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The effect of temperature on enzyme activity: new insights and their implications

Roy M. Daniel · Michael J. Danson · Robert Eiseenthal · Charles K. Lee · Michelle E. Peterson

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Abstract The two established thermal properties of enzymes are their activation energy and their thermal stability. Arising from careful measurements of the thermal behaviour of enzymes, a new model, the Equilibrium Model, has been developed to explain more fully the effects of temperature on enzymes. The model describes the effect of temperature on enzyme activity in terms of a rapidly reversible active-inactive transition, in addition to an irreversible thermal inactivation. Two new thermal parameters, T_{eq} and ΔH_{eq} , describe the active–inactive transition, and enable a complete description of the effect of temperature on enzyme activity. We review here the Model itself, methods for the determination of T_{eq} and ΔH_{eq} , and the implications of the Model for the environmental adaptation and evolution of enzymes, and for biotechnology.

Keywords Equilibrium Model · Temperature · Enzyme · Adaptation · Evolution · Temperature optimum · Stability · Denaturation · T_{eq} · ΔH_{eq}

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Introduction

If an enzyme is assayed for a fixed duration over a range of temperatures, we observe a plot with an apparent optimum (Fig. 1). This observed “optimum temperature” is not an intrinsic enzyme property, since it arises from a mixture of thermal properties, and from assay duration. Nevertheless, this behaviour has led to a long-standing implicit assumption of a two-state model (the Classical Model), where the dependence of enzyme activity on temperature can be described by the effect of temperature on two processes: the catalytic reaction, defined by k_{cat} , and irreversible inactivation (denaturation) defined by k_{inact} . The temperature dependence of these rate constants is characterised, respectively, by the catalytic activation energy ΔG_{cat}^\ddagger , and the activation energy for the irreversible inactivation reaction, $\Delta G_{inact}^\ddagger$.

In this situation the variation in enzyme activity with temperature and time of assay can be described as follows:

$$V_{max} = k_{cat} \cdot [E_0] e^{-k_{inact} \cdot t} \quad (1)$$

where V_{max} = maximum velocity of enzyme; k_{cat} = enzyme’s catalytic constant; $[E_0]$ = total concentration of enzyme; k_{inact} = thermal inactivation rate constant; t = time.

The variation of the two rate constants in Eq. 1 with temperature is given by:

$$k_{cat} = \frac{k_B T}{h} \cdot e^{-\left(\frac{\Delta G_{cat}^\ddagger}{RT}\right)} \quad (2)$$

and

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

Fig. 1 The apparent temperature optimum. A typical graph of enzyme activity versus temperature showing the “temperature optimum” of an enzyme-catalysed reaction. This apparent optimum arises from an unknown contribution of the effect of temperature on activity, thermal stability and on assay duration, since the contribution of denaturation is time dependent as well as temperature dependent

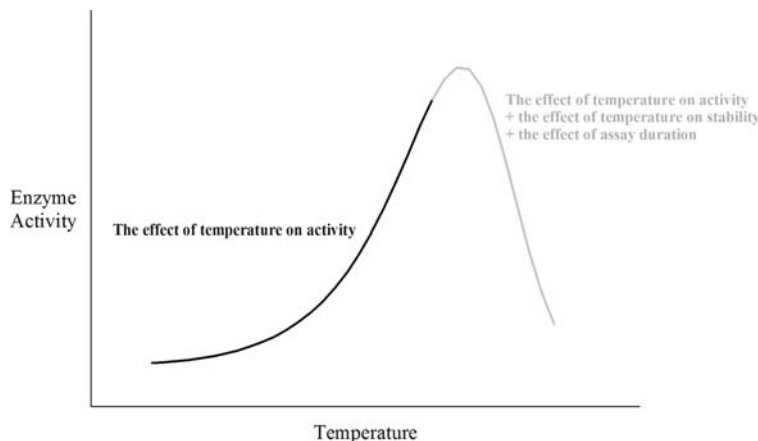
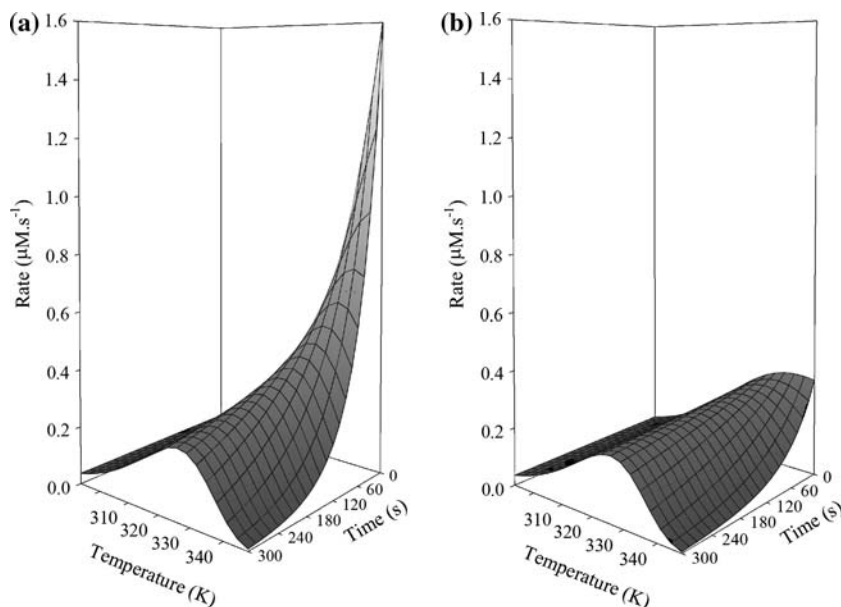


