligomers have been proposed to account for oligomer-to-subunit M_r ratios greater than 6 (e.g. Borkovich and Weiss, 1987a). However the suggestion that the anomalously low ratio of *Iris hollandica* arginase was due to a subunit arrangement that produced an elongated oligomeric enzyme (Boutin, 1982) is improbable as the M_r's of proteins with high axial ratios are typically over-estimated by molecular-sieving methods (Andrews, 1970). Non-specific ionic or hydrophobic interactions with the gel permeation Superose 12 matrix might retard the oligomeric *B. caldovelox* and *Thermus* 4-1A arginases sufficiently to account for the low M_r estimates of oligomeric arginases, but this does not explain low results obtained by other non-denaturing methods. Based on the data in Table 5-2-5, the scarcity

of pentameric enzymes and the number of arginases for which a hexameric structure has been formulated, it is probable that both *B. caldovelox* and *Thermus* 4-1A arginases are homomeric hexamers.

It was hoped that crosslinking experiments on the *B. caldovelox* and *Thermus* 4-1A arginases would eliminate the unlikely possibility that these enzymes are pentamers. However, 5mM dimethyl adipimidate (D 8138, Sigma) or dimethyl suberimidate (D 7636, Sigma) failed to crosslink 0.2mg/ml *B. caldovelox* arginase effectively at either pH7 or pH10. Preliminary glutaraldehyde crosslinking experiments with *B. caldovelox* arginase at pH7 did crosslink the enzyme, but each major band including the subunit band appeared as a doublet suggesting that a small SDS-dissociable peptide may be a component of a proportion of the arginase subunits. It will be necessary to optimize crosslinking and electrophoretic conditions to obtain useful information from glutaraldehyde crosslinking experiments. In particular, the Laemmli buffer system may be not be appropriate for the M_r determination of crosslinked proteins; a continuous phosphate buffer system is recommended by Sigma.

From the entries in Table 5-2-6 it is clear that the M_r estimates for arginase oligomers and subunits depend on the methods used. With the obvious exception of monomeric arginases, all arginases appear to be homomeric oligomers consisting of three, four or six subunits. The subunit M_r's of most arginases are between 30000 and 40000, although SDS-PAGE experiments indicate that subunit M_r's of bacterial arginases are significantly lower than those from eukaryotic organisms. With this fairly uniform subunit M_r, the M_r's of native arginases could be expected to cluster around multiples of 35000 as dictated by subunit structure. Referring to Table 5-2-6, the monomeric arginases (1-3) form the first cluster. Entries 4-17 consist of trimeric (and possibly

tetrameric) arginases, and those enzymes from entry 18 onwards are probably hexameric. The octameric subunit structure suggested for some high M_r arginases (Mora *et al.*, 1966; Reddy and Campbell, 1970) should be regarded with caution as it is based on an assumed subunit M_r of 30000.

The difficulties of determining arginase subunit structures are amply demonstrated by the numerous contradictory studies on rat liver and human liver arginases. For these two enzymes evidence is available to support both trimeric and tetrameric structures. Initial studies supported a tetrameric structure (Hirsch-Kolb and Greenberg, 1968; Carvajal *et al.*, 1971) but Penninckx *et al.* (1974) failed to detect a band that corresponded to a tetramer when glutaraldehyde crosslinked rat liver arginase was subjected to SDS-PAGE. The highest M_r band formed indicated a trimeric structure for rat liver and *Saccharomyces cerevisiae* arginases. This study and that of Berüter *et al.* (1978) found the subunit M_r of liver arginase estimated by SDS-PAGE to be 4000-5000 higher than when estimated by gel permeation chromatography or ultracentrifugation. A recent study found that mammalian liver arginase subunits have M_r's of 39000±1000 by SDS-PAGE and suggested that proteolytic degradation might have caused the lower M_r estimations in earlier studies (Brusdeilins *et al.*, 1985). Indeed, proteolysis *in vivo* (Spolarics and Bond, 1988) and during purification (Borkovich and Weiss, 1987a) has now been implicated in size-heterogeneity of subunits and this heterogeneity may be responsible for discrepancies in subunit M_r data. The reversible dissociation of rat kidney, rat liver and human liver arginases into dimers and monomers (but not trimers) by metal ion chelation with EDTA, acid treatment or modification with *p*-hydroxymercuribenzoate (Hosoyama, 1972; Barańczyk-Kuźma *et al.*, 1976; Carvajal *et al.*, 1982a) is good evidence for a tetrameric structure.

However, unless the high M_r values of eukaryotic arginase subunits (36000-39000) are the result of anomalous behaviour in SDS-PAGE, the current evidence favours a trimeric structure, since a tetrameric structure is inconsistent with these subunit M_r's and oligomeric M_r's of 100000 to 140000.

The available data suggests no particular correlation between oligomeric structure and enzyme source for arginase (Reddy and Campbell, 1970), refuting the proposal by Mora *et al.* (1965) of a relationship between an organism's mode of nitrogen excretion and the M_r of the arginase. Although tertiary structure is sufficient for expression of arginase activity the majority of arginases studied are oligomeric enzymes. This suggests that the oligomeric structure is functionally significant and might be required for regulation of activity. Cooperativity has seldom been observed for arginases but this may be due to kinetic studies being carried out at alkaline pH optima (see Table 5-3-2) rather than a more physiological pH. Carvajal *et al.* (1982b) noted cooperative effects for human liver arginase at pH7.5. Alternatively the regulatory significance for some arginases may lie in the ability of oligomeric arginases to enter into specific combinations with other enzymes as occurs in epiarginasic control (Davis, 1986).

5-3 Enzymatic Properties of Arginases

5-3-1 Development of an assay methodology for arginase

Because activation under non-physiological conditions has the potential to modify the properties of arginase (Pace *et al.*, 1980), the possibility of assaying "native metals" enzymes without preactivation or addition of a cofactor to the assay medium was investigated. In the absence of a divalent metal ion, assays with 2-70mM arginine were

non-linear with time at 70°C and to a lesser extent at 60°C in the pH range 6.5 to 9.6. Non-linearity was more pronounced at lower pH's, e.g. the ornithine formed after 16min represented 65%, 77%, 83% and 91% of that expected from an extrapolation of activity after 2min at pH6.5, 7, 8 and 9.6 respectively (Fig. 5-3-1). Product inhibition did not account for the non-linearity as % deviations were the same at lower arginase concentrations. Addition of Ca^{2+} or Mg^{2+} did not linearize the assays but the appropriate $[Mn^{2+}]$ at a particular assay pH did (e.g. 200μM Mn^{2+} at pH6.5, see Fig. 5-3-1). This effect is probably largely mediated by the maintenance of a constant level of Mn^{2+} saturation of arginase, non-linearity being due to dissociation of the Mn^{2+}-arginase complex during the assay. Assays conducted at pH9.8 or above were linear without addition of Mn^{2+} and the failure of Mn^{2+} to increase activity in assays conducted above pH9.8 may be due to oxidation or hydrolysis of the hydrated ion. The minimum $[Mn^{2+}]$ required to linearize a 16min assay with 20mM arginine at 60°C using *B. caldovelox* "native metals" arginase was determined for several pH values and was plotted against pH in Fig. 5-3-2.

In addition to promoting linear assays, Mn^{2+} ions activated the *B. caldovelox* enzyme. The maximum apparent activation was of the order of 27% at pH9 (see Fig. 5-3-2) and as linearization of the assay (by extrapolation from t=2min activity) accounts for ~11%, the remaining 16% must be due to a rapid activation phenomenon. Both linearization and activation contributed to the shape of graphs of % activation vs $[Mn^{2+}]$ in Figures 5-3-2 and 5-3-3 and the relative contributions varied with pH (linearization accounts for 71% of the apparent activation seen at pH7, 56% at pH8 and ~40% at pH9 for *B. caldovelox* arginase). The main aim of these experiments was to indicate the minimum $[Mn^{2+}]$ required to give linear assays at a particular pH. However, assuming

both linearization and activation phenomena are related to interaction of Mn^{2+} with the active site, apparent K_{diss} values for the Mn^{2+}-arginase complex were calculated by plotting $[Mn^{2+}]/\%$ activation vs $[Mn^{2+}]$, i.e. a Hanes plot. K_{diss} values for *B. caldovelox* arginase were 1.5μM (pH9), 6μM (pH8) and ~45μM (pH7) at 60°C in the presence of 20mM arginine. The K_{diss} values for *Thermus* 4-1A arginase were 1μM (pH9), 8μM (pH8) and 15μM (pH7) under the same assay conditions. Using a similar approach, apparent K_{diss} values of 2μM at pH9.5 (Borkovich, 1985), 8μM at pH9.6 (Vielle-Breitburd and Orth, 1972) and 33μM and 56μM at pH7 at 38°C (Campbell, 1966) have been found for other arginases. The latter value for rat liver arginase agrees well with the K_{diss} of 50μM found for the two low-affinity sites of this enzyme in NMR studies at pH7.5 at 34°C (Hirsch-Kolb *et al.*, 1971).

Under the assay conditions established for *B. caldovelox* "native metals" arginase the reaction was linear with time (for ≥6min) over the range of pH's (pH6.5-9.6) and substrate concentrations (2-70mM) employed. The assay was also linear with enzyme concentration over at least a ten-fold concentration range appropriate to the assay conditions, e.g. 0.55ng/ml-11ng/ml in the assay tube at pH9 with 20mM arginine and 5μM Mn^{2+}. *Thermus* 4-1A "native metals" arginase lost a greater proportion of the Mn^{2+} cofactor during purification and so showed a much greater % activation. Assays of this enzyme in the absence of Mn^{2+} also resulted in negative deviations from linearity but inclusion of Mn^{2+} caused large positive deviations. For this enzyme preactivation would appear to be a prerequisite for an assay that is linear with time.

5-3-2 The pH optimum

The activity of *B. caldovelox* "native metals" arginase was measured at 60°C with 70mM arginine in the presence of the minimum $[Mn^{2+}]$ required for a linear assay at each pH between pH6.5 and pH9.6 (Fig. 5-3-4). The broad pH range for optimal activity centred at pH9 is similar to that found for some plant arginases but lower than the optima for other bacterial arginases (see Table 5-3-1). *B. caldovelox* arginase prepared by purification scheme I yielded a similar pH-activity curve under the same assay conditions. The pH optimum for *Thermus* 4-1A arginase (preactivated as described in Fig. 5-3-8) was pH9.3-9.8, when assayed with 20mM arginine at 60°C with the same concentrations of Mn^{2+} used for *B. caldovelox* arginase.

Pace *et al.* (1980) discussed the factors which might contribute to

Table 5-3-1 pH optima of arginases

Source	pH optimum	Reference
Evernia prunastri		
constitutive	6.5	Legaz & Vicente, 1982
inducible	9.1	Legaz & Vicente, 1980
Artichoke tubers	9.0	Wright *et al.*, 1981
Iris bulb	9.0	Boutin, 1982
Bacillus caldovelox	9.0	
Thermus 4-1A	9.3-9.8	
Equine liver	9.3	Bach & Killip, 1961
	10.2	Long, 1961
Pumpkin cotyledons	9.5	Splittstoesser, 1969
Human liver	9.5	Berüter *et al.*, 1978
Genypterus maculatus	9.5	Carvajal *et al.*, 1987
Rat liver	9.5-9.7	Tarrab *et al.*, 1974
	10	Campbell, 1966
Bacillus licheniformis	9.5-10	Ramaley & Bernlohr, 1966
Staphylococcus aureus	9.6	Soru & Zaharia, 1976
Rabbit liver	9.8	Vielle-Breitburd & Orth, 1972
Bacillus anthracis	9.8-10	Soru, 1983
Pheretima communissima	10	Iino & Shimadate, 1986
Rat small intestine	10	Fujimoto *et al.*, 1976

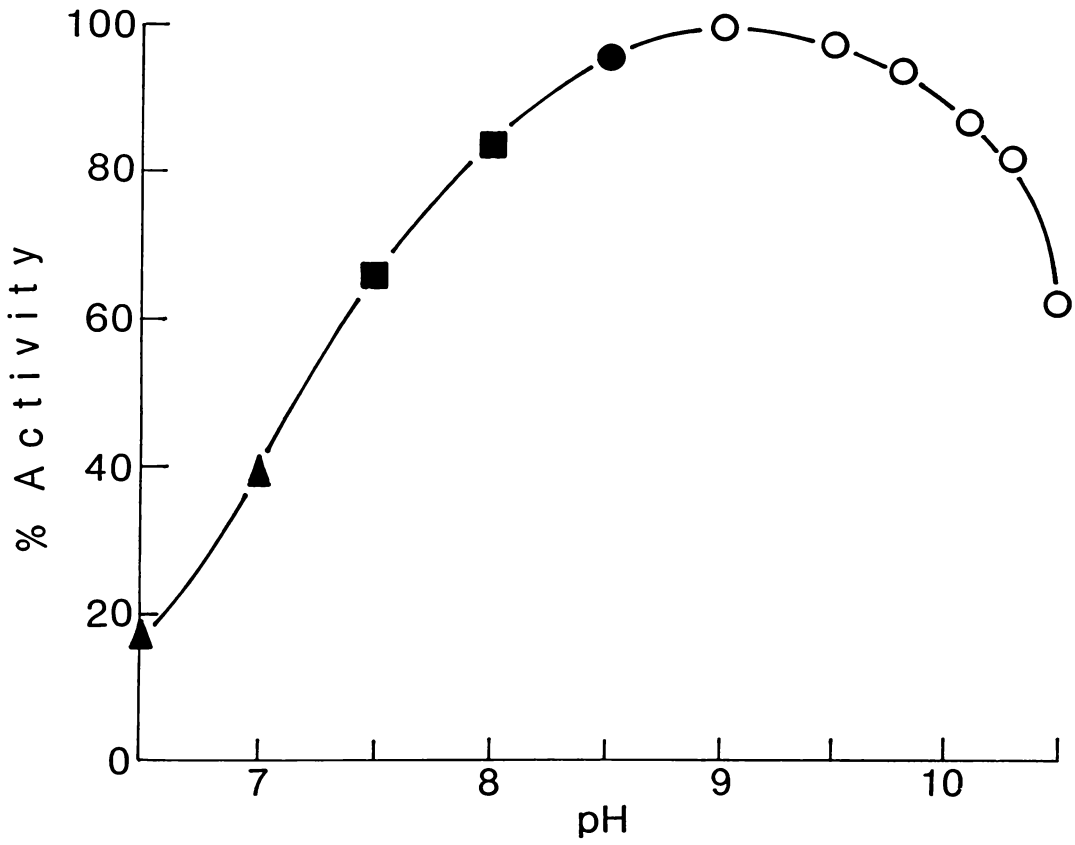

Figure 5-3-4 pH optimum of *B. caldovelox* arginase.

Assays were performed at 60°C using 70mM arginine as substrate. The $[Mn^{2+}]$ present at each pH was as specified in the legend of Fig. 5-3-2. *B. caldovelox* arginase ("native metals", 0.33mg/ml) was diluted 2000x in 25mM CAPS/NaOH buffer, pH9.8 (20°C) and assayed for 6min in 0.1M MOPS/NaOH (▲), 0.1M EPPS/NaOH (■), 0.1M CHES/NaOH (●) and 0.1M CAPS/NaOH (O) buffers at the pH values indicated. Assays (0.5ml) were stopped with 0.2ml 36% (w/v) acetic acid and 0.2ml of this mix added to 0.3ml H_2O for determination of ornithine in the usual way. 100% activity at pH9 was ~4.1kU/mg.

the alkaline pH optima (~pH9-10) of most arginases including the ratio of charged and neutral forms of the substrate. At 60°C the neutral form predominates above pH8.1 suggesting that this is the preferred form of arginine for *B. caldovelox* arginase. However, the pH dependence of both the stability of the arginine-Mn^{2+} complex and the hydrolysis of Mn^{2+}_{aq} prevents a simple interpretation. Hellerman (1937) suggested that differences in the pH-activity profiles of 3 bovine liver metallo-arginases were related to the stability of the cofactor-arginase complexes so that the relatively weak binding of Mn^{2+} by arginase at pH5-6.5 was responsible for the more alkaline pH optimum. This does not appear to be the case for *B. caldovelox* arginase because the pH optimum without Mn^{2+} in the assay medium (pH9-9.5) was not greatly altered from that found when Mn^{2+} was included to stabilize the Mn^{2+}-arginase complex (pH9).

5-3-3 Determination of kinetic parameters

The effect of L-arginine concentration (2-40mM) on *B. caldovelox* arginase activity was determined at pH9 at 60°C with 5μM Mn^{2+}. The data shown in the Michaelis-Menten plot (Fig. 5-3-5) was replotted using the linear transformations of Lineweaver-Burk, Eadie-Hofstee (Fig. 5-3-6) and Hanes (Dixon and Webb, 1979). Close agreement was obtained for these plots which were all linear indicating that the enzyme displays Michaelis-Menten kinetics in this range of substrate concentration. A K_m of 3.4±0.1mM (SD, n=5) with a V_{max} of ~4.4kU/mg was calculated for *B. caldovelox* "native metals" arginase. Complete activation of this enzyme with 0.5mM Mn^{2+} under the conditions described in Fig. 5-3-8 did not alter K_m but increased V_{max} by ~30% to 5.7kU/mg. Under the same assay conditions the K_m of *B. caldovelox* arginase prepared by

purification scheme I was 3.5mM. The *B. caldovelox* "native metals" arginase reaction rate remained constant at substrate concentrations between 50mM and 200mM arginine although the Michaelis-Menten equation predicts a of ~5% increase in rate over this range. This may be due to substrate inhibition which is often reported for arginases (e.g. Mora *et al.*, 1965; Carlisky *et al.*, 1972; Tarrab *et al.*, 1974; Hanlon, 1975) and is typically stronger than that seen here for *B. caldovelox*.

