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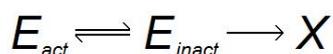
Deactivation of TEM-1 beta-lactamase investigated by isothermal batch and non-isothermal continuous enzyme membrane reactor methods

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The thermal deactivation of TEM-1 β -lactamase was examined using two experimental techniques: a series of isothermal batch assays and a single, continuous, non-isothermal assay in an enzyme membrane reactor (EMR). The isothermal batch-mode technique was coupled with the three-state "Equilibrium Model" of enzyme deactivation, while the results of the EMR experiment were fitted to a four-state "molten globule model". The two methods both led to the conclusions that the thermal deactivation of TEM-1 β -lactamase does not follow the Lumry-Eyring model and that the T_{eq} of the enzyme (the point at which active and inactive states are in thermodynamic equilibrium) is at least 10°C from the T_m (melting temperature), contrary to the idea that the true temperature optimum of a biocatalyst is necessarily close to the melting temperature.

Introduction

The operational stability of a biocatalyst is an important characteristic in determining the suitability of that catalyst in a given process. It is well-understood that catalytic activity tends to increase with temperature, but that in the case of enzymes, elevated temperature also leads to thermal deactivation due to a loss of integrity of the active site. [1, 2] When kept at elevated temperatures for extended time periods, enzymes will undergo denaturation to a permanently inactive state. Some enzymes are capable of recovering catalytic activity after relatively short incubations at elevated temperatures which induce a loss of activity. [3, 4] The equilibrium model captures this phenomenon by including a reversible transition between active and inactive states, and has been shown to be valid for all of the enzymes (approximately 40) on which it has been tested. [5-8]



Scheme 1. Equilibrium Model

By treating the reversible transition between E_{act} and E_{inact} as a van't Hoff-type equilibrium, and the transition from E_{inact} to X as a time-dependent, first-order decay with Arrhenius-type rate constant, one can derive an expression for the observed reaction rate as a function of temperature and elapsed time. [5, 6] Transition state theory is utilized to define the three quantities involved in the deactivation model, according to Equations 1-3. These are k_{cat} (turnover number of enzyme), K_{eq} (equilibrium constant between E_{act} and E_{inact}), and k_{inact} (first-order rate constant for decay from E_{inact} to X). The description of the E_{inact} to X step in the Equilibrium Model is not intended to preclude the presence of multiple, inactive intermediate unfolding states, though it makes no distinction between such states. [8]

$$k_{cat} = \frac{k_B T}{h} e^{-\frac{\Delta G_{cat}^\ddagger}{RT}} \quad (1)$$

$$K_{eq} = e^{\frac{\Delta H_{eq}}{R} \left(\frac{1}{T_{eq}} - \frac{1}{T} \right)} \quad (2)$$

$$k_{inact} = \frac{k_B T}{h} e^{-\frac{\Delta G_{inact}^\ddagger}{RT}} \quad (3)$$

Under isothermal conditions, the values of k_{cat} , K_{eq} , and k_{inact} remain fixed, and depend only on the activation and deactivation parameters of the enzyme at that temperature: ΔG_{cat} (activation energy for enzyme-catalysed reaction), ΔG_{inact} (activation energy for the E_{inact} to X transition), ΔH_{eq} (enthalpy change for the E_{act} to E_{inact} transition), and T_{eq} (temperature at which $[E_{act}] = [E_{inact}]$). When isothermal conditions exist, the expression for the reaction rate V_{max} , as a function of temperature and time, is given by Equation 4, where E_0 is the initial enzyme concentration.

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$$V_{\max} = \left(\frac{k_{cat} E_0}{1 + K_{eq}} \right) e^{-\left(\frac{k_{inact} K_{eq}}{1 + K_{eq}} \right) t} \quad (4)$$

By substituting the results of Equations 1-3 into Equation 4, the values of ΔG_{cat} , ΔG_{inact} , ΔH_{eq} , and T_{eq} may be estimated by fitting experimental data of reaction rate vs. temperature vs. time to the model. [9] The established method for estimating these parameters is to perform an array of activity progress curves (i.e. including isothermal deactivation) while holding the initial enzyme concentration, E_0 , constant. After several temperatures have been examined, all progress curves are fitted to a three-dimensional surface plot using Powell's algorithm to determine the global minimum of the sum of squares deviation between the experimental curves and the model. [9]

Recently, a method was developed for determining the optimum operating temperature for a biocatalyst using a single, continuous assay rather than an array of isothermal experiments. [10,11] This method utilizes an enzyme membrane reactor (for soluble enzyme) or a packed-bed reactor (for immobilized enzyme) to subject a single sample of enzyme to a continuous, positive, most often linear temperature gradient. Since time and temperature are functionally related in such a linear ramp scheme, the reaction rate may be expressed as a function of a single variable (either time or temperature), plus all of the intrinsic activation and deactivation parameters listed in Equations 1-3.