Fig. 2 The temperature dependence of enzyme activity. These plots of rate ($\mu\text{M s}^{-1}$) versus temperature (K) versus time (s) illustrate the effect of temperature on enzyme activity as described by **a** The Classical Model; and **b** The Equilibrium Model



where k_B = Boltzmann's constant; R = Gas constant; T = absolute temperature; h = Planck's constant; $\Delta G_{\text{cat}}^\ddagger$ = activation energy of the catalysed reaction; $\Delta G_{\text{inact}}^\ddagger$ = activation energy of the thermal inactivation process.

Using these equations, and inserting plausible values for $\Delta G_{\text{cat}}^\ddagger$ and $\Delta G_{\text{inact}}^\ddagger$, we can construct a plot of activity against temperature against time (Fig. 2a). At zero time there is no denaturation since denaturation is time dependent. Any experimental discrepancies from this model have been difficult to quantify because activity and stability are usually measured under different conditions.

The equilibrium model

Recently a number of workers have observed apparent temperature optima in short duration assays. In particular, work by Thomas and Scopes (1998) has shown a lower

activity at high temperatures for some enzymes than can be accounted for by denaturation. To attempt to explain this, a new model has been formulated, the Equilibrium Model (Daniel et al. 2001), wherein the active enzyme (E_{act}) is in equilibrium with an inactive form (E_{inact}), and it is the inactive form that undergoes irreversible thermal inactivation to the denatured state (X). The equilibrium is described by an equilibrium constant (K_{eq});



In the Equilibrium Model, the dependence of enzyme activity on temperature has an additional component, namely the effect of temperature on the equilibrium position between active and inactive forms of the protein. This temperature dependence is characterised in terms of the enthalpy of the equilibrium, ΔH_{eq} , and a new thermal parameter, T_{eq} , which is the temperature at which the

concentrations of E_{act} and E_{inact} are equal. The variation of K_{eq} with temperature is given by;

$$\ln(K_{\text{eq}}) = \frac{\Delta H_{\text{eq}}}{R} \left(\frac{1}{T_{\text{eq}}} - \frac{1}{T} \right), \quad (5)$$

where ΔH_{eq} is the enthalpic change associated with the conversion of active to inactive enzyme, and T_{eq} is the temperature at the mid-point of transition between the two forms. At T_{eq} , $[E_{\text{act}}] = [E_{\text{inact}}]$; hence $K_{\text{eq}} = 1$, $\Delta G_{\text{eq}}^{\ddagger} = 0$, and $\Delta H_{\text{eq}} = T_{\text{eq}} \Delta S_{\text{eq}}$, where ΔS_{eq} is the change in entropy associated with the equilibrium. We can thus regard T_{eq} as the thermal equivalent of K_M , being the temperature at which half of the enzyme is active.