The K_m of "native metals" arginase was also determined at pH7 in 0.1M MOPS/NaOH buffer over a substrate concentration range of 2-70mM arginine in the presence of 0.1mM Mn^{2+} using 500x diluted 0.33mg/ml enzyme. Plots of the data using linear transformations of the Michaelis-Menten equation were linear (results not shown) and a K_m of 25mM was calculated. This is a 7.4-fold increase in the K_m seen at pH9. With few exceptions (e.g. Bedino, 1977, Carvajal *et al.*, 1982b) arginases show Michaelis-Menten kinetics at both neutral pH and the pH optimum (e.g. Middelhoven, 1969; Carvajal *et al.*, 1984, 1987; Iino and Shimadate, 1986). At near neutral pH, K_m values are typically 2 to 6-fold higher than those determined at the pH optimum. It is possible that a decreased concentration of the neutral form of substrate arginine at the lower pH is responsible but exceptions to the trend (e.g. Carvajal *et al.*, 1987) suggest that other factors are involved. The K_m's and specific activities of several arginases are listed in Table 5-3-2. Despite the wide range of reported values (spanning ~4 orders of magnitude) the most reliable studies indicate that mammalian liver and bacterial arginase K_m's are between 1mM and 10mM when determined at the pH optimum. The K_m of *B. caldovelox* arginase at pH9 is close to that found for three other bacterial arginases. The V_{max} of preactivated *B. caldovelox* "native metals" arginase is the highest specific activity value reported for an arginase. K_m values for

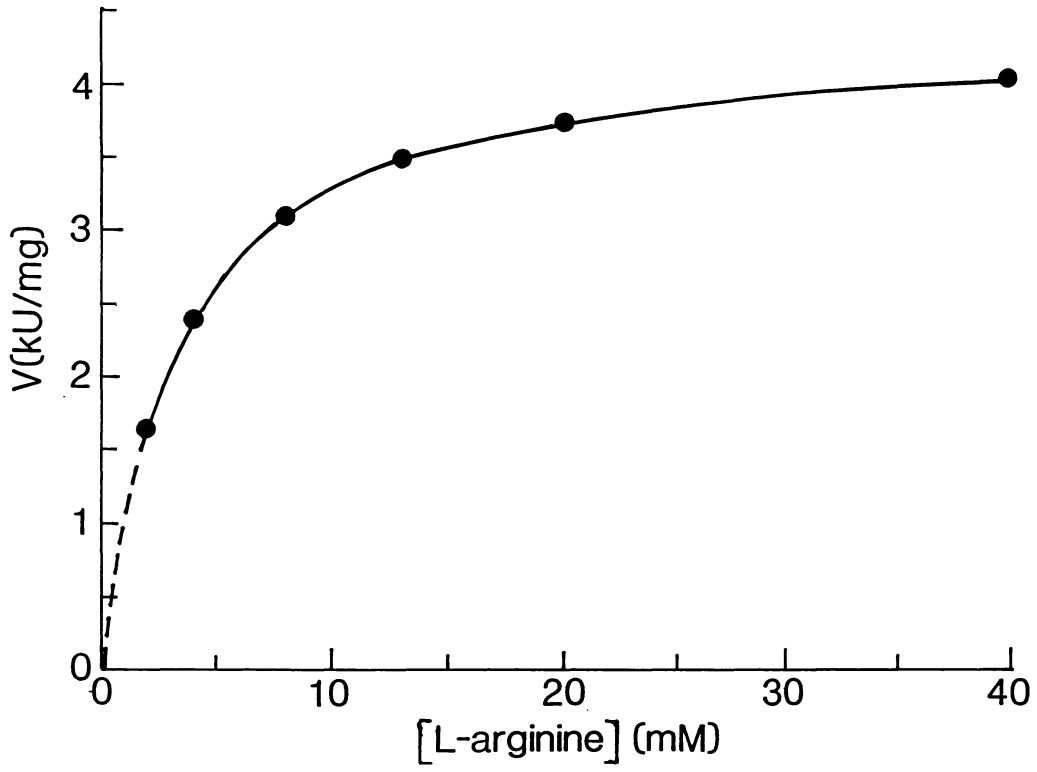

Figure 5-3-5 Michaelis-Menten plot of *B. caldovelox* arginase.

B. caldovelox arginase ("native metals", 0.33mg/ml) was diluted 2000× in 50mM CAPS/NaOH buffer, pH9.8 (20°C), and assayed in 0.1M CAPS/NaOH buffer, pH9 (60°C) at 60°C with 2mM to 40mM L-arginine in the presence of 5μM Mn^{2+}.

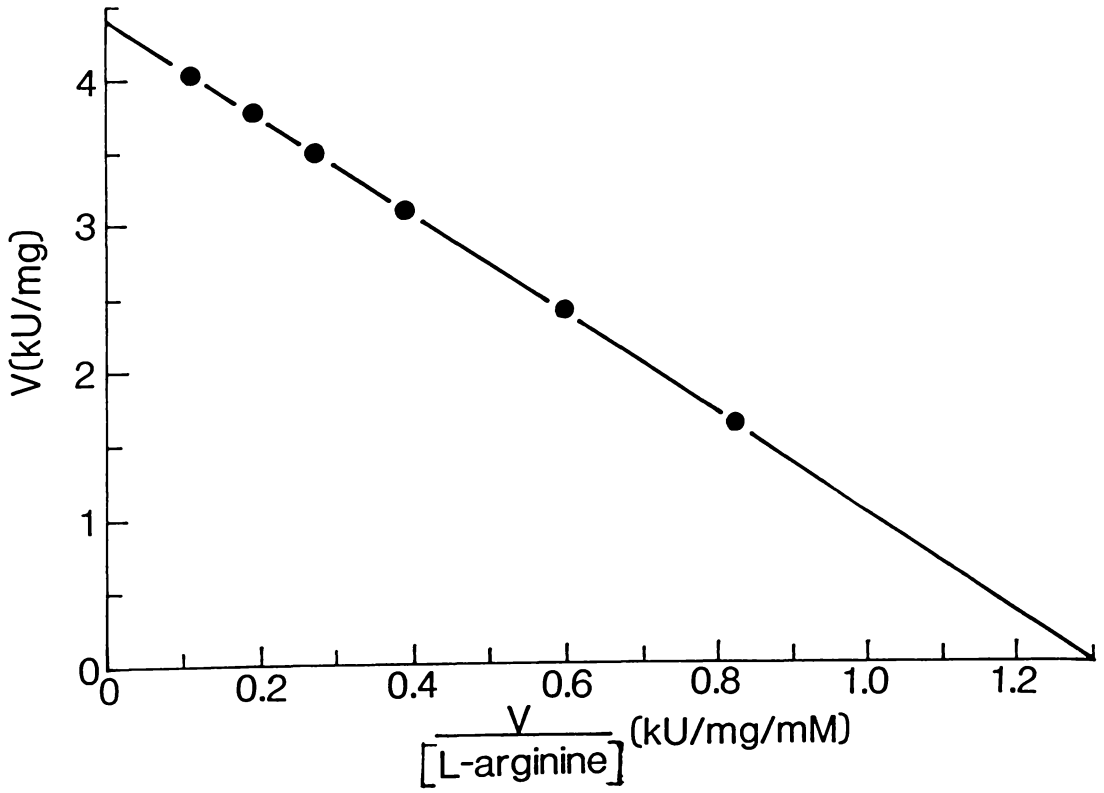

Figure 5-3-6 Eadie-Hofstee plot of Michaelis-Menten data.

Table 5-3-2 Kinetic parameters of arginases

Source	K_m (mM)	V_{max} (U/mg)	T (°C)	Reference
Pumpkin cotyledon	0.026			Splittstoesser, 1969
Evernia prunastri				
inducible	0.2			Legaz & Vicente, 1980
constitutive	2.5			Martin-Falquina & Legaz, 1984
Bovine liver	~0.35			Rosenfeld *et al.*, 1975
	0.95	790*	25	Harell & Sokolovsky, 1972
	4			O'Grady & Joyce, 1981
	10 (pH7.5)			
	15.3			Carlisky *et al.*, 1972
Saccharomyces cerevisiae	1.0 (pH8)			Penninckx, 1975
		890*	30	Penninckx *et al.*, 1974
Rat liver	1			Pace *et al.*, 1980
	~2 (pH7.5)			
mitochondrial	1			Cheung & Raijman, 1981
	2.5			Schimke, 1962
	3-6	5000*	37	Tarrab *et al.*, 1974
	4.5			Iino & Shimadate, 1986
	9.5 (pH7.5)			
	5.5			Campbell, 1966
	6.8			Kaysen & Strecker, 1973
	20-40			Mora *et al.*, 1966
		2790*	25	Hirsch-Kolb & Greenberg, 1968
Rabbit liver	1.3	2460*	37	Vielle-Breitburd & Orth, 1972
Staphylococcus aureus	1.8	200*	37	Soru & Zaharia, 1976
Bacillus licheniformis	2.2			Ramaley & Bernlohr, 1966
		1030*	37	Simon & Stalon, 1976
Bacillus anthracis	2.9	267*	37	Soru, 1983
Bacillus caldovelox	3.4	5700	60	
	25 (pH7)			
Human heart	5.5			Barańczyk-Kuźma *et al.*, 1980
Arachis hypogea	5.7			Desai, 1983
Chicken liver (high act.)	8			Rossi & Grazi, 1969
(norm. act.)	93			
	100-200			Mora *et al.*, 1966
Bacillus subtilis	~8			Issaly & Issaly, 1974
Pheretima communissima	8.5	3500*	37	Iino & Shimadate, 1986
	54 (pH7.5)			
Human liver	10.5	2090*	37	Berüter *et al.*, 1978
Rat kidney	18			Kaysen & Strecker, 1973
Rat small intestine	19			Fujimoto *et al.*, 1976
Bullfrog kidney	20			Carlisky *et al.*, 1972
CTAB solubilized	~130			Carlisky, 1972
Neurospora crassa	131	2610*	37	Borkovich & Weiss, 1987a
	100-200			Mora *et al.*, 1966
Artichoke tubers	145			Wright *et al.*, 1981
Pista pacifica	160	79*	25	O'Malley & Terwilliger, 1974
Pig liver		10*	25	Sakai & Murachi, 1969
Iris bulb		791*	30	Boutin, 1982

*Specific activity of purified (≥90% pure) arginase.

K_m values were measured near the pH optimum unless otherwise indicated.

arginases prepared from the same source can vary widely between studies. Much of this variation may be due to differences in the degree of saturation with a metal ion cofactor and different assay conditions (including the substrate concentration range) and assay methods. Pace *et al.* (1980) suggested that lower K_m values were obtained by the direct UV assay of arginase activity than by colorimetric methods.

5-3-4 Substrate and inhibitor specificity

Arginase is usually a highly specific enzyme and *B. caldovelox* arginase is no exception. Of the potential substrates tested only L-canavanine was hydrolysed at a rate >1% of that seen for L-arginine (Table 5-3-3). Many studies report the hydrolysis of the arginine analogue canavanine by arginase at rates ranging from 38% for rat kidney arginase (Kaysen and Strecker, 1973) to 0.6% for iris bulb arginase (Boutin, 1982). The 4.7% rate for *B. caldovelox* is similar to that found for a number of arginases (e.g. Campbell, 1966; O'Malley and Terwilliger, 1974; O'Grady and Joyce, 1981). There are only two reports of arginases that do not hydrolyse L-canavanine (Wright *et al.*, 1981; Iino and Shimadate, 1986). Hydrolysis of L-homoarginine is less common and generally less rapid than L-canavanine hydrolysis (values range from ~5% (Wright *et al.*, 1981; Boutin, 1982) to 0.36% (Kaysen and Strecker, 1973)) indicating the importance of correct chain length for effective catalysis. D-arginine hydrolysis by arginase has only been reported for rabbit small intestine arginase (Fujimoto *et al.*, 1976). It is possible that the hydrolysis of D-arginine by *B. caldovelox* "native metals" arginase is due to a contaminating arginine racemase activity or contamination of the substrate by L-arginine. Attempts to inhibit possible racemase activity by preincubation with 10mM hydroxylamine (an inhibitor of pyridoxal phosphate enzymes) did not

Table 5-3-3 Substrate/inhibitor specificity of *B. caldovelox* arginase

Substrate/inhibitor[a]	% activity	% inhibition	
		5mM arginine	70mM arginine
L-arginine	100	-	-
L-canavanine	4.7	6	0
D-arginine	0.70	0	0
L-homoarginine	0.47	4	4
L-argininic acid	0.09	0	0
N_{ω}-nitro-L-arginine	0.07	3	0
N_{α}-acetyl-L-arginine	0	0	0
L-argininamide	0	0	0
Agmatine	0	7	2
Creatine	0	30	10
4-guanidinobenzoic acid	0	2	0
N^G,N^G-dimethyl-L-arginine	0	3	0
Guanidinoacetic acid	0	5	1
γ-guanidinobutyric acid	0	28	12
L-α-amino-β-guanidino-propionic acid	0	32	12
2-oxoarginine	0	35	12
L-leucine	-	14	7
L-isoleucine	-	36	14
L-valine	-	30	12
L-lysine	-	34	14
L-ornithine	-	71	34
L-proline	-	4	2
Thiourea	-	26	25
Phenylthiourea	-	55	40
L-cysteine	-	20	8

[a]Compounds were used as substrates at a concentration of 20mM and the % activity of that seen on 20mM L-arginine is listed. Compounds were used as inhibitors at a concentration of 5mM and the % inhibition of assays with 5mM L-arginine and 70mM L-arginine is listed.

B. caldovelox arginase ("native metals", 0.33mg/ml) was diluted 2500× in 0.05M CAPS/NaOH buffer pH9.5 (20°C) and assayed for 6min in 0.1M CHES/NaOH buffer pH8.5 (60°C) containing 5μM Mn^{2+} and 20mM of the substrates listed. A 25× dilute enzyme solution was used for those substrates that were not significantly hydrolysed with the 2500× diluted enzyme solution. Urea or ornithine was detected as was appropriate for each substrate.

For % inhibition experiments 1500× or 2500× diluted 0.33mg/ml enzyme was assayed using 5mM arginine or 70mM arginine respectively in the presence of 5mM inhibitor. 5μM Mn^{2+} was included in all assays. Assays with 70mM arginine were stopped and processed as described in Fig. 5-3-4. Urea, alanine, aspartic acid, asparagine, glutamic acid, glutamine, glycine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine, 4-aminobutyric acid, ε-aminocaproic acid, NH_4Cl, creatinine and putrescine (all at 5mM) caused <4% inhibition in assays with 5mM arginine.

decrease the amount of ornithine formed from D-arginine. Urea was produced from incubations of arginase with L-arginine methyl ester but activity increased with increasing periods of substrate pre-equilibration, indicating that ester hydrolysis was occurring under the assay conditions. It was not possible to test citrulline as a potential substrate by the normal colorimetric methods because of the positive reaction citrulline gives with both ornithine and urea reagents. However, analysis of stopped assay mixtures by RP-LC showed that no ornithine was formed from L-citrulline. The same result was found for bovine liver arginase (O'Grady and Joyce, 1981).

The inability of *B. caldovelox* arginase to hydrolyse agmatine or L-argininamide shows that the α-COOH group of arginine is essential for catalysis. Similarly, modification or substitution of the α-NH_2 group (L-argininic acid, N_α-acetyl-L-arginine, 2-oxoarginine) or the guanidino group (citrulline, N_ω-nitro-L-arginine, N^G,N^G-dimethyl-L-arginine) prevented or severely limited the rate of hydrolysis so demonstrating the importance of these groups. Changes to the length of the carbon chain (L-homoarginine, L-α-amino-β-guanidino-propionic acid) or the substrate stereochemistry (D-arginine) both caused a large decrease in the hydrolytic rate. The conclusion that the three functional groups and the correct chain length and conformation of the substrate arginine are all required for efficient catalysis has been borne out in other studies (Greenberg, 1960; Roche, 1960; Campbell, 1966). These constraints on substrate structure are not necessarily absolute. For example, *B. caldovelox* arginase hydrolysed L-argininic acid at 0.09% of the rate seen with L-arginine and for bovine liver arginase the relative rate was ~0.03% (Hunter and Woodward, 1941).

In most respects the inhibition pattern seen for the *B. caldovelox* enzyme is typical of arginases with ornithine, lysine and

branched-chain amino acids inhibiting the enzyme (e.g. Kaysen and Strecker, 1973; Fujimoto *et al.*, 1976; Carvajal *et al.*, 1984; Iino and Shimadate, 1986) although the degree of inhibition by proline is small compared to other studies. Inhibition of *B. caldovelox* arginase by L-cysteine and thiourea compounds may be related to their Mn^{2+}-complexing properties and for thiourea the inhibition is not competitive. The weak inhibition by putrescine compared to ornithine demonstrates that the α-COOH group is required for effective inhibition and this is consistent with the results of a recent study on bovine liver arginase (Subrahmanyam and Reddy, 1986). Unusual aspects of *B. caldovelox* inhibition include the poor inhibition by L-homoarginine (the +1C analogue) compared to L-α-amino-β-guanidino-propionic acid (the -2C analogue) and the inhibition by 2-oxoarginine, but not by

Table 5-3-4 Inhibition of arginase by ornithine

Source	K_i (mM)	pH	Reference
Saccharomyces cerevisiae	0.25	8.0	Penninckx, 1975
	~1.6		Middelhoven, 1969
	1.4	7.5	
Bacillus caldovelox	0.55		
	4.4	7.0	
Snail hepatopancreas	0.7		Campbell, 1966
Bovine liver	1.3		Pace & Landers, 1981
	1.5		Subrahmanyam & Reddy, 1986
	3	7.5	Kuchel *et al.*, 1975
	4.1		Hunter & Downs, 1945
	7.4		Carlisky *et al.*, 1972
Rat liver	1.3		Campbell, 1966
	4.4		Carlisky, 1972
Bacillus subtilis	2	8.5	Issaly & Issaly, 1974
Bullfrog kidney	4.9		Carlisky *et al.*, 1968
Concholepas concholepas	6.2		Carvajal *et al.*, 1984
Genypterus maculatus	9.3	7.5	Carvajal *et al.*, 1987

Unless otherwise indicated values were determined at or near the pH optimum.