Because certain enzymes are known to lose catalytic activity before the onset of complete unfolding (i.e. the global loss of secondary structure often associated with the T_m of the enzyme) [1], the model used with the continuous temperature ramp experiment assumes that the biocatalyst follows a four-state deactivation model. This four-state model is similar to the equilibrium model but allows for two separate inactive states. The native state, N , is in equilibrium with a "molten globule" state, M , which has undergone slight conformational changes that render it inactive. Here, states N and M are taken to be functionally equivalent to the E_{act} and E_{inact} , respectively, of the equilibrium model. The four-state model includes a second equilibrium between M and another unfolded state, U , which has unfolded in the sense that it has experienced a widespread loss of secondary structure. This "molten globule" unfolding model is shown in Scheme 2.



Scheme 2. Four-state "molten globule" deactivation model

The model in Scheme 2 assumes that the enzyme must first enter the unfolded state U before it may become permanently denatured and that there are no pathways for denaturation directly from the native or molten globule states. Scheme 2 uses the same parameter as the equilibrium model, K_{eq} , to describe the van't Hoff equilibrium between states N and M . The first-order denaturation constant for state U , k_{inact} , is also the same for both models. The molten globule model, however, contains the additional parameter, $K_{mg} = [U]/[M]$, where:

$$K_{mg} = e^{\frac{\Delta H_{mg}}{R} \left(\frac{1}{T_{mg}} - \frac{1}{T} \right)} \quad (5)$$

Hence, the four-state model assumes a heterogeneous population of inactive enzyme and seeks to capture both the T_{eq} and the traditional T_m of the enzyme. Both of these values, which do not necessarily coincide with each other, are important when designing and characterizing a stable biocatalyst. [8]

When biocatalyst deactivation data is collected in a system which is operated as a continuous stirred-tank reactor, the degree of substrate conversion, X , is given as a function of temperature according to Equation 6.

$$X = \left(\frac{V}{[S]_0 v} \right) k_{cat} [N] \quad (6)$$

Equation 6 contains the system parameters V (reactor volume), $[S]_0$ (substrate feed concentration), and v (feed volumetric flowrate). Both k_{cat} and $[N]$ vary over the course of the experimental run as temperature is ramped up, and are readily expressed as functions of either time or temperature in the case of a linear heating ramp (the relationship $T = T_0 + mt$ applies, where m is the rate of increase of the reactor temperature). The value of k_{cat} is given by Equation 1, and the value of $[N]$ is derived by combining the definitions of K_{eq} and K_{mg} , the mass balance on species N , M , U , and D , and the first-order denaturation rate law which states $d[D]/dt = k_{inact}[U]$.

$$[D] = E_0 - [N] - [M] - [U] \quad (7)$$

$$\frac{d[D]}{dt} = \frac{d}{dt} (E_0 - [N] - K_{eq}[N] - K_{mg}K_{eq}[N]) \quad (8)$$

$$k_{inact}K_{mg}K_{eq}[N] = \frac{d}{dt} (E_0 - [N] - K_{eq}[N] - K_{mg}K_{eq}[N]) \quad (9)$$

Upon rearrangement, and application of the chain rule, Equation 9 is transformed into a first-order differential equation for which $K_1 = d(K_{eq})/dt$ and $K_2 = d(K_{mg})/dt$. The rate of disappearance of native enzyme at any time (or temperature) according to Scheme 2 is given by Equation 10.

$$\frac{d[N]}{dt} = \left(\frac{-k_{inact}K_{mg}K_{eq} - K_2K_{eq} - K_1K_{mg} - K_1}{1 + K_{eq} + K_{mg}K_{eq}} \right) [N] \quad (10)$$

Unlike the three-state, isothermal Equilibrium Model in which all of the equilibrium constants and the deactivation rate constant are constant through the course of an experimental run, this four-state, non-isothermal model equation is not readily integrated analytically. However, provided that the continuous temperature ramp experiment is started at any temperature well below the T_m of the enzyme, such that $K_{mg} \ll K_{eq}$, Equation 10 may be solved numerically by assuming an initial value of $[N]_0 = E_0 / (1 + K_{eq})$.