L-argininic acid. It may be that the greater structural diversity of arginase inhibitors is due in part to the competitive inhibitors (ornithine and lysine) and noncompetitive or mixed-type inhibitors (branched-chain amino acids and proline) acting at different sites, as has been proposed for rat kidney and liver arginases (Carvajal and Cederbaum, 1986).

The K_i(orn) for *B. caldovelox* arginase was determined at pH9 (Fig. 5-3-7) and pH7 by the method of Dixon (1953). The results are given in Table 5-3-4. Inhibition of arginase by ornithine was competitive at pH9 (data not shown). Virtually all arginases are inhibited by ornithine and there are very few exceptions to the competitive type of inhibition. Urea is not an inhibitor (e.g. Long, 1961; Carlisky *et al.*, 1972) except in the cases of two arginases from *Evernia prunastri* (Legaz and Vicente, 1983; Martin-Falquina and Legaz, 1984) which are activated by ornithine (K_a 0.13mM (inducible) and ~1.1mM (constitutive)) and inhibited by urea (K_i 2.6mM).

K_i(orn) increases 8-fold from pH9 to pH7 and a similar increase is seen in the K_m so at both pH values the K_m:K_i(orn) ratio is ~6. The inhibition of *B. caldovelox* arginase by ornithine is likely to be of physiological significance during growth on arginine when the extracellular concentration of ornithine can exceed the arginine concentration (see Section 3-3-3).

5-3-5 Inhibitors and activators of arginase

B. caldovelox "native metals" arginase was assayed for 6min at pH8.5 at 60°C with 70mM arginine and 5μM Mn^{2+} and the effects of various additives on enzyme activity assessed. 1mM $AgNO_3$ and 5mM NaF caused 26% inhibition, 10% (w/v) EtOH caused 20% inhibition and 5mM *o*-phenathroline, 5mM trisodium citrate, 5mM tetrasodium pyrophosphate

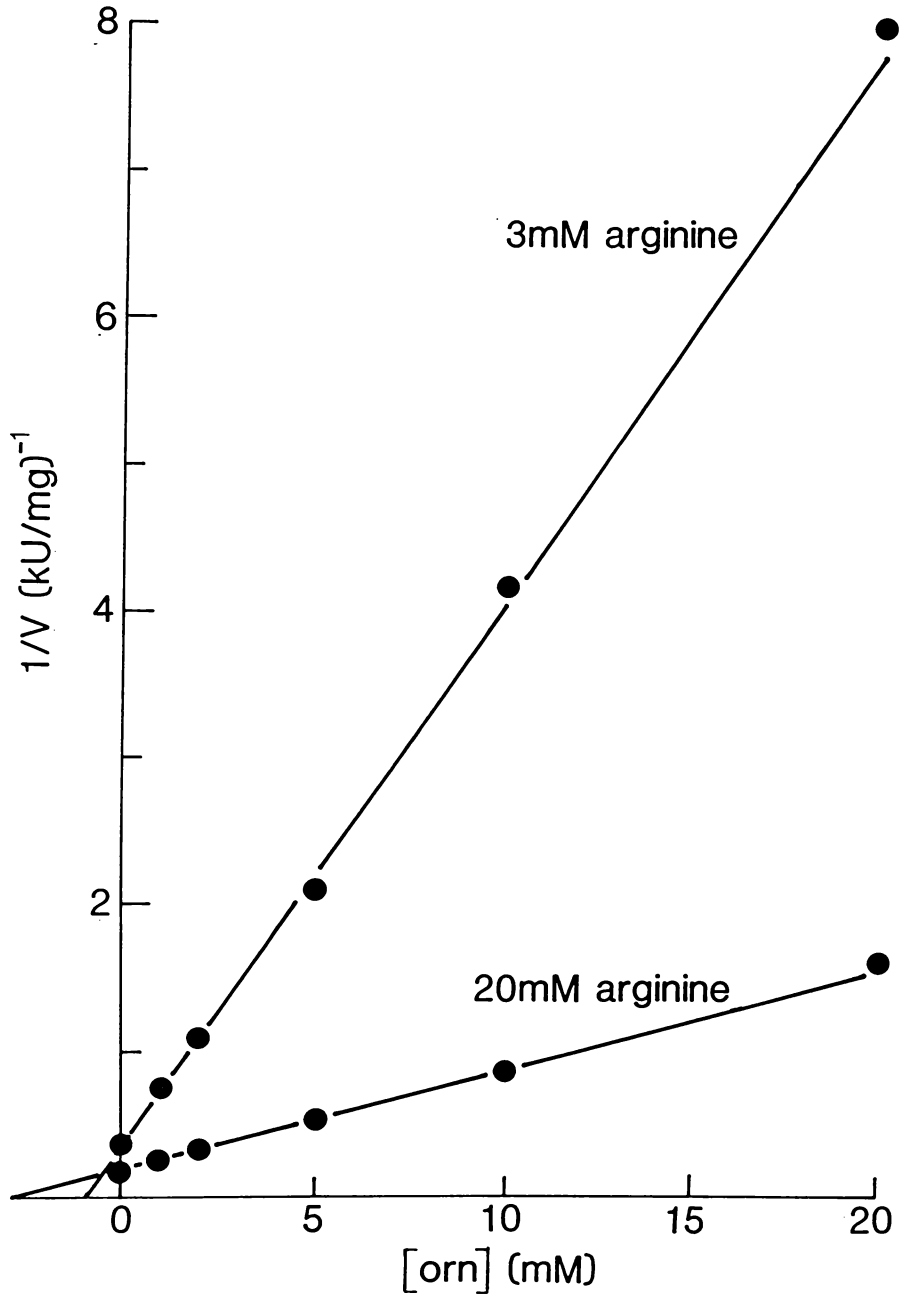

Figure 5-3-7 Dixon plot of arginase inhibition by ornithine at pH9.

B. caldovelox arginase ("native metals", 0.33mg/ml) was diluted 1500× in 0.1M CAPS/NaOH buffer pH9.9 (20°C) and assayed with 3mM or 20mM arginine solutions containing 5μM Mn^{2+} and the ornithine concentrations indicated for 6min at pH9 at 60°C. The amount of urea formed was determined colorimetrically.

and 1mM sodium tetraborate resulted in ~12-15% inhibition. Slight activations of 6% and 11% were seen with 5mM KBr and 5mM KI respectively. 5mM diethyldithiocarbamate, 5mM Na_2HPO_4, 5mM sodium sulphite, 5mM NaN_3, 5mM $NaHCO_3$, 5mM KCl, 1mM *p*CMBS (*p*-chloromercuribenzenesulphonic acid), 10% (w/v) Triton X-100, 10% (w/v) glycerol, 10% (w/v) acetonitrile and 1mg/ml BSA had no effect on activity. These results are consistent with those reported for many arginases from mesophilic sources. Borate is considered a potent noncompetitive inhibitor of arginase (Pace and Landers, 1981) and the slight (12-15%) inhibition by this and other compounds is probably related to their Mn^{2+}-complexing/precipitating properties. The lack of inhibition by *p*CMBS is typical (e.g. Campbell, 1966; Mora *et al.*, 1966; Kaysen and Strecker, 1973; O'Malley and Terwilliger, 1974; Carvajal *et al.*, 1982b, 1984), although some arginases including two bacterial enzymes are strongly inhibited by this thiol group reagent (Mora *et al.*, 1966; Soru and Zaharia, 1976; Wright *et al.*, 1981; Soru, 1983). Greenberg *et al.* (1956) demonstrated that thiol groups and disulphide bonds play no part in equine liver arginase activity.

5-3-6 Activation energies of arginases

The reaction rate of preactivated *B. caldovelox* and *Thermus* 4-1A arginases was measured at pH9 over a 40-80°C temperature range (Fig. 5-3-8). The activation energies of arginases listed in Table 5-3-5 fall between 30 and 52kJ/mole and values for the thermophilic arginases do not extend this range. *B. caldovelox* arginase activity decreased above ~70°C under the assay conditions used. As the rate of thermal denaturation at 70°C is still slow compared to the 6min assay time (see Section 5-4-2) other factors may be responsible, e.g. an increase in K_m at temperatures >65°C. *Thermus* 4-1A arginase is stable to at least 80°C

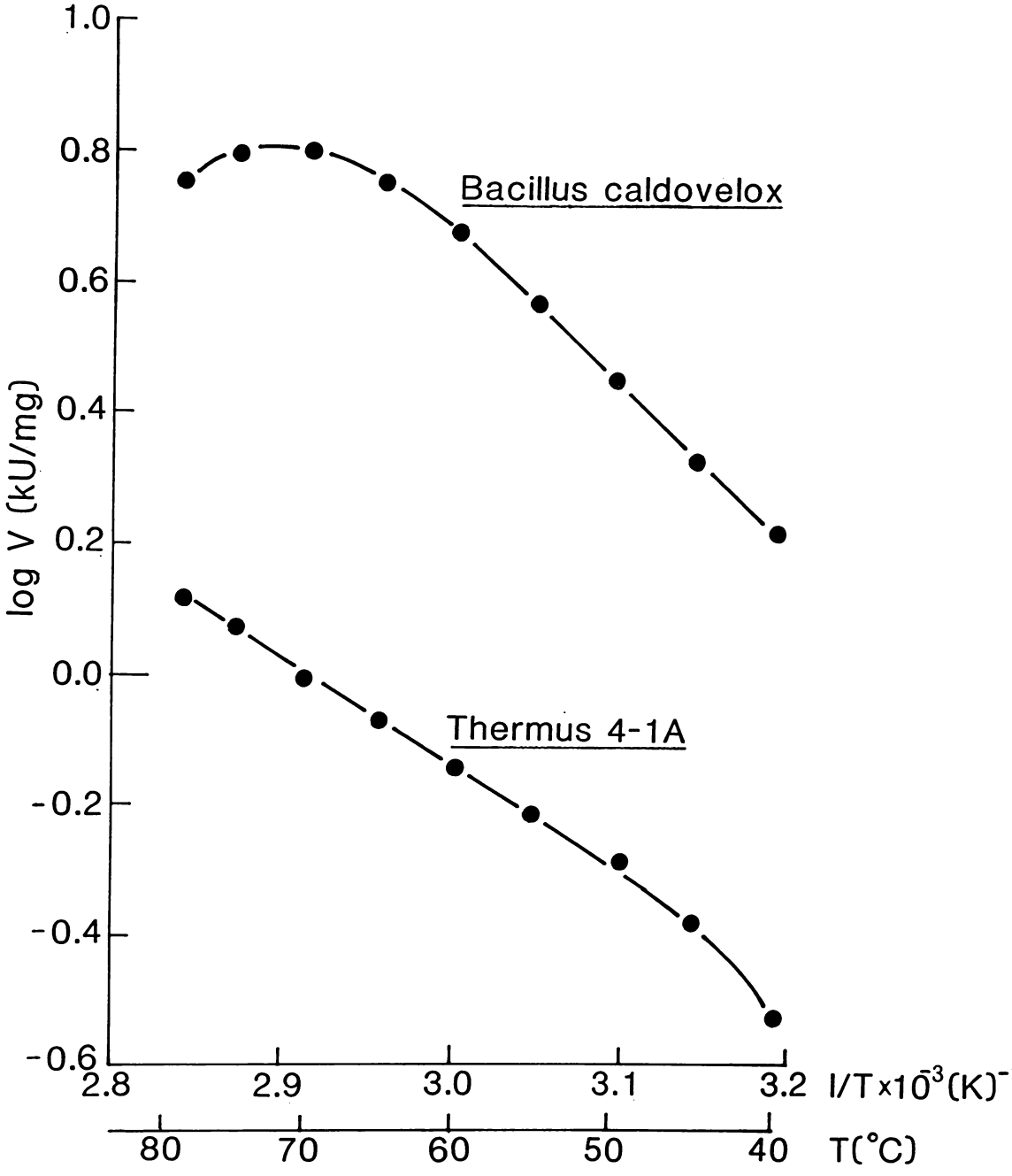

Figure 5-3-8 Arrhenius plots for *B. caldovelox* and *Thermus* 4-1A arginases.

B. caldovelox arginase ("native metals", 13μg/ml) was activated at 75°C at pH7 with 0.5mM Mn^{2+} in the presence of 1mg/ml BSA for 6h. *Thermus* 4-1A arginase ("native metals", 30μg/ml) was activated under the same conditions for 45min. The preactivated arginases were diluted appropriately in 0.1M CAPS/NaOH buffer pH9.9 (20°C) and assayed for six minutes at pH9 with 20mM arginine in the presence of 5μM Mn^{2+}. The pH of each buffered substrate solution was adjusted at the temperature of use. The slope of each line was calculated by linear regression of 40-60°C data and 50-80°C data for *B. caldovelox* and *Thermus* 4-1A arginases respectively.

under the assay conditions.

Table 5-3-5 Activation energies of arginases

Source	E_a (kJ/mole)	Reference
Thermus 4-1A	30.2	
Rat liver (35-60°C)	31	Campbell, 1966
(10-35°C)	49.3	
(mol. form 2, pH7)	35.1	Tarrab *et al.*, 1974
(mol. form 1, pH7)	50.2	
Staphylococcus aureus	31.8	Soru & Zaharia, 1976
Concholepas concholepas	32.6	Carvajal *et al.*, 1984
Pista pacifica	33.9	O'Malley & Terwilliger, 1974
Snail hepatopancreas	37.5	Campbell, 1966
Human erythrocyte	39.7	Cabello *et al.*, 1961
Human liver	41.8	
Bacillus caldovelox	46.3	
Genypterus maculatus	48.5	Carvajal *et al.*, 1987
Bacillus anthracis	51.5	Soru, 1983

Unless otherwise indicated activation energies were estimated at or near the optimum pH for activity.

5-3-7 Binding of arginase to L-arginine-agarose matrices

Two L-arginine-sepharose affinity chromatography matrices were tested for the ability to bind *B. caldovelox* and *Thermus* 4-1A arginases. L-arginine was attached directly to cyanogen bromide activated 4% agarose though the α-NH_2 group (6μmoles/ml, A 1018, Sigma) or through a 10C spacer arm to epoxy activated 4% cross-linked agarose (1.4μmoles/ml, A 8405, Sigma) also by the α-NH_2 group. Both these matrices bound arginase completely at low ionic strength (25mM Tris/HCl, pH8.3 (5°C)) at 5°C. The arginases were not eluted by 5, 10 or 20mM L-arginine but ~2000U (assayed at pH9 (60°C) with 20mM arginine and 5μM Mn^{2+}) of purified *B. caldovelox* arginase immobilized on a 1ml column of the directly-coupled L-arginine-agarose matrix processed most of the arginine in a 20mM solution to ornithine and urea at ~5°C, pH8.4

as it passed through the column. 20 and 50mM L-ornithine did not elute the enzymes (diluted fractions were assayed for urea production) and this eliminated the unlikely possibility that an ornithine-agarose matrix produced by enzymatic degradation of the arginine ligand was binding the arginase. The enzymes were eluted by buffers containing 0.2-0.3M KCl. The most likely explanation for the elution of arginase by buffers of moderate ionic strength and not by arginine or ornithine is that the positively charged guanidino group of arginine is acting as an anion exchanger rather than as an affinity ligand.

5-4 Metal Ion Cofactors and Stability of Arginase

5-4-1 Substitution of metal ions in arginase

The effect of incubating crude *B. caldovelox* arginase and partially purified *Thermus* 4-1A arginase with the chelating agent CDTA and subsequent reactivation with various divalent metal ions on enzyme activity was assessed. For Mn^{2+}-reactivation of CDTA-inactivated *B. caldovelox* and *Thermus* 4-1A arginases recoveries of activity were generally <50% and if CDTA-inactivated *B. caldovelox* arginase was dialysed prior to reactivation recoveries did not exceed 15%. These values are low compared to arginases from mesophilic sources for which reactivation recoveries of 70-100% are common (e.g. Hosoyama, 1972; Carvajal *et al.*, 1984). Greater attention to the preparation and storage of apoarginase coupled with the more appropriate reactivation conditions suggested by other work (e.g. incubation at moderate temperatures (up to ~60°C) for several hours with a low concentration of the metal ion (~1mM) in the presence of 1mg/ml BSA at pH7) might improve reactivation recoveries.

Post-GP-HPLC (TSK-Gel) *Thermus* 4-1A arginase (2mg/ml, 74U/mg when

assayed at pH7 at 70°C with 20mM arginine in the presence of 0.1mM Mn^{2+}) was prepared essentially by purification scheme I from *Thermus* 4-1A fermenter batch culture cells grown for extracellular protease production on *Thermus* medium (Hickey and Daniel, 1979) supplemented with 3g/l TP and 3g/l YE. The partially purified arginase was incubated at 70°C with 15mM neutral CDTA in 25mM MOPS/NaOH buffer pH7 (70°C). After 40min the activity (assayed as above but without 0.1mM Mn^{2+}) had decreased to ≤1% of the original activity. This inactivated arginase was diluted 100-fold into 10mM solutions of metal ions in 0.2M acetic acid/NaOH buffer pH5.2, 0.2M MES/NaOH buffer pH6.3 or 0.2M MOPS/NaOH buffer pH7.4 and incubated for 12h at 20°C. In each case the highest pH compatible with the stability of a particular metal ion was used. The reactivation obtained with each metal ion is shown in Table 5-4-1. Many of the metal ion solutions used to reactivate *Thermus* 4-1A were prepared under anaerobic conditions with deaerated buffers to minimize oxidation and precipitation. The enhanced stability of the VO^{2+} ion under anaerobic conditions lead to increased % reactivation compared to that seen in aerobic solution. Cations activating *Thermus* 4-1A arginase can be divided into strong activators (VO^{2+} (anaerobic), Mn^{2+}, Ni^{2+}, Cd^{2+}) and weak activators (Mg^{2+}, V^{3+}, Fe^{2+} (anaerobic), Co^{2+} (anaerobic), Mo(III) (anaerobic)). The precipitations noted in Table 5-4-1 may have inhibited reactivation by some metal ions. When the Fe^{2+} and Mo(III) metallo-arginase solutions were reassayed after 30min exposure to air the percentage reactivation fell to ~3%.

At 70°C an 8min incubation of *B. caldovelox* arginase (cell-free extract) with 15mM CDTA in 25mM MOPS/NaOH buffer pH7 (20°C) caused complete inactivation of the enzyme when assayed at pH7 without Mn^{2+}. The inactivated enzyme was diluted 150-fold into 10mM solutions of metal ions buffered at pH5.2, 6.3, or 7.4 (as above) and incubated at

70°C for 10min. 25μl of these metallo-arginase preparations were assayed as described for reactivated *Thermus* 4-1A arginase. The only

Table 5-4-1 Reactivation of *Thermus* 4-1A apoarginase by metal ions

Metal ion	Reactivation pH	% reactivation[a]
no metal	7.4	<1
Mg^{2+}, Sr^{2+}	7.4	15
La^{3+}	6.3	8
V^{3+} [b]	5.2	26
VO^{2+}	5.2	20
VO^{2+} [b]	5.2	100
Fe^{2+} [b]	7.4	25
Mn^{2+}	5.2	63
Mn^{2+}	6.3	100
Mn^{2+}	7.4	100
Mn^{2+} [b]	7.4	100
Co^{2+}	7.4	24
Ni^{2+}	7.4	130
Mo(III) [b]	7.4	25
Cd^{2+}	7.4	113

[a]Expressed as a percentage of the reactivation seen after 12h incubation with 10mM Mn^{2+} at pH7.4 at 20°C in aerobic solution (~50% recovery of original activity). Less than 1% reactivation (at pH7.4 unless otherwise indicated) was seen with Ca^{2+}, Mo(VI), Pd^{2+} (ammonia and chloride complexes), Ag^{+}, Ba^{2+}, W(VI), Pt^{4+}, Al^{3+} (pH6.3), Zn^{2+} (pH6.3), Gd^{3+} (pH6.3), Ho^{3+} (pH6.3), Yb^{3+} (pH6.3), Lu^{3+} (pH6.3), Pb^{2+} (pH6.3), Cr^{3+} (pH5.2), Cu^{2+} (pH5.2), In^{3+} (pH5.2) or Au^{3+} (pH5.2, partial precipitation). Reactivated arginase preparations (10μl) were assayed with 20mM L-arginine for 5min at 70°C at pH8.5 in a total volume of 0.5ml.

[b]10mM solutions of these metal ions were prepared in boiled, $N_{2(g)}$ sparged buffer in an anaerobic chamber (90% $N_{2(g)}$, 10% $H_{2(g)}$). Solutions of inactivated arginase were reactivated for 12h at 20°C in the anaerobic chamber and sealed in air-tight tubes before removing the solutions from the chamber for assaying. No reactivation by Ti^{3+} (pH5.2, precipitated), V^{3+} (pH7.4, green colloidal precipitate), Cr^{2+} (pH7.4, orange precipitate) or Pt^{2+} (pH7.4, heavy black precipitate) was seen under these conditions. A solution of 0.1M Na_2MoO_4 and 0.3M DTT diluted 10× in pH7.4 buffer gave a yellow 10mM Mo(III) solution. 30mM DTT alone did not reactivate arginase. Fe^{2+} solution precipitated some green Fe(II) hydroxide.

All assays were conducted in degassed buffered substrate which was exposed to air after equilibration at 70°C. An assay blank was run for each metal ion but only Au, Pt and Pd ions caused a significant increase in the absorbance at 515nm. The salts used to prepare the metal ion solutions are listed in Section 2-9.

metal ions to reactivate *B. caldovelox* arginase were Mn^{2+} (~55% recovery of the original activity, set at 100% for comparison with other metal ions), Ni^{2+} (200%), Co^{2+} (100%) and Cd^{2+} (60%). That fewer metal ions activate *B. caldovelox* arginase than *Thermus* 4-1A arginase may be due to the poor stability of some metallo-arginases or metal ions at 70°C under the aerobic incubation conditions used for reactivation. Mg^{2+}, Ca^{2+}, VO^{2+} (pH5.2), Cr^{3+} (pH5.2), Cu^{2+} (pH5.2), Zn^{2+} (pH6.3), Sr^{2+}, Ba^{2+}, La^{3+} (pH6.3) and Lu^{3+} (slight precipitation) gave no apparent reactivation and Fe^{2+} oxidised rapidly and precipitated.

With the exception of Sr^{2+}, La^{3+}, V^{3+} and Mo(III) all of the metal ions that reactivated the thermophilic arginases are activators of one or more arginases from a mesophilic source. Because of the variety of procedures used to prepare, activate and assay arginases from different sources it is difficult to distinguish genuine species differences from procedural artifacts in the patterns of metal ion activation. Arginase from axolotl liver has been subjected to treatment with the widest range of metal ions (Palacios *et al.*, 1969). Ni^{2+}, Co^{2+}, Mn^{2+}, Fe^{2+}, and Zn^{2+} activated this enzyme but Cd^{2+} and Mg^{2+} were inhibitory. Bullfrog kidney (cytoplasmic), bovine liver, rat liver and earthworm arginases showed the most common pattern of activation, i.e. by Mn^{2+}, Co^{2+}, Ni^{2+} and Cd^{2+} (Carlisky, 1972; Dahlig and Porembska, 1977; Iino and Shimadate, 1986). Zn^{2+} does not activate these enzymes. Excessive concentrations of the metal ion (e.g. 50mM) or inappropriate incubation conditions may be responsible for the failure of one or more of the four commonly activating metal ions to activate in some studies, e.g. Co^{2+} for rat liver arginase (Campbell, 1966), as another study reports activation by 1mM Co^{2+} (Tarrab *et al.*, 1974). Subunit reassociation of oligomeric enzymes can be prevented by high concentrations of a

cofactor (Jaenicke, 1987) and there is some evidence that for each apoarginase and metal ion there is an optimum metal ion concentration (<<50mM) for the reactivation process (e.g. Anderson, 1945; Roche *et al.*, 1953).

Fe^{2+} is known to be a good activator of some arginases under the appropriate conditions (Hellerman and Perkins (1935-1936); Tarrab *et al.*, 1974). It is probable that Fe^{2+} belongs with Mn^{2+}, Co^{2+} and Ni^{2+} as a common activator of arginase but the instability of Fe^{2+} in solution has prevented more extensive studies. Mg^{2+} was a weak activator of *Saccharomyces cerevisiae* arginase and Mn^{2+}, Fe^{2+}, Co^{2+} and Ni^{2+} also activated, but Zn^{2+}, Ca^{2+}, Sr^{2+}, Cd^{2+} and La^{3+} did not (Middelhoven, 1969).

There appears to be no correlation between the properties of an activating metal ion (e.g. ionic radius, coordination geometry, Lewis acidity, substrate-metal ion complex stability) and its apparent catalytic efficiency. The molecular mechanism by which the metal ion permits catalysis may not be related to these parameters in arginase. However, it is possible that other factors such as differences in cofactor saturation of apoarginase attained during activation or a lack of optimization of assay and activation procedures for each metallo-arginase are masking a correlation. The above experiments were performed before information on enzyme stability was available and before the activation and assay methodologies for arginase had been fully developed, therefore these are only preliminary results. A more detailed study into the effects of metal ion substitution on *B. caldovelox* arginase activity is being carried out (J. M. Whittaker, pers. commun.).

5-4-2 Arginase thermostability

The thermostability of *B. caldovelox* arginase has proven to be dependent upon a number of factors and a brief overview of the content of this section will be given first for clarity. During preliminary thermostability experiments it became evident that under certain incubation conditions the observed inactivation of *B. caldovelox* arginase was not due to thermal denaturation but rather to the loss of Mn^{2+} ions from the active site. Efforts to eliminate this detrimental effect on activity by including Mn^{2+} in incubation solutions caused two further complications. The first was the Mn^{2+}-mediated activation of *B. caldovelox* "native metals" arginase during incubation, solved by full preactivation of the arginase with Mn^{2+}. The second involved Mn^{2+}-mediated inactivation of the enzyme, apparently caused by toxicity of unchelated Mn^{2+} and minimized by various chelating agents. Once these effects had been eliminated it was possible to examine the influence of other compounds on the thermostability of *B. caldovelox* arginase. A full account of the studies outlined above now follows.

Initial thermostability experiments studied the effect of pH on the half-life of "native metals" *B. caldovelox* arginase. At 60°C, in the absence of added Mn^{2+}, there was a decrease in apparent thermostability with decreasing pH (see Table 5-4-2). It will be seen later, however, (Section 5-4-3) that the quaternary structure of even the *B. caldovelox* apoarginase is stable at pH7 at 60°C. Thus the loss of activity is not due to thermal denaturation of the enzyme, but rather to the partial dissociation of the active Mn^{2+}-arginase complex. The more rapid inactivation at lower pH values results from an increase in the rate of this dissociation. It was hoped that dissociation of the Mn^{2+}-arginase complex could be prevented by maintaining an arginase-saturating $[Mn^{2+}]$ in the incubation buffer. Addition of 0.1mM

Mn^{2+} increased the half-life at pH7 (60°C) from 4.5min to 18min but this thermostability was still unexpectedly poor given the structural stability of the apoenzyme and the temperature at which *B. caldovelox* was grown (70°C). Poor thermostability was not due to heavy metal impurities in the buffer salts of the AR grade manganous salts used as extraction of concentrated salt solutions with 0.001% dithizone (D 5130, Sigma) in chloroform (SLR, Fisons) as described by Morrison (1979) did not detect any impurities.

An alternative explanation for the poor thermostability is that the hydrated Mn^{2+} ion (Mn^{2+}_{aq}) or its hydrolysis products can

Table 5-4-2 Effect of pH and Mn^{2+} on *B. caldovelox* thermostability.

Incubation conditions	$[Mn^{2+}]$ (mM)	1mg/ml BSA	$t_{1/2}$ (min)
60°C, 0.1M CAPS/NaOH pH9.8	0	-	107*
60°C, 0.1M CAPS/NaOH pH9	0	-	107*
60°C, 0.1M CHES/NaOH pH8.5	0	-	60
60°C, 0.1M EPPS/NaOH pH8	0	-	26
60°C, 0.1M MOPS/NaOH pH7	0	-	4.5
" " "	0.1	-	18
" " "	0	+	16
" " "	0.1	+	>5000
85°C, 0.1M CAPS/NaOH pH9	0.1	+	8
85°C, 0.1M CHES/NaOH pH8.5	0.1	+	25
85°C, 0.1M CHES/NaOH pH8	0.1	+	100
85°C, 0.1M EPPS/NaOH pH7.5	0.1	+	380
85°C, 0.1M MOPS/NaOH pH7	0.1	+	900
85°C, 0.1M MOPS/NaOH pH6.5	0.1	+	200

*First-order inactivation kinetics, i.e. graphs of log(% activity remaining) vs time are linear.

All experiments used *B. caldovelox* "native metals" arginase at a concentration of 0.13-0.2μg/ml in the incubation solution. 10μl of 13μg/ml preactivated *B. caldovelox* arginase or ~12-15μl of 13μg/ml unactivated *B. caldovelox* arginase was mixed with 1ml of the incubation solution equilibrated at the incubation temperature and a t=0min sample was taken 5-20min later, the exact time depending on the incubation temperature. $MnSO_4$ was used as a source of Mn^{2+} in these experiments. See Figures 5-4-1, 5-4-3 and 5-4-4 for further experimental details.

inactivate arginase at 60°C. It can be seen from Table 5-4-2 that the combination of 1mg/ml BSA with 0.1mM Mn^{2+} had a strong synergistic effect on arginase thermostability (no loss of activity after 24h at pH7 at 60°C), although each additive increased thermostability only slightly when used separately. This combination may function by maintaining *B. caldovelox* arginase in a Mn^{2+}-saturated state without exposing the enzyme to high concentrations of unchelated Mn^{2+}. The conditions probably duplicate most closely the *in vivo* environment of the enzyme in which the concentration of unchelated transition metal ions is certainly very low. Under incubation conditions that minimized dissociation of the active Mn^{2+}-arginase complex and Mn^{2+}-toxicity effects the pH optimum for thermostability was ~pH7 at 85°C (see Table 5-4-2). This differs markedly from the value of ≥pH9 obtained at 60°C in the absence of Mn^{2+} which actually indicates the pH optimum for stability of the Mn^{2+}-arginase complex rather than arginase thermostability.

The above experiments had shown that *B. caldovelox* arginase was very thermostable at near-neutral pH in the presence of Mn^{2+} and BSA. The pH and $[Mn^{2+}]$ were therefore varied in the presence of 1mg/ml BSA at 75°C to find the best conditions for fully activating *B. caldovelox* "native metals" arginase (Fig. 5-4-1). It was hoped that preactivation would eliminate the deviations from first-order inactivation kinetics seen in the presence of Mn^{2+} due to activation in the initial stages of incubation. It can be seen from Fig. 5-4-1 that at pH7 with 0.1mM Mn^{2+} and at pH8 with either 0.1mM or 0.5mM Mn^{2+} an initial activation was followed by a decrease in activity. At pH7 with 0.5mM, 1mM or 2mM Mn^{2+} the activation phenomenon was not deleteriously affected by prolonged incubation. From this study the preactivation procedure selected for use in subsequent thermostability experiments was a 6h incubation at

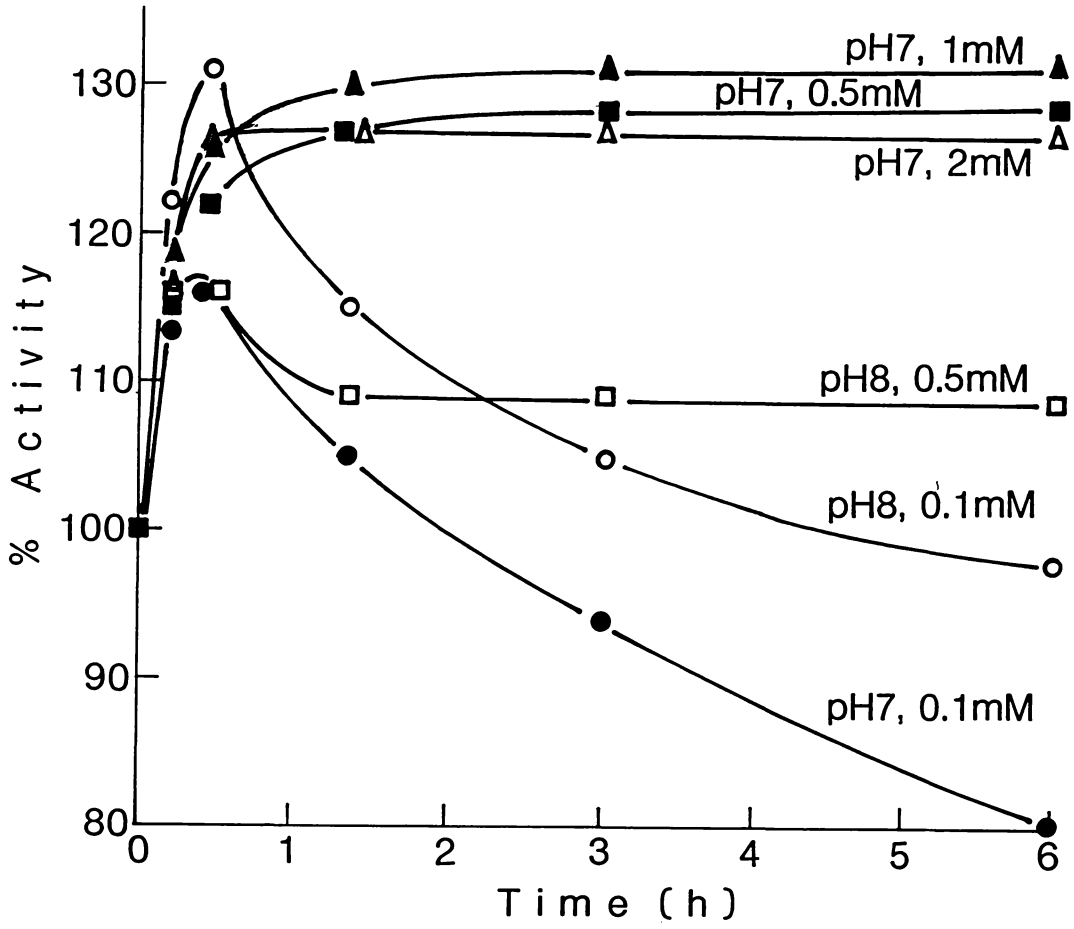

Figure 5-4-1 Activation of *B. caldovelox* arginase with Mn^{2+}.

B. caldovelox arginase ("native metals", 0.33mg/ml) was diluted 3333× into 1ml solutions of 0.1M MOPS/NaOH buffer pH7 (75°C) or 0.1M CHES/NaOH buffer pH8 (75°C) containing 1mg/ml BSA and the [Mn^{2+}] indicated. The solutions were incubated at 75°C in 1.5ml Wheaton sample vials (catalogue no. 225170, Wheaton Scientific) sealed with Teflon-faced silicone septa. 25μl samples were taken with a 25μl glass syringe (product code 25-FN-GP, SGE) at the times indicated and assayed at pH9 at 60°C with 20mM arginine in the presence of 5μM Mn^{2+} for 6min. The carryover of BSA from the incubation mix into the assay tube (up to 50μg/ml) did not alter enzyme activity or interfere with determination of ornithine. Mn^{2+} carryover had a slight effect on activity in some cases but this was corrected for by expressing all activity as a percentage of t=0min activity which was measured immediately before heating for each of the incubation conditions.