Essentially, the continuous ramp method uses variable K_{eq} , k_{cat} , and k_{inact} , while the Equilibrium Model (in isothermal batch-mode) allows these all to be held constant. Both methods, however, assume the intrinsic activation and deactivation parameters (ΔG_{cat} , ΔG_{inact} , ΔH_{eq} , T_{eq}) remain nearly constant over the entire temperature range of interest.

In this work, we have studied the thermal deactivation of an enzyme, TEM-1 β -lactamase from *Escherichia coli*, using both the isothermal batch techniques and the continuous EMR temperature ramp, on the same batch of enzyme, in order to

assess the relative strengths of the two methods. Previously, a metallo- β -lactamase from *Bacillus cereus* has been studied using the batch-mode method with the equilibrium model, but a Class A β -lactamase such as TEM-1 has not been investigated to date using this method. [6, 12, 13]

Experimental Section

Materials

TEM-1 β -lactamase (E.C. 3.5.2.6) was over-expressed in *Escherichia coli* and purified from the expression medium according to the method of Rogers et al. [11] Penicillin G potassium salt was purchased from USB Corp. (Cleveland, OH). Coomassie Protein Assay Reagent was obtained from Thermo Scientific (Rockford, IL). All other chemicals used were of analytical grade.

Batch-mode Experimental Setup

Enzyme activity was measured using a Thermospectronic Helios γ -spectrophotometer equipped with a Thermospectronic single-cell Peltier-effect cuvette holder. This system was networked to a computer installed with Vision32 (Version 1.25, Unicam) software for recording absorbance vs. time data. Quartz cuvettes with a 5mm path length were used during the course of all experiments, due to their favourable optical and heat transfer properties. The cuvette was kept isothermal during the course of each experimental run and a Cole-Parmer Digi-Sense thermocouple thermometer was used to ensure there was no temperature gradient larger than 0.1°C within the cuvette, or from the beginning to the end of the run. Runs were discarded and repeated if a temperature gradient was detected. The thermometer and the temperature control bath were calibrated using a Cole-Parmer NIST-traceable glass thermometer.

Assay Conditions

The reaction mixture consisted of an aqueous solution of 50 mM sodium phosphate buffer and 4.4 mM Penicillin G at pH 7.0. For each run, 1.0 mL of reaction mixture was added to the cuvette, along with a 2-mm diameter teflon-coated stir wire, and the system was allowed to thermally equilibrate for a minimum of 5 minutes. A stock solution of enzyme, with concentration determined via Bradford assay (measurement of A_{280} in the presence of Coomassie Protein Assay Reagent), was used to prepare a sample with a final concentration of 32 $\mu\text{g/mL}$. After thermal equilibration, 5 μL of the enzyme solution (for a total enzyme concentration of 5.5 nM) was injected directly into the cuvette using a positive displacement pipettor and the contents of the cuvette were agitated rapidly for 3 seconds using the preheated stir wire. After stirring was completed, the software began recording absorbance data.

The hydrolysis of Penicillin G by TEM-1 β -lactamase was observed by monitoring the absorbance shift at 252 nm. At this wavelength, the unreacted substrate exhibited an extinction coefficient of $\epsilon_{252} = 682 \text{ M}^{-1} \text{ cm}^{-1}$ and the fully hydrolyzed mixture exhibited an extinction coefficient of $\epsilon_{252} = 213 \text{ M}^{-1} \text{ cm}^{-1}$. This difference of 469 $\text{M}^{-1} \text{ cm}^{-1}$ between the substrate and product remained constant throughout the entire temperature range of this study. The combination of substrate concentration, cuvette

path length, and enzyme loading was chosen such that the starting absorbance never exceeded 1.5 AU (to preclude instrument saturation), and the substrate conversion never exceeded $\sim 40\%$ at any of the temperatures examined. Hence, the substrate concentration was never lower than $\sim 2.6 \text{ mM}$; a value over 100 times the published K_M of the enzyme for Penicillin G at 25°C. [14] The linearity of the instrument response over the absorbance range 0.5-1.5 AU was confirmed by running a dilution series of unreacted substrate solution ($R^2 = 0.995$). Reactions were allowed to proceed for 120 seconds, with data points acquired at 2-second intervals.