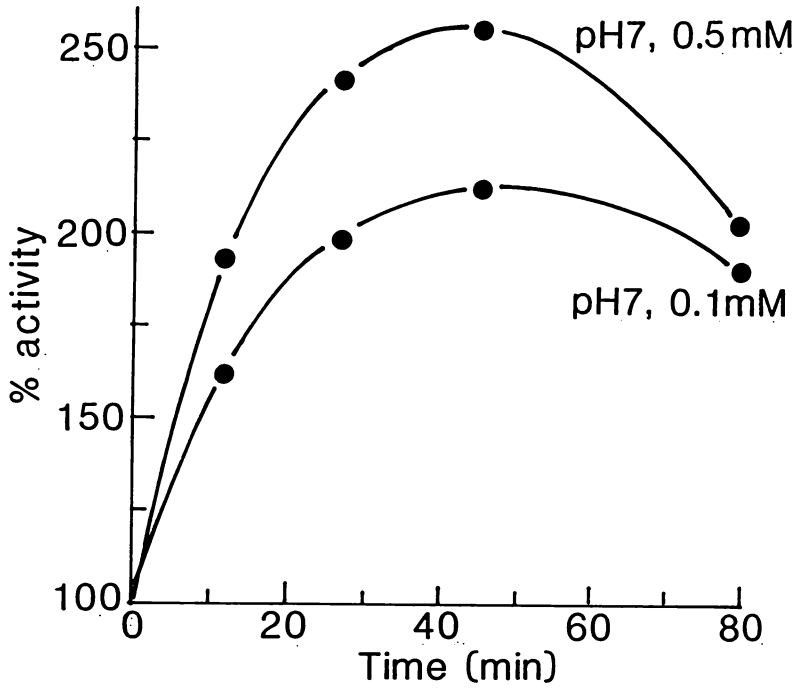

Figure 5-4-2 Activation of *Thermus* 4-1A arginase with Mn^{2+}.

Thermus 4-1A arginase ("native metals", 0.15mg/ml) was diluted 200x into 1ml solutions of 0.1M MOPS/NaOH buffer pH7 (75°C) containing 1mg/ml BSA and the $[Mn^{2+}]$ indicated. Solutions were incubated, sampled and assayed as described in Figure 5-4-1.

75°C at pH7 with 0.5mM Mn^{2+} in the presence of 1mg/ml BSA. Enzyme activity and Mn^{2+} were stable under these conditions and BSA remained soluble, unlike the incubations with 1mM and 2mM Mn^{2+}. Preactivated *B. caldovelox* arginase (typically 13μg/ml) was stored at 0-5°C and used within 24h of preactivation. Preactivation of *Thermus* 4-1A "native metals" arginase (Fig. 5-4-2) was less successful because this enzyme was not stable to the activating conditions.

The thermostability of preactivated *B. caldovelox* arginase was then studied under various conditions. At 95°C and pH7 the inactivation of preactivated *B. caldovelox* arginase obeyed first-order kinetics in the presence of 0.1mM-1mM Mn^{2+} and either 1mg/ml BSA or 25mM aspartic acid (results listed in Table 5-4-3 and presented graphically in Fig. 5-4-3). The loss of arginase activity under conditions for which the highest thermostability was observed is probably largely due to thermal denaturation, whereas inactivation under conditions which afforded suboptimal half-lives (<100min) would have been due partly to dissociation of the Mn^{2+}-arginase and/or Mn^{2+} toxicity. For example, the decrease in thermostability from 0.1mM to 1mM Mn^{2+} with BSA (Fig. 5-4-3A) can be explained in terms of an increasing concentration of toxic unchelated Mn^{2+} as the ~0.09mM Mn^{2+}-binding capacity of 1mg/ml BSA (Mildvan and Cohn, 1963) is exceeded. 25mM aspartic acid was more effective at stabilizing *B. caldovelox* arginase in the presence of high concentrations of Mn^{2+} and this may reflect a greater Mn^{2+} binding capacity. The K_{diss}(pH7, 95°C) of the Mn^{2+}-aspartic acid complex is 2.5mM (calculated from data in Martell and Smith, (1976)) and with 25mM aspartic acid the $[Mn^{2+}_{aq}]$ is approximately one tenth of the total $[Mn^{2+}]$ under these conditions. Addition of 1mg/ml BSA to a 0.5mM Mn^{2+}/25mM aspartic acid solution providing optimum stability at 95°C, pH7, did not improve thermostability and this suggests that BSA and

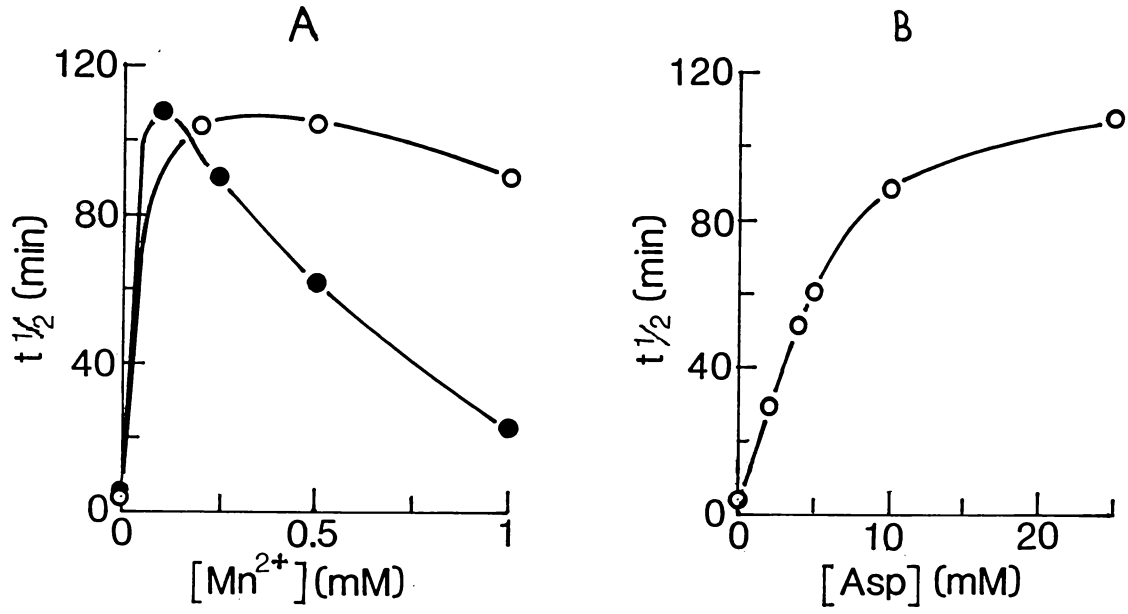

Figure 5-4-3 *B. caldovelox* arginase thermostability: effect of Mn^{2+}, BSA and aspartic acid.

10μl volumes of *B. caldovelox* arginase ("native metals", 13μg/ml, preactivated at 75°C at pH7 with 0.5mM Mn^{2+} and 1mg/ml BSA for 6h) were injected into 1ml solutions (equilibrated at 95°C in sealed 1.5ml Wheaton vials) of 0.1M MOPS/NaOH buffer pH7 (95°C) containing Mn^{2+} and BSA (A 4378, Sigma) or aspartic acid at the concentrations indicated below and in the Figure. After a further equilibration of the enzyme for 10-15min before the t=0min sample the solutions were sampled and assayed for residual activity as described in Figure 5-4-1. In these and all subsequent experiments in which aspartic acid was used 1.1 moles of NaOH was added with each mole of the free acid to maintain a pH of ~7.

A. Solutions contained either 1mg/ml BSA (●) or 25mM aspartic acid (O) and the $[Mn^{2+}]$ indicated. Because of carryover from the preactivated arginase solution the lowest $[Mn^{2+}]$ was 5μM.

B. All solutions contained 0.5mM Mn^{2+}.

aspartic acid both stabilize arginase by the same mechanism, i.e. chelation of excess Mn^{2+}. BSA (1mg/ml) precipitated with 0.5mM Mn^{2+} at 95°C, pH7 but did not precipitate in the presence of 25mM aspartic acid in the above system, so aspartic acid also protects BSA from precipitation again, presumably, by chelation of Mn^{2+}.

Other chelators of Mn^{2+} were also found to stabilize arginase. When incubated with 0.5mM Mn^{2+} at pH7 (95°C) in the presence of 5mM chelator the order of effectiveness of stabilization of preactivated *B. caldovelox* arginase was L-methionine < L-tryptophan < L-glycine < L-valine < L-asparagine < diaminoethane < L-histidine < L-aspartic

Table 5-4-3 Effect of BSA and aspartic acid in combination with Mn^{2+} on *B. caldovelox* arginase thermostability at pH7 at 95°C.

Additions to 0.1M MOPS/NaOH buffer pH7 (95°C)			$t_{1/2}$ (min)
$[Mn^{2+}]$ (mM)	1mg/ml BSA	[Aspartic acid] (mM)	
0.5	-	0	5
0.005	+	0	7
0.1	+	0	107*
0.25	+	0	90*
0.5	+	0	61*
1.0	+	0	22*
0.005	-	25	6
0.2	-	25	103*
0.5	-	25	106*
1.0	-	25	90*
0.5	-	2	28*
0.5	-	4	51*
0.5	-	5	60*
0.5	-	10	88*
0.5	-	25	105*

*First-order inactivation kinetics, i.e. graphs of log(% activity remaining) vs time are linear.

10μl of 13μg/ml preactivated *B. caldovelox* arginase was mixed with 1ml of 0.1M MOPS/NaOH buffer pH7 (95°C) equilibrated at 95°C and containing the additives indicated. The use of 0.5mM $MnCl_2$ rather than $MnSO_4$ at 95°C with 25mM aspartic acid made no difference to arginase thermostability. See Fig. 5-4-3 for further experimental details.

acid. When 0.49mM EDTA (i.e. less than equivalent concentration) was used as a chelator the half-live was ~85min but with excess EDTA (0.7mM) this was decreased to <2min. The phenomenon of synergistic ligand stabilization has also been seen for *Bacillus caldolyticus* glutamine synthetase using Mn^{2+} in combination with various substrate ligands (Merkler *et al.*, 1988).

It is of interest that the toxicity of $[Mn^{2+}]$ above 0.1mM at 95°C with 1mg/ml BSA (Table 5-4-3) is not apparent at 75°C (Fig. 5-4-1) and 0.1mM Mn^{2+} is not as effective in stabilizing *B. caldovelox* arginase at 75°C as at 95°C. These observations may reflect a disproportionate increase in Mn^{2+}-toxicity with temperature and changes in the relative contributions of Mn^{2+}-saturation and other forces (e.g. hydrophobic interactions) to the net free energy of stabilization as temperature increases. The temperature dependence of K_{diss} of the Mn^{2+}-arginase and Mn^{2+}-BSA complexes might also contribute to these results.

The thermostabilization of *B. caldovelox* arginase by saturation with Mn^{2+} is consistent with the well recognized stabilizing effect of ligand/cofactor binding (Klibanov, 1983; Jaenicke, 1987; Merkler *et al.*, 1988). The thermostability of other arginases is enhanced in the presence of Mn^{2+} or when the enzyme is preactivated with Mn^{2+} (e.g. Soru and Zaharia, 1976; Soru, 1983; Rossi *et al.*, 1983) and stability is higher still when Mn^{2+} is used in conjunction with a chelating buffer such as maleic acid (Greenberg *et al.*, 1956) or glycine (Campbell, 1966; Kaysen and Strecker, 1973).

The above experiments show that Mn^{2+}-mediated activation/inactivation effects on *B. caldovelox* arginase during thermostability experiments at 95°C, pH7 were prevented by complete preactivation of the enzyme with Mn^{2+} and by conducting the incubations with 0.5mM Mn^{2+} and 25mM aspartic acid. This stabilized the

Mn^{2+}-arginase complex against dissociation while minimizing the toxic effects of unchelated Mn^{2+}. Adherence to these conditions allowed observations to be made solely of thermal denaturation phenomena. Figure 5-4-4 shows the effect of some additives on the thermostability of *B. caldovelox* arginase. The half-life found for these and other additives are recorded in Table 5-4-4, glycerol being the only additive that enhanced thermostability. This stabilization is probably related to preferential hydration resulting from the exclusion of glycerol from the protein domain (Gekko and Timasheff, 1981). Preferential hydration can cause localized phase separation between the protein and the aqueous glycerol solution and so stabilize the water-protein complex

Table 5-4-4 Effect of various compounds on *B. caldovelox* arginase thermostability.

Additive to 0.1M MOPS/NaOH buffer pH7 (95°C) containing 0.5mM Mn^{2+} and 25mM aspartic acid	$t_{1/2}$ (min)
none	55*[a]
none	106*
10% (v/v) glycerol	150
20% (v/v) glycerol	165
1mg/ml BSA	104*
5mM DTT	87*
5mM $MgCl_2$	52*
5mM $CaCl_2$	36
10% (v/v) EtOH	24
0.1M NaF	75*
0.1M KCl	75*
0.1M KBr	66*
0.1M KI	45*
0.5M KCl	40*

*First-order inactivation kinetics, i.e. graphs of log(% activity remaining) vs time are linear.
[a]Arginase was not preactivated.

Unless otherwise indicated preactivated *B. caldovelox* "native metals" arginase was used in the above experiments. See Table 5-4-2 for further experimental details.

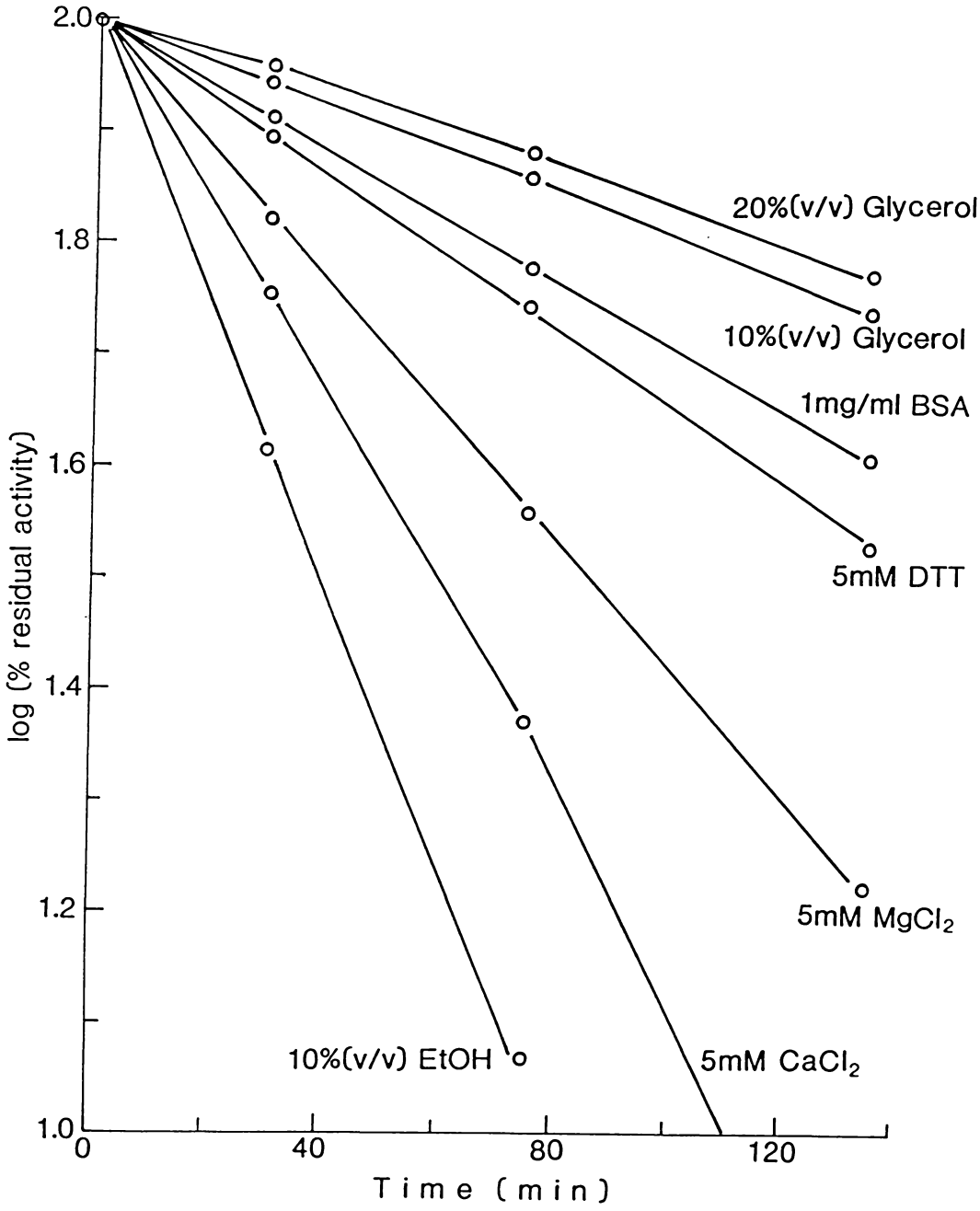

Figure 5-4-4 The effect of additives on *B. caldovelox* arginase thermostability.

1ml volumes of a 0.1M MOPS/NaOH buffer pH7 (95°C) containing 25mM aspartic acid and 0.5mM Mn^{2+} in addition to the additives indicated were equilibrated and injected with 10μl 13μg/ml preactivated *B. caldovelox* arginase then incubated, sampled and assayed as described in Figure 5-4-1 with a 15min equilibration of the enzyme before the t=0min sample. A control solution (i.e. no additive) had the same thermostability as was found for the BSA additive. The line representing inactivation with 5mM $CaCl_2$ connects with a fourth data point.

against thermal unfolding (Lee and Lee, 1987). Both Mg^{2+} and Ca^{2+} caused a more rapid inactivation of the enzyme. While not catalytically active, these metal ions may exchange with Mn^{2+} in the active site. The observed inactivation could therefore actually be due to a combination of decreased catalytic activity from metal ion substitution and a decrease in the thermostability of the substituted species. All salts of the halide ions destabilized the enzyme and this was not due to ionic strength alone as the larger halide ions caused greater destabilization at equal concentrations.

Although a comparison with other data is difficult due to differences in experimental protocols used, it would appear that the *B. caldovelox* arginase is the most thermostable purified arginase reported to date. For comparison, half-lives were for *Bacillus anthracis* arginase 80min at 60°C (calculated from data *in* Soru, 1983), for equine liver arginase 45min at 75°C (Greenberg *et al.*, 1956) and for *Arachis hypogea* cotyledon arginase 12min at 80°C (Desai, 1983). Greenberg *et al.* (1956) used incubation conditions very similar to those used in here, i.e. complete preactivation and incubation in the presence of a saturating $[Mn^{2+}]$ in a chelating buffer (maleic acid) at pH7.