Batch-mode Data Analysis

Triplicate assays were performed at each temperature, and the absorbance vs. time data were converted to reaction rate vs. time data. The average of the three rates at each time point was used to construct a single progress curve of reaction rate vs. elapsed assay time for each given temperature. The progress curves were smoothed using a Loess transformation (SigmaPlot 2001 for Windows) to eliminate noise from the data, and the smoothed curves for all temperatures were initially fitted to the equilibrium model using MATLAB, with initial parameter estimates of $\Delta G_{cat} = 85 \text{ kJ/mol}$, $\Delta G_{inact} = 100 \text{ kJ/mol}$, $\Delta H_{eq} = 120 \text{ kJ/mol}$, $T_{eq} = 320 \text{ K}$. The fitting parameters resulting from the first MATLAB run were used as improved initial estimates for a second MATLAB run, which provided the final values. [9]

Continuous-mode EMR Experimental Setup

The conversion vs. time data for the continuous temperature ramp experiment were collected in an enzyme membrane reactor according to the method of Rogers et al. [11] A single sample of enzyme was charged to a reactor with a working volume of 10.0 mL and a solution of 200 mM sodium phosphate buffer and 20 mM Penicillin G was continuously fed to the reactor at a rate of 1.25 mL/min. After a 1-hr equilibration period at the initial temperature of 295 K, the reactor temperature was progressively increased to 330 K using a linear temperature ramp of 2 K/hr.

The substrate conversion was monitored by measuring the optical rotation of the reactor effluent stream via an in-line polarimeter with a sample cell which was kept at 25°C by external water bath. At 25°C, the specific optical rotation of the substrate mixture was $\alpha_{589} = 107^\circ \text{ M}^{-1}$ while that of the product was $\alpha_{589} = 57^\circ \text{ M}^{-1}$. To account for background thermal degradation of the substrate, a calibration curve was constructed by first running pure unreacted feed and then completely hydrolysed product through the entire temperature ramp.

Continuous-mode Data Analysis

The conversion vs. time data were smoothed with the MATLAB Curve Fitting Toolbox, using a Robust Loess method with span = 85. The smoothed experimental data were fitted according to Equations 6 and 10, using an iterative solving procedure to minimize the sum of squared residuals by adjusting the fitting parameters. Two different numerical techniques were used to solve the IVP of Equation 10: an implicit Euler method with a time interval of $\Delta t = 120$ seconds and an area-under-the-curve approximation per the trapezoidal rule, with a time interval of $\Delta t = 120$ seconds.

In addition, a model was generated which omitted the folding state, U , and assumed only a three-state model equivalent to the equilibrium model. An attempt was then made to fit the continuous EMR data to this three-state model for purposes of comparison, using the area-under-the-curve approximation per the trapezoidal rule for the numerical integration.

Results and Discussion

Batch-mode Results

A total of ten progress curves for the enzyme were measured, each in triplicate, over the temperature range from 292 K to 332 K. The temperature spacing between progress curves was varied through the course of the experiments, and two of the progress curves were measured at high temperatures such that the observed initial reaction rate had fallen below its peak value (i.e. the temperature was above T_{opt}). The experimental data for rate vs. temperature vs. time, overlaid with the rate predicted by the MATLAB routine according to Equation 4, is shown in Figure 1.

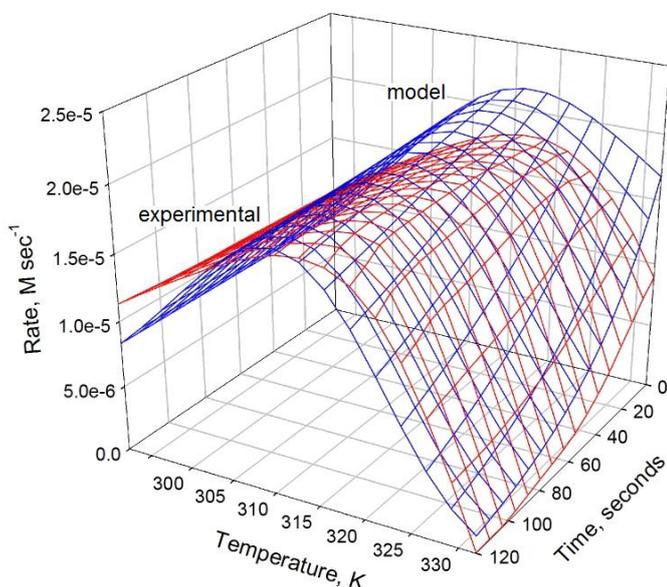


Figure 1. Isothermal Batch-mode (experimental vs. model)

Figure 2 depicts the experimental data from the continuous EMR temperature ramp experiment, overlaid with three different model predictions: the four-state molten globule model using the implicit Euler technique, the four-state molten globule model using the trapezoidal rule, and the three-state equilibrium model. The values of the deactivation parameters which were found by the various experimental methods and model fitting procedures are shown in Table 1.

Comparison of Models to Experimental Data

Figures 1 and 2 show the experimental data for the batch-mode and continuous methods, respectively, overlaid with the predictions of the models. One conspicuous feature of the experimental data for both the isothermal batch and temperature-ramp methods is that the early (low-temperature) segment of the curve shows a near-linear increase of activity with temperature. Absent any deactivation effects in this regime, one would expect the curve to be concave upward, more closely resembling an Arrhenius-type activation profile. The near-linear increase

observed in the isothermal batch-mode data suggests the onset of activity loss at a temperature well below the traditional T_m of TEM-1 β -lactamase, which is 51.5°C as evidenced by interpretation of the circular dichroism spectrum. [15] It is clear that a different effect must be responsible for the observed activity loss in the vicinity of 310-315 K, more than 10°C below the T_m . This is contrary to the widely-held idea that the optimum operating temperature of a biocatalyst is very close (within a degree or two) of its melting temperature, but is expected for an enzyme following the Equilibrium Model. [10, 16] This may only hold true for those enzymes for which global secondary structure and active site integrity are compromised simultaneously.

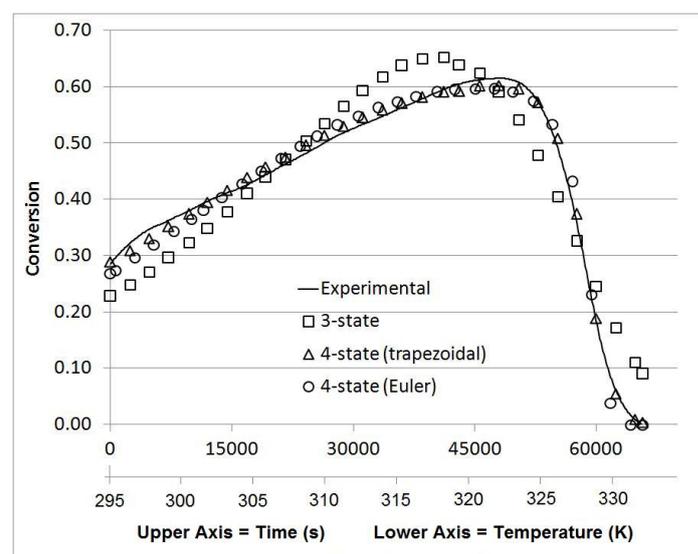


Figure 2. Continuous Temperature-ramp EMR (fitted to models)

The three-state deactivation model resulted in a relatively poor fit to the continuous non-isothermal data from the EMR. Similar to the fit of the Equilibrium Model to the batch results in Figure 1, the three-state deactivation model, numerically integrated via the trapezoidal rule, tends to underestimate the enzyme activity on the low-temperature end of the EMR experiment, anticipating an Arrhenius-type increase as temperature rises. It can be clearly seen that the predicted slope of the rate vs. temperature trend, in the low-temperature regime, is significantly greater than that of the experimental data, for both the three-state batch and three-state temperature-ramp models.

Notably, however, the three-state isothermal Equilibrium Model (MATLAB routine) resulted in a relatively good fit to the batch data in the high temperature regime, particularly near the T_{eq} of 314 K, while the three-state EMR model was unable to converge upon a good fit in this regime. One explanation for this could be the large difference in time scales between the two experimental methods. At temperatures a few degrees below the T_{eq} , denaturation during the course of the experimental run may be nearly invisible in the two-minute isothermal batch assay. The three-state EMR model, however, is forced to include the cumulative effects of denaturation over a much longer time (the time between each data point in the EMR model is 120 seconds, the same length as the entire progress curve of the batch model).