5-4-3 Maintenance of quaternary structure and subunit reassociation

Mn^{2+} is essential for maintenance of the quaternary structure of many animal arginases (e.g. Carvajal *et al.*, 1971; O'Malley and Terwilliger, 1974; Barańczyk-Kuźma *et al.*, 1976, 1980; Dahlig and Porembska, 1977; Boutin, 1982). One exception is bovine liver apoarginase which retains its native oligomeric structure (Harell and Sokolovsky, 1972). Mn^{2+} is also required for reassociation of inactive mammalian arginase subunits (apo-monomers) prepared by acid treatment or metal ion chelation (e.g. Vielle-Breitburd and Orth, 1972;

Barańczyk-Kuźma *et al.*, 1976; Carvajal *et al.*, 1977). To examine the structural role of Mn^{2+} in *B. caldovelox* arginase the enzyme was subjected to three treatments (pretreatment numbers 3, 4 and 5 in Table 5-4-5) to inactivate the enzyme by removing metal ions and the effect on activity and structure was examined. After 120min of pretreatments 3 and 4, <1.5% and < 0.5% of the original activity remained respectively (Fig. 5-4-5). At pH2.5 (pretreatment number 5) 2% of the original activity remained after 10min and no activity was detected at 40min. These apoarginase preparations were chromatographed on an analytical

Table 5-4-5 Effect of pretreatment on quaternary structure of arginase

Sample pretreatment	% of total peak area		
	Aggregate	Oligomer	Monomer
1. 50mM CAPS/NaOH buffer pH9.9, 20°C, 5min	0	100	0
2. 2-fold diluted eluting buffer pH9.5, 20°C, 5min	0	100	0
3. 50mM acetic acid/NaOH buffer pH4.4 containing 40μM CDTA, 20°C, 165min	0	98	2
4. 50mM MOPS/NaOH buffer pH7 (60°C) containing 7.5mM CDTA, 60°C, 120min	0	98	2
5. 15mM sodium phosphate buffer pH2.5 containing 40μM CDTA, 20°C, 10min	5	2	93
6. As for 5 above, but for 72min	~25	<1	75
7. As for 5 above, then 20mM sodium tetraborate (14mM NaOH, 25mM CDTA) buffer pH9.5, 20°C for 20min	2	89	9
8. As for 7 above, but for 40min at pH9.5	1	93	6

B. caldovelox native metals arginase (0.7mg/ml) was mixed with an equal volume of buffer to produce the solution conditions described above and incubated for the times and temperatures specified before injection for GP-FPLC. Typically 10μl samples were injected and the detector was set at 0.1AUFS. Sample **4** was cooled to 20°C and held at this temperature for 20min before injection. Samples **7** and **8** were first treated at pH2.5 at 20°C for 10min then mixed with an equal volume of 40mM sodium tetraborate buffer containing 28mM NaOH and 25μM CDTA pH9.5 and incubated at 20°C for the time specified.

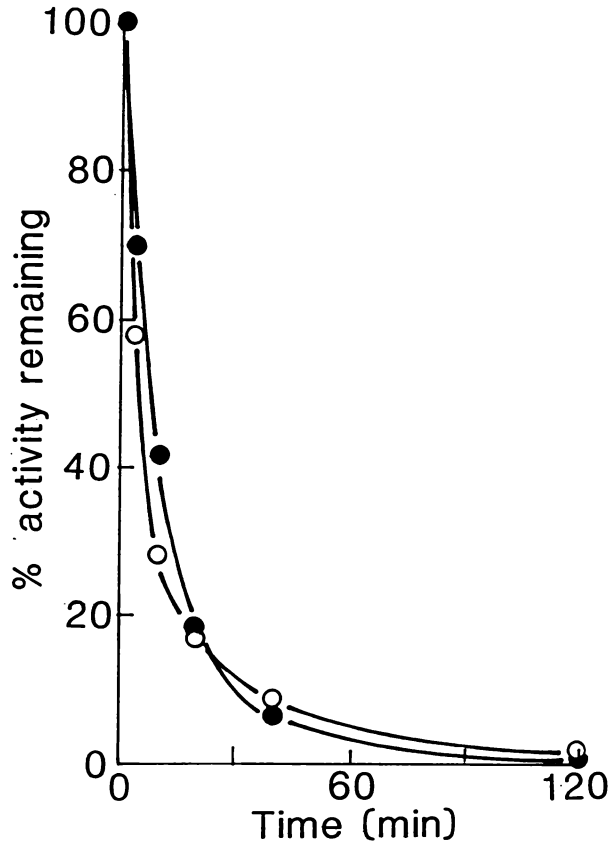

Figure 5-4-5 Inactivation of arginase for GP-FPLC experiments.

B. caldovelox arginase ("native metals", 0.35mg/ml) was incubated at pH4.4 at 20°C (○) or at pH7 at 60°C with CDTA (●) (see pretreatment numbers 3 and 4 respectively in Table 5-4-5). Samples taken to measure residual activity were diluted 1000× in 0.1M CAPS/NaOH buffer pH9.9 (20°C) and assayed 1min later at pH9 at 60°C with 20mM arginine in the presence of 2μM CDTA for 6min. When residual activity was <10% the samples were diluted 100× before assay. Enzyme diluted at pH9.9 was used for the t=0min activity measurement.

GP-FPLC column (described in Section 2-6-2) equilibrated with a running buffer consisting of 5mM sodium tetraborate containing 3.5mM NaOH, 50mM K_2SO_4 and 15μM CDTA pH9.5 (20°C). The eluent was monitored at 205nm and all injected protein appeared in three peaks. Two of these corresponded to the monomer and oligomer shown in Figure 5-2-7 and the third was a high M_r aggregate which eluted in the void volume. The results of peak area integration are presented in Table 5-4-5.

Pretreatments 1 and 2 demonstrated the complete stability of the oligomeric structure at alkaline pH before and during GP-FPLC. The results of pretreatments 3 and 4 indicated that the oligomeric structure of *B. caldovelox* apoarginase is stable at pH7 at 60°C and pH4.4 at 20°C. The intersubunit forces of *B. caldovelox* apoarginase are clearly greater than those of mesophilic arginases which dissociate when Mn^{2+} is removed by chelation or exposure to low pH. This is paralleled by the findings for octameric enolase from the thermophile *B. stearothermophilus* which also has stronger intersubunit forces than its dimeric mesophilic counterparts (Veronese *et al.*, 1984). Pretreatments 5 and 6 showed that oligomeric arginase dissociates rapidly at pH2.5 and that little if any reassociation occurs during GP-FPLC at pH9.5. The formation of a high M_r aggregate of monomers with time at pH2.5 was also demonstrated.

Acid-induced dissociation of the Mn^{2+}-arginase complex (as measured by inactivation) is largely complete before significant subunit dissociation occurs. This is in accord with the conclusion of Rossi *et al.* (1983) that "Mn^{2+} ions in [bovine liver] arginase play a fundamental role in its function and stability, but not in its [quaternary] structure". The reassociation of *B. caldovelox* arginase subunits at pH9.5 in the absence of Mn^{2+} (pretreatments 7 and 8) is also consistent with this conclusion. As the formation of a structured

monomer capable of reassociation is usually the rate-determining step in subunit reassociation processes (Jaenicke, 1987), the presence or absence of a Mn^{2+} requirement for reassociation of arginase apo-monomers may be related to the structural stability of the competent apo-monomer. Thus for apo-monomers of mesophilic arginases the binding of Mn^{2+} may be required to stabilize the conformation necessary for reassociation (Carvajal *et al.*, 1977, 1982a), while the less flexible *B. caldovelox* arginase subunits might retain this native subunit structure during dissociation at pH2.5 at 20°C or regain it rapidly without Mn^{2+} at pH9.5.

No peak with a M_r between 34000 and 172000 was seen in any GP-FPLC experiments with *B. caldovelox* "native metals" arginase. A monomer-oligomer equilibrium set up in 50mM citric acid/NaOH buffer pH4 failed to show intermediates of the dissociation pathway when analysed by GP-FPLC at pH9.5 suggesting that any intermediates are not stable under these conditions. An SDS-PAGE experiment (see Section 5-2-4) indicated that a trimeric species is an intermediate of SDS-induced dissociation. A trimeric intermediate was also seen by O'Malley and Terwilliger (1974) for the hexameric arginase from *Pista pacifica* whereas dimeric intermediates occur in the dissociation and reassociation of other arginases (Hosoyama, 1972; Barańczyk-Kuźma *et al.*, 1976; Boutin, 1982).

Dilute *B. caldovelox* "native metals" arginase was incubated at pH's ranging from 2.5 to 9.9 at 25°C and the residual activity measured (Fig. 5-4-6). The rate of inactivation increased with decreasing pH and at pH2.5 all activity was abolished after 7min. Competition between H^+ and Mn^{2+} for the donor atoms of metal-binding ligands is the probable reason for dissociation of the Mn^{2+}-arginase complex although a pH-dependent change in protein conformation cannot be ruled out. The

poor stability of *Saccharomyces cerevisiae* and human liver and erythrocyte arginases below ~8.5 has been attributed to dissociation of the metal ion-arginase complex (Cabello *et al.*, 1961; Middelhoven, 1969).

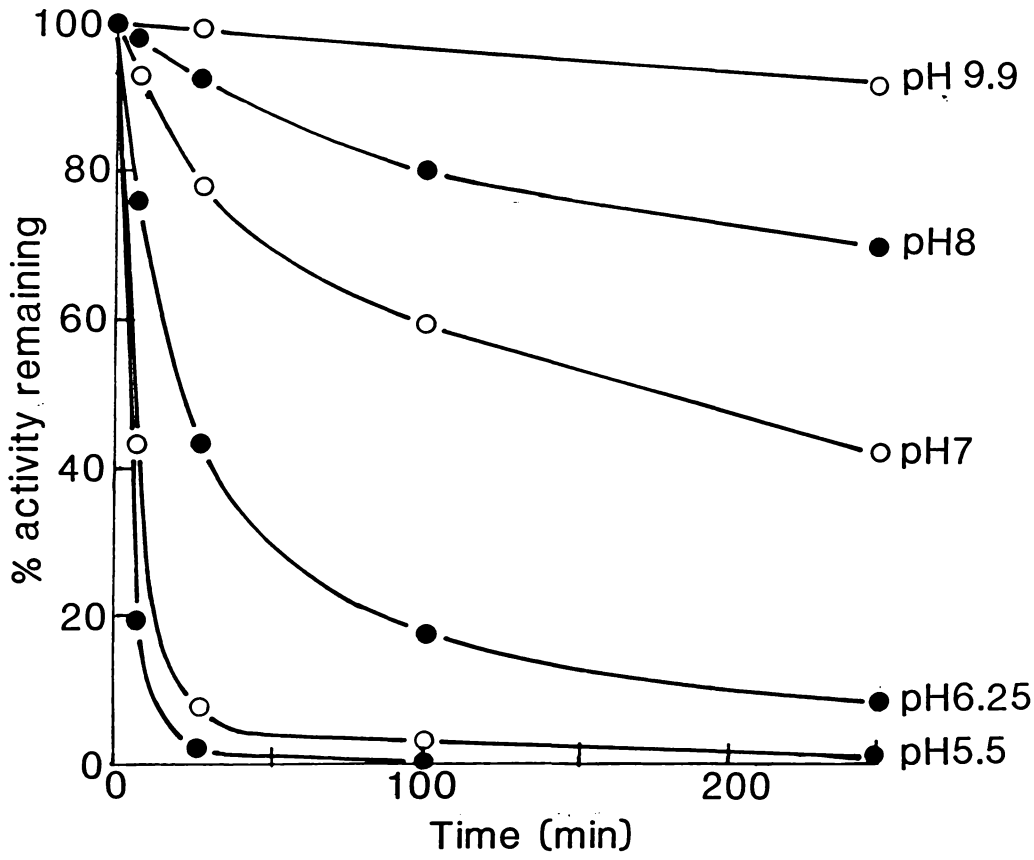

Figure 5-4-6 Effect of pH on the stability of *B. caldovelox* arginase.

B. caldovelox arginase ("native metals", 0.33mg/ml) was diluted 20× in 10mM CAPS/NaOH buffer pH9.9 (20°C) and then diluted 116× into 0.1M CAPS/NaOH buffer pH9.9, 25mM EPPS/NaOH buffer pH8, 25mM MOPS/NaOH buffer pH7, 25mM MES/NaOH buffer pH6.25, 25mM acetic acid/NaOH buffer pH5.5 and pH4.75, 25mM formic acid/NaOH buffer pH4 or 15mM potassium phosphate buffer pH2.5. The solutions were incubated at room temperature (20-22°C) and 25μl samples assayed for residual activity as described in Figure 5-4-1. At pH4 5% of the initial activity remained after 7min and at pH2.5 no activity could be detected after 7min.

CHAPTER 6

FINAL DISCUSSION

At the outset of this study there were very few examples of arginine catabolism by extremely thermophilic bacteria recorded in the literature. The screening project initiated for arginase, arginine deiminase and arginine decarboxylase activities detected each of these in thermophilic bacteria. The RP-LC method used provided a sensitive assay for these activities and catabolic ornithine carbamyltransferase and ornithine aminotransferase activities in cell-free extracts. This procedure has the potential to detect a large number of enzymes involved in amino acid and primary amine metabolism and could reveal new metabolites not detected by conventional screening methods. The distribution of the arginine-catabolizing enzymes was consistent with the pattern seen in mesophiles, i.e. arginase activity was restricted to aerobic thermophiles and arginine deiminase activity was found in thermophilic anaerobes. Arginase activity was induced by arginine and repressed under conditions of oxygen limitation. The arginase pathway operating in all thermophilic *Bacillus* and *Thermus* strains is similar to that found in mesophilic *Bacillus* species such as *B. licheniformis* and *B. subtilis*. Unlike these mesophiles, however, the aerobic thermophilic *Bacillus* strains apparently lack the ability to synthesize enzymes of the arginine deiminase pathway under conditions of oxygen limitation. It is notable that induction of arginine-catabolic enzymes in the thermophilic archaebacteria was slight or non-existent although inappropriate media compositions may be responsible for this. The only unexpected finding of the RP-LC screening experiments was the high

specific activity of ornithine aminotransferase in cell-free extracts of the the anaerobic archaebacterium Tok12 S.1. In mesophilic bacteria this enzyme activity is associated with enzymes of the aerobic arginase and arginine succinyltransferase pathways which dissimilate the carbon and nitrogen of arginine. Further work is needed to determine the fate of arginine in this organism and if arginine is utilized as an energy source.

With regard to purification of the arginase from *B. caldovelox*, concern that the heat treatment step in purification scheme I might have modified the structural or kinetic properties of the enzyme does not seem justified. After being purified to a similar degree by schemes I and II (i.e. with and without a heat treatment step respectively) the enzyme had the same subunit size and the oligomers had identical electrophoretic mobilities and similar IEF banding patterns. The N-terminal amino acid was the same for both preparations as was the pH optimum and K_m (at pH9, 60°C) for arginine. Because as little as 100μM Mn^{2+} has a detrimental effect on activity at 60°C in the absence of a chelating substance the high concentration of protein (26mg/ml) present before heat treatment was started must have protected the enzyme against inactivation during heating at 70°C with 50mM Mn^{2+}.

The comment of Hellerman and Perkins (1935-1936) on the activation of arginase by metal ions that "comparisons of the results of different investigators in this field must take account of the varying experimental conditions employed" is also valid for comparisons of arginase molecular weight, subunit structure, subunit dissociation studies and kinetic parameters. The experimental conditions can be considered to include those used to purify the arginase, as well as in the activation and assay of the enzyme. For example, kinetic properties of arginases depend on the degree of saturation with a metal ion

cofactor and vary with the particular cofactor used. The decrease in cofactor saturation that occurs during purification accounts for differences between the properties of purified arginases and those in crude extracts, e.g. the 10-fold increase in K_m during purification of artichoke tuber arginase (Wright *et al.*, 1981). Borkovich and Weiss (1987a) noted that the apparent "unsaturation" of purified *Neurospora crassa* arginase might have compromised the kinetic analyses that yielded the very high K_m values of 131mM for this enzyme. The K_m and pH optimum of the *B. caldovelox* "native metals" arginase preparation did not alter when the enzyme was fully activated with Mn^{2+}, suggesting that the "native metals" form is similar to the Mn^{2+}-activated form, and that the slight subsaturation of the former preparation is insufficient to affect these properties. Mn^{2+} was required in the assay medium of *B. caldovelox* arginase for linear assays.

The use of Mn^{2+} as opposed to another activating transition metal ion is justified on the basis of the metal ion composition of the "native metals" enzyme which showed Mn^{2+} to be the predominant metal present. For most arginases the assumption that Mn^{2+} is the *in vivo* cofactor and its use in *in vitro* studies has been based only on the consistently high degree of activation or reactivation that can be achieved with this cation. However this evidence alone is insufficient to identify the *in vivo* cofactor; in several cases other cations are equally good (or better) activators. Additionally, the reactivation process is monitored at an alkaline (non-physiological) pH, is only optimized for Mn^{2+} and the % recovery of reactivation can vary with different metal ions (Palacios *et al.*, 1969; Carvajal *et al.*, 1984; Iino and Shimadate, 1986). A further reason for using Mn^{2+} is its comparative stability at alkaline pH. As pH-activity profiles and values of physiologically important parameters such as K_m, V_{max} and K_i

(ornithine) depend on the metal ion cofactor (Hellerman and Stock, 1936; Middelhoven, 1969) it is clearly important that if *in vitro* studies are to be physiologically relevant the appropriate metal ion(s) be used in an assay mix and, if necessary, in the activation procedures. Pace *et al.* (1980) noted that in most kinetic studies of arginase the enzyme was activated and assayed under non-physiological conditions. The preactivation conditions developed for *B. caldovelox* arginase were not entirely suitable for *Thermus* 4-1A as it was not stable under these conditions. Unless a purification procedure can be developed in which *Thermus* 4-1A arginase retains a greater proportion of its *in vivo* cofactors then study of the Mn^{2+} form (obtained by preactivation before assay) will be necessary.