Thus, at a moderate temperature which is apparently “non-denaturing” as seen in the shorter batch assay, a significant amount of time has passed in the continuous EMR run and there

is already much less active enzyme present than if the enzyme were being exposed to that temperature for the first time. Indeed, it has been previously shown in the literature that insufficient monitoring time, particularly at higher temperatures, can disguise the equilibrium character of inactivation and thus make it difficult to fit data to a model which would remain consistent in longer assays. [17] Effects such as uncooperative unfolding and grace-period inactivation can influence the accuracy of such short-term assays.

rate constant k_{inact} may appear to be quite different during a two-minute assay than over the course of several hours.

The ΔG_{cat} value of 54.2 kJ/mol predicted by the continuous four-state trapezoidal model gives a turnover number of 1530 s^{-1} at 25°C , which is in excellent agreement with the literature value of 1500 s^{-1} for TEM-1 using Penicillin G as a substrate. [22] The predicted ΔG_{cat} was slightly lower in the three-state batch-mode Equilibrium Model, at 53.6 kJ/mol. This corresponds to a turnover

Table 1. Estimation of Deactivation Parameters by Isothermal Batch-mode Tests and Continuous temperature-ramp EMR Methods

Method	ΔG_{cat} (kJ/mol)	ΔH_{eq} (kJ/mol)	T_{eq} (K)	ΔH_{mg} (kJ/mol)	T_{mg} (K)	ΔG_{inact} (kJ/mol)	Σ (residual) ²
Batch Isothermal	53.6	98.5	313.8	-	-	92.1	-
EMR (four-state, Euler)	54.0	64.6	317.9	716.5	328.6	101.2	0.1835
EMR (four-state, trap.)	54.2	74.9	309.2	699.7	324.7	100.6	0.0497
EMR (three-state, trap.)	54.8	69.4	330.5	-	-	101.0	1.7198

There is ample evidence in the literature that several strains of β -lactamase follow a four-state unfolding mechanism. [18-20] It has been previously established via guanidinium chloride denaturation experiments that TEM-1 β -lactamase does not follow a simple "one-step, two-state" folding mechanism, but rather exhibits a thermodynamically stable intermediate state in equilibrium with the native and unfolded states, likely influenced by the cis-trans isomerization of Xaa-Pro peptide bonds. [21] This intermediate state, described in the literature, is modelled here to correspond to the molten globule state of the four-state model.

The batch-mode isothermal Equilibrium Model and the non-isothermal four-state temperature ramp models, remarkably, were in good agreement with respect to the location of the T_{eq} , though the batch-mode Equilibrium Model predicted a higher ΔH_{eq} . This may be explained because the three-state model is converging on an intermediate value somewhere between the enthalpy change necessary to enact a conformational change at the active site (the ΔH_{eq} of the four-state model), and the enthalpy change associated with the onset of unfolding (the ΔH_{mg} of the four-state model). The four-state model makes a distinction between these two transitions, in which the molten globule state M exhibits no activity but also does not readily become permanently denatured, while the unfolded state is much more readily denatured, an effect which corresponds to the sudden loss of activity in the vicinity of T_{mg} . In addition, the nature of the Equilibrium Model equation is such that ΔH_{eq} is subject to relatively high errors. [6, 9] Discrepancies between the two models may be due to the fact that the Equilibrium Model treats the $E_{inact} \rightarrow X$ transition as a single event, although not necessarily via a single product. [7] Alternatively, it may be due to the fact that, for this enzyme, data for the Equilibrium Model has only been collected for two minutes. The value of T_{mg} predicted by the temperature-ramp four-state trapezoidal model (the better fit of the two four-state models) is 51.7°C , which coincides almost exactly with the documented T_m of the enzyme as measured by circular dichroism. [15]

The values for ΔG_{inact} predicted by the four-state continuous model were somewhat higher than that of the three-state batch-mode Equilibrium Model. Again, this may be attributable to the fact that there is a very large time-scale difference between the two experiments, and the prediction of the first-order denaturation

number of 2550 s^{-1} at 25°C . The discrepancy between these two values could be a consequence of experimental error associated with the Bradford assay in determining the initial enzyme concentration, or it may be due to determinations of k_{cat} having been made under conditions for which not all the enzyme is active. [7, 23] However, the difference is not far from the sum of the fitting and experimental errors expected for the determination of Equilibrium Model parameters. [9]

The four-state continuous temperature-ramp models predict the ΔH_{mg} to be nearly an order of magnitude higher than the ΔH_{eq} value, which is in agreement with the notion that the conformational shift needed to render the enzyme inactive is slight compared to the complete unfolding of the enzyme. [7, 8, 24, 25] The value of 700 kJ/mol for the enthalpy change upon unfolding is in the typical range for globular proteins such as β -lactamase I from *Bacillus cereus*, which exhibits a denaturation enthalpy of 646 kJ/mol, although this is low compared with subtilisin. [26]

The two numerical integration methods used with the four-state model (implicit Euler method and trapezoidal rule) predicted nearly the same values for ΔG_{cat} , ΔH_{mg} , and ΔG_{inact} . The two methods produced slightly different predictions for the T_{mg} , which may be due to the different behaviour of the two integration techniques in the vicinity of T_{mg} . The trapezoidal rule method, which resulted in a better overall fit, was particularly more effective than the implicit Euler method at tracking the experimental data in this regime where the rate of change of conversion with respect to time is high. The significant difference between the two models was in their predictions of ΔH_{eq} and T_{eq} . The implicit Euler method showed a higher T_{eq} with a lower energy barrier, while the trapezoidal method showed the converse arrangement. Nevertheless, both methods confirmed that ΔH_{eq} was about an order of magnitude less than the ΔH_{mg} indicated by both the four-state continuous experiment and CD data from literature and that the T_{eq} was at least 10°C lower than T_{mg} (i.e. melting temperature T_m).

Conclusion

While the three-state Equilibrium Model (Scheme 1) did not result in a good fit to the data obtained via the continuous temperature

ramp method, it resulted in a relatively good fit to the isothermally-obtained batch data, though the predicted value of ΔG_{cat} was slightly low and the abbreviated increase of actual reaction rate with temperature was not fully captured by the model. The four-state deactivation model (Scheme 2), especially when the steep changes around T_{mg} were tracked by a routine employing the trapezoidal rule rather than Euler's method, resulted in an excellent fit to the data collected via the continuous temperature ramp EMR experiment, and furthermore showed good agreement with the batch-mode equilibrium model regarding the location of T_{eq} , the temperature of transition between native, active enzyme and conformationally-compromised, inactive enzyme.

While the four-state model contains a higher number of fitting parameters and should admittedly result in an improved fit over the three-state model, there is, as discussed earlier, copious evidence in the literature which indicates TEM-1 unfolds via a four-state mechanism. Both the three-state equilibrium and four-state continuous models were effective at pinpointing the transition between active and inactive enzyme. The finding of two distinct transitions in the temperature-ramp EMR model corroborates the previously established notion that T_{eq} is separate from T_{opt} , with the latter depending on a mix of T_{eq} , ΔG_{cat} , and ΔH_{eq} . [5-7] Both are important to engineers who wish to characterize and design biocatalysts. It is clear that the difference in timescale of these two experimental modes may affect the parameters predicted by model fitting. Denaturing effects which may be ignored during an assay lasting only a few minutes will have a greater effect on models which seek to capture the cumulative deactivation over several hours. A high degree of uncooperativity in the process of transitioning between native and denatured states is likely one source of discrepancy between the two experimental methods.

The discontinuous Equilibrium Model batch method has a string of advantages. The collection of an array of isothermal progress curves, while labour-intensive compared to the continuous temperature-ramp experiment, can be fitted to a model which is mathematically straightforward in that all temperature-dependent equilibrium constants remain unchanged during progress curves. The batch method also uses smaller quantities of substrates, buffers, and other reagents and can be performed with commonplace instrumentation and equipment (i.e. no need to configure a CSTR). In the current study with TEM-1 β -lactamase, the equilibrium model was equally as capable as the more complex four-state continuous model in identifying the T_{eq} of the enzyme. So far, all enzymes for which Equilibrium Model data has been collected obey the model. [7, 8]

The temperature-ramp method, while it is faster and requires minimal direct monitoring, and furthermore utilizes a single injection of enzyme (minimizing systematic error), requires a model which relies on numerical integration techniques due to the complicated behaviour of native enzyme population when temperature increases with time. However, the ability of the continuous EMR system, in conjunction with the four-state deactivation model, appears to be a powerful tool for examining multiple thermodynamic transitions (N to M as well as M to U), both of which are of great importance in biocatalyst design, in a

single experiment. This technique can likely be extended to other biocatalysts which do not deactivate according to a simple two-state cooperative mechanism.

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