As all arginases bind a metal ion cofactor it is likely that a substantial proportion of the increase in thermostability of *B. caldovelox* arginase compared to the arginases from mesophiles originates from increases in stabilizing interactions unrelated to the binding of Mn^{2+}. Mn^{2+} has both stabilizing and inactivating effects on *B. caldovelox* arginase. A low concentration of unchelated Mn^{2+} is required to maintain the enzyme in a saturated state with respect to the cofactor and this state has maximal activity and thermostability. Increasing the $[Mn^{2+}]$ actually destabilizes the enzyme because of metal ion toxicity. Even under incubation conditions that yield the greatest thermostability for *B. caldovelox* arginase at 95°C it is possible that significant inactivation is due to the toxicity of Mn^{2+} and that the optimum concentration of free Mn^{2+} is a compromise between the stabilizing effect of Mn^{2+} and its toxicity. The thermostability of *B. caldovelox* arginase under optimal conditions of Mn^{2+} and aspartic acid concentration at pH7 ($t_{1/2}$ = 105min at 95°C) is comparable to values obtained for other enzymes from extremely thermophilic bacteria (e.g.

Barnes and Stellwagen, 1973; Higa and Ramaley, 1973; Fujita *et al.*, 1976).

The ability of transition metal ions to inactivate enzymes at elevated temperatures should be considered when comparing the apparent thermostability of a metal activated/stabilized enzyme measured in a crude extract to that found for the pure enzyme. The higher concentration of protein and the presence of nucleic acids in the crude extract might enhance thermostability by regulating the concentration of the free metal ion. As purification progresses it would be reasonable to expect the thermostability to decrease as these protective substances are removed. The thermostability of *B. caldovelox* arginase in the presence of Mn^{2+} in crude extracts was much greater that that seen for the purified enzyme with Mn^{2+} until a suitable chelator was added. A protein with high thermostability does not have decreased susceptibility to the simple chemical reactions/combinations of metal ions with surface residues which inactivate enzymes and the increased rate of such reactions at high temperatures may cause inactivation. The stabilizing effect of aspartic acid and Mn^{2+} on *B. caldovelox* arginase suggests that the synergistic ligand stabilization of substrate and metal ions observed in other studies is partly due to regulation of the concentration of free metal ion.

The mechanism by which Mn^{2+} stabilizes arginase against thermal inactivation is unknown. Mn^{2+} may be involved in maintaining the native subunit tertiary structure required for maximizing the attractive interactions between subunits. It is also possible that Mn^{2+} contributes directly to these forces by binding in a position between the surfaces of adjacent subunits. Further GP-FPLC experiments using a buffer system compatible with Mn^{2+} are needed to determine if Mn^{2+} perturbs the monomer-hexamer equilibrium seen at pH4. Although

dissociation of the Mn^{2+}-arginase complex is largely complete before acid-induced subunit dissociation occurs this does not mean that Mn^{2+} cannot influence the oligomer-subunit equilibrium. The zinc metalloenzyme alkaline phophatase from *E. coli* also loses Zn^{2+} at acid pH before major structural changes occur, yet Zn^{2+} does influence the oligomer-monomer equilibrium of this enzyme (Coleman, 1971).

It is unlikely that *B. caldovelox* arginase will be of use in the depletion of blood arginine for cancer therapy because of the high K_m and poor stability of the Mn^{2+}-arginase complex at physiological pH. The enzyme may, however, be of value in enzyme replacement therapy as it exhibits many kinetic properties that are similar to the mammalian arginases and is active at 37°C. The use of an immobilized form of the enzyme in extracoporeal shunts would probably be the most practical application. This reduces the immune response, and immobilization can stabilize the Mn^{2+}-arginase complex (Aguirre and Kasche, 1983).

Comparisons of the properties of an enzyme from mesophilic bacteria with the thermophilic counterpart typically show that, with the exception of (thermo)stability, the properties are similar (e.g. Freeze and Brock, 1970; Barnes and Stellwagen, 1973; Ramaley and Hudock, 1973; Walsh *et al.*, 1974; Fujita *et al.*, 1976; Rasnick and Powers, 1978; Allgood and Perry, 1986; Patchett *et al.*, 1987; Sung *et al.*, 1988). This similarity was evident between the thermophilic arginases studied here and the arginases of mesophilic bacteria. Many similarities were also seen between the thermophilic arginases and arginases from eukaryotic organisms. Indeed, apart from the greater thermostability and higher M_r, many properties of the *B. caldovelox* arginase are similar to those of mammalian liver arginases. Table 6-1 summarizes a few characteristics of *B. caldovelox* arginase and provides a comparison with arginases from a mesophilic bacterium (*Bacillus*

anthracis), a eukaryotic microbe (*Neurospora crassa*) and rat liver.

Table 6-1 A comparison of the characteristics of *B. caldovelox* arginase with other arginases

Property	*Bacillus caldovelox*	*Bacillus anthracis*	*Neurospora crassa*	Rat liver
Multiplicity	1	1	2?	≥2
Inducible	Yes	-	Yes	-
pH optimum	9.0	9.8-10	-	~9.7
pI	(5.2) 5.4 (5.5)	-	-	9.3
Molecular weight (thousands)				
Oligomer	176	160	266	105-121
Subunit	32	-	~38	31- 39
Subunit structure	Hexamer	-	Hexamer	Trimer/tetramer
K_m (mM) (pH9-9.5)	3.4	2.9	131	1-5
V_{max} (U/mg) (pH9-9.5)	5700 (60°C)	267 (37°C)	2610 (37°C)	3000-5000 (37°C)
K_i (ornithine) (mM)	0.55	-	-	1.3
Activating cations	Ni>Mn=Co>Cd	-	-	Mn,Ni,Co,Cd
Specificty; activity on				
L-canavanine	4.7%	-	-	~5%
L-homoarginine	0.5%	-	-	0.4%
D-arginine	0.7%	-	-	0
Activation energy (kJ/mole)	46.3	51.5	-	33/50

References: *Bacillus anthracis*, Soru (1983); *Neurospora crassa*, Davis (1986), Borkovich and Weiss (1987a, 1987b); Rat liver, Campbell (1966), Hirsch-Kolb and Greenberg (1968), Hirsch-Kolb *et al.* (1970), Reddy and Campbell (1970), Kaysen and Strecker (1973), Penninckx *et al.* (1974), Tarrab *et al.* (1974), Pace *et al.* (1980), Brusdeilins *et al.* (1985).

APPENDIX 1

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DETERMINATION OF AGMATINE, ARGININE, CITRULLINE AND ORNITHINE BY REVERSED-PHASE LIQUID CHROMATOGRAPHY USING AUTOMATED PRE-COLUMN DERIVATIZATION WITH *o*-PHTHALALDEHYDE

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SUMMARY

A method is presented for the pre-column derivatization of agmatine, arginine, citrulline and ornithine with *o*-phthalaldehyde–2-mercaptoethanol, and subsequent separation of the derivatives by reversed-phase liquid chromatography. Fluorescent response is linear from 10 to 150 pmol of injected analyte and detection limits range from 28 to 100 fmol. Response factors relative to the internal standard, homocysteic acid, were 1.16 (agmatine and arginine), 1.03 (citrulline) and 0.34 (ornithine). The applicability of the method to the measurement of arginase, arginine deiminase, arginine decarboxylase and other enzyme activities in bacterial extracts was examined.

INTRODUCTION

The fluorogenic pre-column derivatization of compounds possessing primary amino groups with *o*-phthalaldehyde–thiol (OPA–thiol) reagents followed by reversed-phase liquid chromatography (RPLC) has become a popular method for the analysis of amino acids [1–6], various metabolic products [7,8] and drugs [9]. Recently, this technique has also been used for the sensitive detection and assay of specific enzymes by quantitation of reaction products [10–13]. During a project to detect arginine-catabolising enzymes in extracts of thermophilic bacteria, the need arose to simultaneously measure enzymatically formed agmatine, citrulline and ornithine in the presence of excess substrate arginine in stopped-assay mixtures. The use of RPLC and pre-column derivatization with OPA to analyse amines in physiological fluids and tissues has shown the feasibility of separating arginine, citrulline and ornithine derivatives by this method [1,3,14,15]. Although there are few studies which record the elution behaviour of OPA derivatives of agmatine, the work of Griffin et al. [16] suggested that of

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the amines investigated in this study only ornithine would elute close to agmatine. The above method therefore appeared most suitable. In developing the procedure described in this paper, we also examined the mixing of OPA reagent and sample during automated pre-column derivatization and the effect high concentrations of arginine had on the response and elution behaviour of other analytes.

EXPERIMENTAL

Apparatus

Analyses were performed using a Waters Assoc. liquid chromatograph equipped with two M510 pumps, a WISP 710B autoinjector, an M420-AC fluorescence detector ($\lambda_{ex}=338$ nm, $\lambda_{em}=425$ nm) with the span control set at maximum and a gain of 16 unless otherwise indicated, an M720 system controller and an M730 data module. A Nova-Pak C_{18}, 5-μm, 150 mm$\times$4.6 mm I.D. stainless-steel column (Waters Assoc., Milford, MA, U.S.A.) enclosed in an M1122 oven (Waters Assoc.) set at 30°C was used for chromatographic separations.

Buffers and eluents

All water used was from a Milli-Q water purification system (Millipore, Milford, MA, U.S.A.). Potassium borate buffer (0.2 *M*, pH 9.4 at 20°C) was prepared by dissolving 3.092 g boric acid (Aristar grade, BDH, Poole, U.K.) in water and adjusting the pH with a saturated solution of potassium hydroxide (Analar grade, BDH) in a final volume of 250 ml. The buffer was passed through a 0.2-μm filter (Gelman Sciences, Australia) and stored at 4°C. A stock solution of eluent A was prepared by dissolving 13.609 g anhydrous potassium dihydrogenphosphate (analytical-reagent grade, Ajax Chemicals, Sydney, Australia) in 950 ml of water and adding potassium acetate (AR grade, Peking Chemical Works, Peking, China) to adjust the pH to 5.93 at 20°C in a final volume of 1 l. After filtering through a 0.45-μm membrane filter (Millipore) this solution was stored at 4°C in the dark in a sterile glass container. Eluent A was freshly prepared by a ten-fold dilution of stock A with water, vacuum-degassed by filtration before use and helium-sparged during use. Eluent B was a 4:3:3 (v/v/v) mixture of acetonitrile, methanol (both HPLC grade, Waters Assoc.) and water. Each component was filtered through a 0.22-μm aqueous Durapore filter (Millipore) before mixing, and eluent B was degassed for 25 min in an ultrasonic bath before use.

Reagents and solutions

Amino acids and agmatine sulphate were supplied by Sigma (St. Louis, MO, U.S.A.) except for L-arginine (chromatographically homogeneous, BDH) and L-ornithine hydrochloride (BDH and Sigma). Urea (AR grade) was obtained from Ajax Chemicals and ammonium chloride (GR grade) from Merck (Darmstadt, F.R.G.). Stock solutions (20 m*M*) of single amino acids and amines were prepared in 50 m*M* nitric acid (Aristar grade, BDH). Sample solutions were prepared for derivatization by mixing with an equal volume of 0.2 m*M* homocysteic acid in 4% (w/v) perchloric acid (AR grade, BDH), diluting ten-fold with filtered water and placing 100 μl in a Waters Assoc. small-volume-insert sample

vial. The samples were centrifuged (10 000 *g*, 5 min) before placing them onto the WISP autoinjector sample carousel.

Research-grade 2-mercaptoethanol (ME) was obtained from Serva (Heidelberg, F.R.G.). OPA (Sigma) was vacuum-sublimed before use. The OPA–ME derivatizing reagent was prepared by dissolving 50 mg OPA in 1 ml of filtered methanol, then adding 53 μl of ME and 9 ml of 0.2 *M* potassium borate buffer (pH 9.4) and was stored at 4 °C for not more than two days before use.

Chromatographic procedure

The WISP autoinjector was programmed to charge the sample needle with 7 μl of the OPA–ME derivatizing reagent, then 5 μl of sample, followed by immediate injection. Concomitantly with injection, the flow-rate was raised from 0 to 0.2 ml/min for 0.2 min, then reduced to 0.1 ml/min for 1 min. After injection (1.2 min) the flow-rate was increased linearly to 1 ml/min over 2.3 min at 20% eluent B in the mobile phase. This was followed by a 1.5-min linear gradient to 27% B and a 10.5-min isocratic step, then an 11.5-min linear gradient to 100% B. After 4 min at 100% B, reequilibration was initiated by a 9-min linear reverse gradient to 20% B and completed when 18 ml of this eluent had been passed through the column (see Fig. 1). The flow-rate was then reduced to 0 over 3 min and held for 1 min in preparation for the next injection. Total time between two injections was 60 min.

RESULTS AND DISCUSSION

The procedure described here separated the four derivatized amines (Fig. 1A). Elution times (see Table I) were highly reproducible, although a high concentration of arginine in the sample decreased the reproducibility. The isocratic step in the elution gradient was required to separate citrulline and arginine when high concentrations of arginine were present in the sample (Fig. 1B), and this led to broadening of these peaks. The presence of 5 nmol arginine in the sample also resulted in an additional peak which eluted before agmatine. The internal standard, homocysteic acid, eluted well away from the analyte peaks.

While an internal standard compensates for variation in sample volume and mixing of sample and derivatizing reagent between injections, the most important effect in our study was to compensate for a drop in OPA–ME reagent strength. Typically, a 9% decrease in sensitivity was observed over the first 4 h after placing fresh OPA–ME reagent in the sample compartment. Subsequently, sensitivity decreased by about 1% per hour. The initial loss of reagent effectiveness is probably due to the oxidation of thiols in the reagent [17]. This decreases the ME/OPA ratio below 2, which is the optimum for reaction rate [18], and thus for fluorescent response [19]. This oxidation was accelerated in the WISP sample compartment, which has a normal operating temperature of 30 °C. At this temperature significant evaporation from sample vials was observed over a 24-h period.

When peak areas were normalised with respect to homocysteic acid, the coefficients of variation ($n=5$) at the 50-pmol level were 7% for ornithine, 2% for agmatine, 0.9% for citrulline and 0.5% for arginine. Poor reproducibility for or-

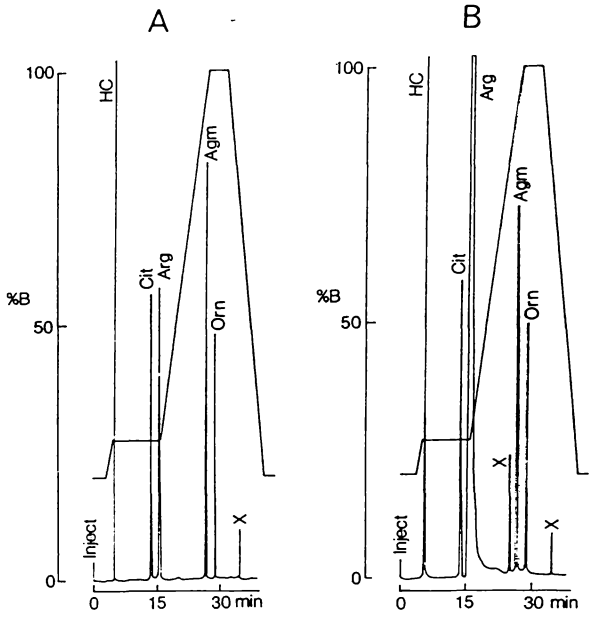

Fig. 1. Elution profiles of 50 pmol of OPA-ME-derivatized homocysteic acid (HC), citrulline (Cit), agmatine (Agm) and ornithine (Orn) in the presence of (A) 50 pmol arginine (Arg) and (B) 5 nmol arginine. X = Unknown peak. Gradient profile superimposed.

nithine has been observed previously [1]. An unidentified peak eluting at 33.8 min occurred in all analyses.

Elution times and response factors for agmatine, citrulline and ornithine relative to homocysteic acid determined at the 50-pmol level in the presence of 50 pmol and 5 nmol of arginine were not significantly different (Tables I and II).

Linearity of response in the 10–150 pmol range (corresponding to a concentration range of 0.04–0.6 m*M* in samples prior to addition of the internal standard solution) was excellent for all four analytes, the critical coefficient (r^2) being greater than 0.997 in every case. This was also the case for citrulline, agmatine and ornithine over the same concentration range in the presence of 5 nmol of arginine. At these high arginine concentrations the molar ratio of OPA to total derivatizable amino groups drops to approximately 40. According to Lindroth and Mopper [20], linearity of response can only be expected if OPA is present in at

TABLE I

ELUTION TIMES

Amount of arginine in injected sample	Elution time (mean ± S.D., $n = 5$) (min)				
	Homocysteic acid	Citrulline	Arginine	Agmatine	Ornithine
50 pmol	5.08 ± 0.02	13.36 ± 0.03	15.22 ± 0.04	25.98 ± 0.03	28.07 ± 0.00
5 nmol	5.12 ± 0.06	13.42 ± 0.10	15.18 ± 0.20	26.04 ± 0.06	28.09 ± 0.09

TABLE II

RESPONSE FACTORS RELATIVE TO HOMOCYSTEIC ACID

Amount of arginine in injected sample	Response factor (mean ± S.D., $n=5$)			
	Citrulline	Arginine	Agmatine	Ornithine
50 pmol	1.03 ± 0.01	1.16 ± 0.007	1.16 ± 0.02	0.34 ± 0.02
5 nmol	1.06 ± 0.01	–	1.20 ± 0.04	0.35 ± 0.01

least a 100- to 200-fold excess over analytes. However, our results are consistent with those of Cooper et al. [19], who have shown that OPA-to-amino acid ratios as low as 18 produce a linear response under correct reaction conditions.

The low fluorescent response of ornithine has been observed previously [3,15] and it is suggested that quenching due to the presence of two fluorescent isoindole structures in the derivative is responsible [21]. To test the purity of arginine, citrulline, agmatine and ornithine, 5 nmol of each were injected. The former three each gave a single major peak, but the elution profile of ornithine consisted of two major and at least seven minor peaks compared with the single peak seen for injections of picomole amounts of ornithine. The largest of the additional peaks eluted at 19.5 min with an area equivalent to the ornithine peak at 28 min. These multiple peaks were observed for both Sigma and BDH ornithine and may be due to incomplete derivatization of sample ornithine when present at high levels, with the rapidly formed, more highly fluorescent [21] and less hydrophobic mono-derivative of the δ-NH_2 group of ornithine accounting for the largest additional peak and eluting at 19.5 min.

Detection limits and sample–reagent mixing during derivatization

The method described was highly sensitive, with all analyte peaks readily detected at the 100-fmol level (Fig. 2). Apart from the analyte peaks, a number of contaminant peaks and baseline shifts became apparent at this sensitivity. The largest interfering peak, which may in part be due to the OPA–ammonia deriva-

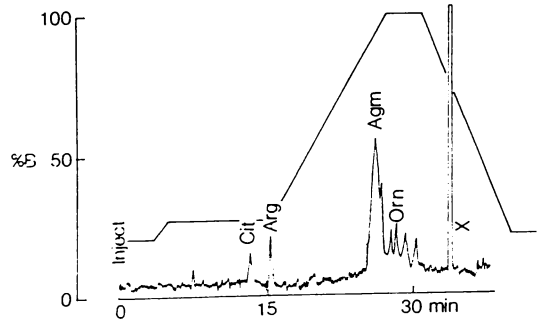

Fig. 2. Elution profile of 100 fmol of OPA-ME-derivatized citrulline (Cit), arginine (Arg), agmatine (Agm) and ornithine (Orn). Detector setting: gain = 128. X = Unknown peak. Gradient profile superimposed.

tive, coeluted with agmatine and caused a relatively high detection limit of about 100 fmol for agmatine. The detection limits for arginine, citrulline and ornithine calculated by the method of Knoll [22] with a peak width multiple of 10 were 28, 38 and 60 fmol, respectively. These are comparable with other automated pre-column derivatization analyses using OPA [1,2,23,24].

The use of automated on-line pre-column derivatization with OPA decreases the extent of decay of OPA derivatives and allows highly reproducible reaction timing. However, to achieve the high sensitivity and reproducibility the method is capable of, good mixing between the OPA–thiol reagent and sample in automated procedures is required. A number of workers have used purpose-built devices or pumping systems to ensure complete mixing [2,7,23,24], while others have employed the WISP autoinjector used in this study and a mixing chamber containing glass beads between the WISP and column [1,4,5]. One group [3] describes a successful pre-column derivatization procedure using the WISP without a mixing chamber, in which 10-μl volumes of OPA reagent and sample were "mixed in the sample needle for 2 min" before injection. Such mixing could not occur by diffusion, as the tubing forming the WISP sample needle has an internal cross-sectional area of approximately $2 \cdot 10^{-3}$ cm^2. A 10-μl sample occupies a length of 5 cm in this tubing.

In the absence of any additional pre-column mixing aids, we found that programming the flow-rate for a slow passage (0.1 ml/min) through the Nova-Pak column frit and onto the column resulted in adequate and reproducible mixing of the OPA reagent and sample. The success of this approach to mixing relies on the OPA and sample solutions being spread by the column frit to form narrow zones and the speed of the derivatizing reaction. Before the sample and OPA solutions pass onto the column packing they are spread by the column frit to zones of approximately 0.5 mm thickness. Mixing of the zones by diffusion is now possible, and this is assisted by the mechanical mixing that occurs by passage through the 2 μm mesh of the filter insert (Waters Assoc.) and the perforated packing retainer plate (Waters Assoc.). Mixing and reaction may continue as the solutions enter the column packing. The reaction time in this study was less than 1 min, but this is sufficient for complete OPA derivatization when factors such as the OPA-to-thiol ratio, reaction pH and OPA-to-amine ratio are optimised [19]. In addition, the reaction was conducted at 30°C in our experiments. A 1-min reaction time may even be excessive for ornithine, as the OPA derivative of this amino acid is very unstable in the reaction mixture [14,19,20]. This instability emphasises the importance of the precisely timed derivatization reactions afforded by automating the procedure.

We are currently applying the method described here to the simultaneous measurement of arginase, arginine deiminase and arginine decarboxylase activities in bacterial extracts (Fig. 3). These enzymes catabolise arginine to ornithine, citrulline and agmatine, respectively. As the former two enzymes also produce urea and ammonia, respectively, we examined the behaviour of these products in our RPLC system. Urea was not detected at the 5-nmol level. The OPA derivatives of ammonia and agmatine coeluted, but the response factor of the ammonia derivative (0.02 relative to homocysteic acid) was too low to interfere with the

Figure 7-1 Representative RP-LC elution profile of Tok12 S.1 showing arginine deiminase and arginine decarboxylase activities.

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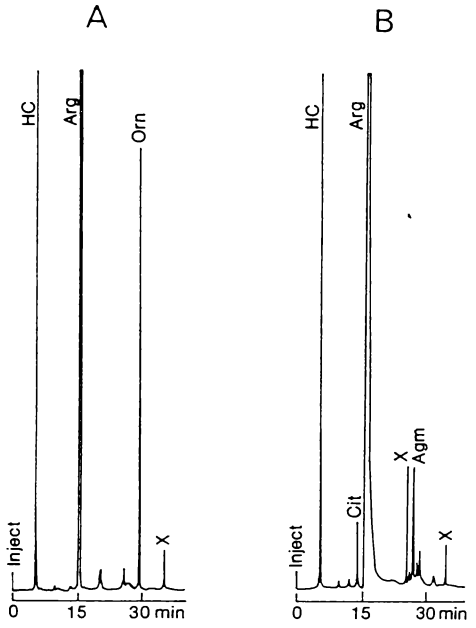

Fig. 3. Elution profiles of deproteinized stopped-assay supernatants resulting from the incubation of cell-free bacterial extracts with buffered 20 m*M* L-arginine. (A) *Bacillus licheniformis* DSM13* extract incubated for 10 min at pH 7.0 at 37°C. Assay solution was diluted 200-fold and a 5-μl sample was derivatized. (B) Tok12S1 (an extremely thermophilic, anaerobic, sulphur-dependent archaebacterium) extract incubated for 10 min at pH 6.2 at 80°C. Assay solution was diluted 20-fold and a 5-μl sample was derivatized. Peaks: HC = homocysteic acid; Arg = arginine; Orn = ornithine; Cit = citrulline; Agm = agmatine; X = unknown peak.

quantitation of agmatine. However, high levels of arginase activity have precluded the detection of the agmatine derivative because it coelutes with one of the minor additional peaks resulting from large amounts of sample ornithine. The method has been successfully used to detect two other enzymes of arginine catabolism, namely ornithine aminotransferase by measurement of glutamate (elution time 5.55 min, response factor 0.9) formed from ornithine and 2-oxoglutarate, and catabolic ornithine carbamyltransferase by measurement of ornithine formed from citrulline in a phosphate buffer.

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Additional points regarding the use of the RP-LC method

Before connecting the column the WISP was purged with eluent A at 1ml/min until a value of 04 or less was obtained for system message code 63, which measures the compressibility of the liquid in the WISP. It was important that all system components coming into contact with the OPA-ME solution were clean, or additional peaks appeared in the elution profile. It was also beneficial to run at least one blank analysis with OPA-ME reagent and water as the sample before commencing a set of analyses, as this helped to clean the system and re-equilibrate the column to the eluents and gradient after storage. Reproducible preparation of solvent B was crucial to maintaining reproducible elution times. Because both degassing of organic solvent mixes by vacuum filtration or continuous helium sparging can alter solvent compositions sonication in a cleaning bath was the method of choice.

Intially the Nova-Pak column generated a high back pressure that was subject to fluctuations of up to ±5% of total back pressure. The high back pressure was due to a dirty frit at the top of the column which was cleaned by sonication in methanol. The pressure fluctuation was caused by small air bubbles trapped in the pump heads. For the solvent system employed, these could develop during periods of zero flow, and were more likely to occur if the solvents had not been adequately sparged/degassed. Eluent B was more prone to developing bubbles, and this was one of the reasons for running pump B at at least 10% of total flow throughout the chromatographic cycle. Trapped air was removed by loosening/removing the upper check valve and flushing the pump chamber with solvent applied at high flow rates from a pump or via a syringe through an open solvent drawoff valve. A visual inspection of the Teflon washer onto which the check-valves seat often revealed small

air bubbles.

While the use of guard columns to protect analytical columns from sample impurities is to be recommended in most cases and is probably especially important for columns with a small pore size like the Nova-Pak column, a silica-based C_{18} guard column caused severe broadening of the peaks and so was not used. After perchloric acid precipitation, centrifugation and dilution of stopped-assay solutions (Section 2-3) the samples were clean enough to derivatize and load directly onto the column.

APPENDIX 2

Titration Curves of Buffers and L-Arginine at 70°C

Solutions of the free acid forms of MOPS, EPPS, CHES and CAPS buffers (0.1M) were titrated at 70°C with 2M NaOH (Fig 7-2). The pH values of the calibrating buffers used are indicated. First derivative plots of the titration curves yielded pK_a's at 70°C (with the known pK_a (20°C) values in parentheses) of 6.7 (7.2), 7.5 (8.0), 8.6 (9.55) and 9.5 (10.4) for MOPS, EPPS, CHES and CAPS buffers respectively. Temperature coefficients ($\Delta pK_a/°C$) calculated for each buffer in the temperature range of 20°C to 70°C were 0.01 for both MOPS and EPPS, and ~0.019 for CHES and CAPS buffers. These values agree with the literature values with the exception of CHES which has been assigned a value of 0.011 (Dawson *et al.*, 1986). In view of the structural relatedness of the sites of ionization of the buffering amine group within the buffer pairs MOPS/EPPS and CHES/CAPS, and the dissimilarity between the pairs, a temperature coefficient of ~0.019 seems more likely for CHES and this value was used when calculating the amount of NaOH needed for CHES buffers at temperatures other than 70°C.

Figure 7-3 shows the curves obtained when 0.21M solutions of L-arginine (free base) were titrated with solid MOPS, EPPS or CHES buffers (free acid forms) or NaOH at 70°C. The pH of an untitrated arginine solution was ~9.85. By making up 0.21M L-arginine solutions of the desired pH at a given temperature using the calibration curves and diluting these solutions with a 0.1M buffer of the same pH a range of substrate concentrations of a given pH (pH6.2-9.9) at a particular temperature could be prepared. Above pH9.9 the guanidino group of arginine is a good buffer at elevated temperatures.

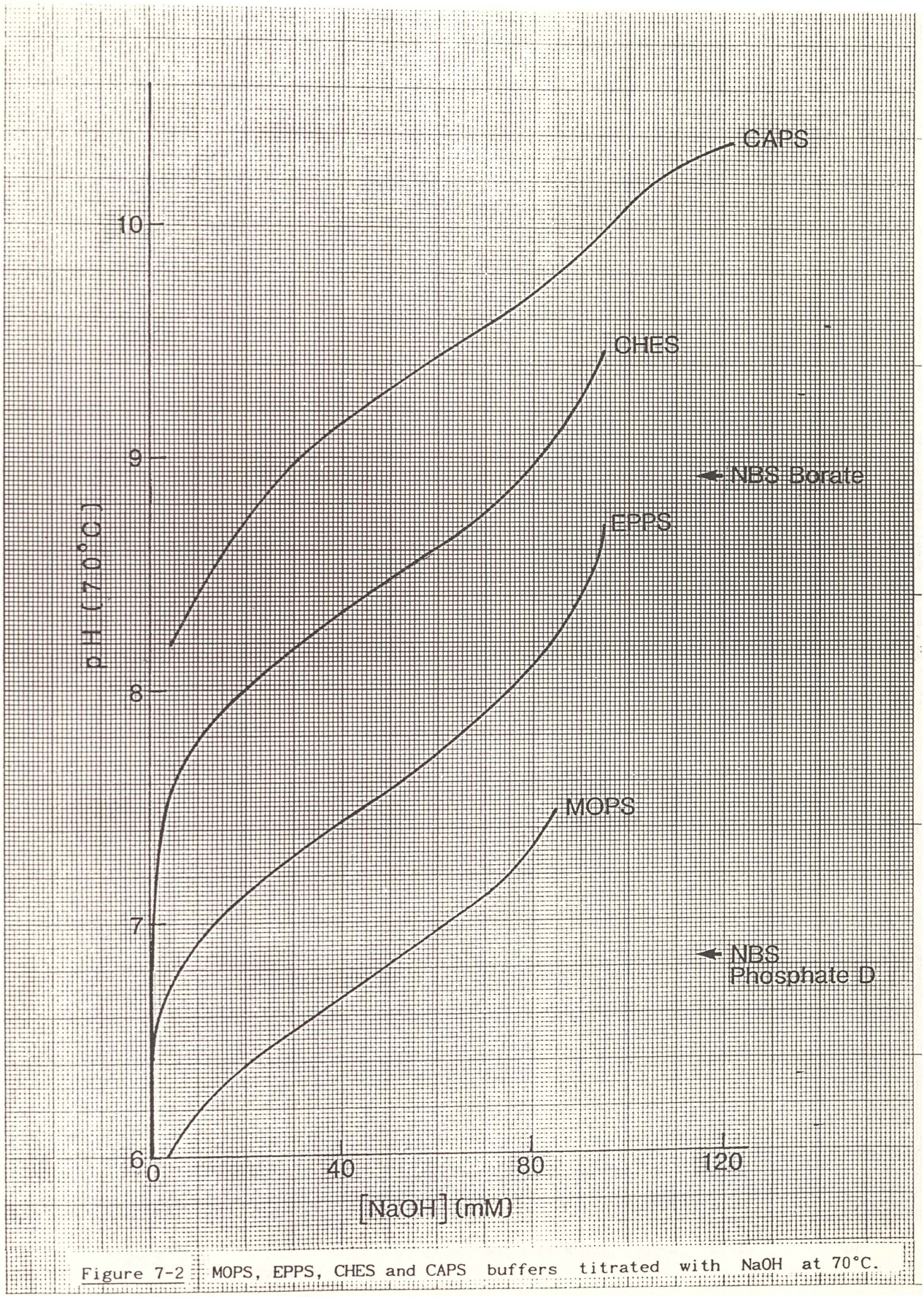

Figure 7-2 MOPS, EPPS, CHES and CAPS buffers titrated with NaOH at 70°C.

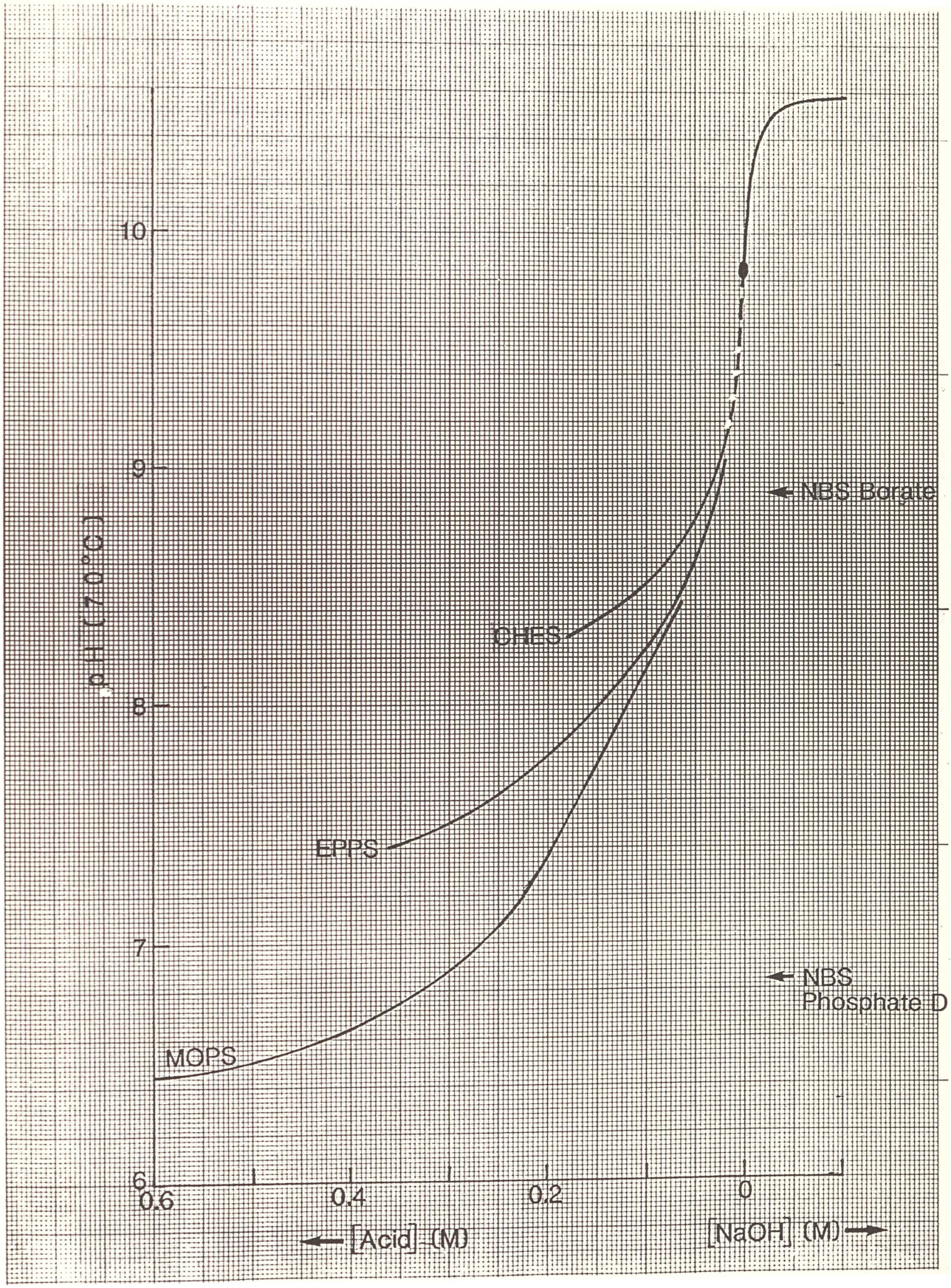

Figure 7-3 0.21M L-arginine titrated with MOPS, EPPS, CHES or NaOH at 70°C.

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