We have developed a mathematical formulation for the Equilibrium Model by incorporating the two new parameters, where the overall dependence of velocity on temperature with time can be described by the relationship:

$$V_{\text{max}} = \frac{k_B T \cdot e^{\left(-\frac{\Delta G_{\text{cat}}^{\ddagger}}{RT} \right)} \cdot E_0 \cdot e^{\left(\frac{k_B T \cdot e^{\left(-\frac{\Delta G_{\text{inact}}^{\ddagger}}{RT} \right)} \cdot e^{\left(\frac{\Delta H_{\text{eq}} \left(\frac{1}{T_{\text{eq}}} - \frac{1}{T} \right)} \right)} \right)}{h \left(1 + e^{\left(\frac{\Delta H_{\text{eq}} \left(\frac{1}{T_{\text{eq}}} - \frac{1}{T} \right)} \right)} \right)}}{h \left(1 + e^{\left(\frac{\Delta H_{\text{eq}} \left(\frac{1}{T_{\text{eq}}} - \frac{1}{T} \right)} \right)} \right)}. \quad (6)$$

If we insert plausible values for the four thermal parameters, $\Delta G_{\text{cat}}^{\ddagger}$, $\Delta G_{\text{inact}}^{\ddagger}$, T_{eq} , and ΔH_{eq} , into the Model, the effect of incorporating an additional inactive species of the enzyme, in reversible equilibrium with the active form, is evident from a plot of enzyme activity vs. temperature vs. time (Fig. 2b). This shows a temperature optimum at zero time, which is not seen in the Classical Model, implying a new mechanism for the reduction in activity as the temperature is raised further, since denaturation is time-dependent. Moreover, depending on the value of T_{eq} with respect to the temperatures at which significant inactivation is observed during the time course of the assay, there is a much smaller change in the apparent temperature optimum with time than in the Classical Model. In effect, the Equilibrium Model provides a “thermal buffer” that protects the enzyme from thermal inactivation.

We have verified this Model (Peterson et al. 2004) by carrying out detailed studies of the variation of enzyme activity with time and temperature of a number of

enzymes, to construct 3D profiles experimentally (Fig. 3a), and, by feeding these data into our mathematical model, derived values for all four thermal parameters, i.e. T_{eq} , ΔH_{eq} , $\Delta G_{\text{cat}}^{\ddagger}$, $\Delta G_{\text{inact}}^{\ddagger}$, and a graphical value for T_{opt} (Fig. 3b).

Every enzyme we have studied so far (>30) shows this behaviour (Peterson et al. 2004; Lee et al. 2007; M.E. Peterson, C. Monk and R.M. Daniel, unpublished results); i.e. all show “tent” type graphs with an activity temperature optimum at zero time, when no denaturation can have occurred, indicating that T_{eq} is unrelated to stability. Note that in these experiments denaturation is measured under the same conditions as activity. Parameters calculated from experiment can be reinserted into the Model to regenerate a 3D graph that closely matches that obtained using the actual experimental data. The experimental data obtained cannot be fitted to the Classical Model.

From the zero time plot, a graphical value for the temperature at which maximum activity occurs, T_{opt} , can be obtained. The relationship of T_{eq} to T_{opt} can be described by the equation:

$$T_{\text{opt}} \approx T_{\text{eq}} (1 - \alpha T_{\text{eq}}). \quad (7)$$

where

$$\alpha \approx \frac{R}{\Delta H_{\text{eq}}} \ln \left(\frac{\Delta H_{\text{eq}} - \Delta G_{\text{cat}}^{\ddagger}}{\Delta G_{\text{cat}}^{\ddagger}} \right) \quad (8)$$

and α is small (such that $\alpha T_{\text{eq}} \ll 1$). Thus, in general, for enzymes whose thermal activity dependence follows the Equilibrium Model, T_{opt} will be close in value to T_{eq} ; for 90% of the enzymes we have studied to date, they are within 6°C of each other.

Other models have been proposed from time to time to account for the effect of temperature on enzyme activity and stability. The idea of a temperature-dependent reversible equilibrium between active and inactive forms of an enzyme was proposed by Sizer (1944), but Sizer’s model did not include the possibility of time-dependent, irreversible inactivation. Time-dependent inactivation was considered by Wright and Schomaker (1948), who studied the inactivation of diphtheria antitoxin by heat. They proposed a model that comprised two interconvertible forms of active antibody, one susceptible to irreversible inactivation, and the other protected. They made no assumption of a rapid equilibrium, and treated the system as one consisting of three first-order reactions. They recognised that the model provided a reservoir of native antibody during a significant fraction of the time course. However, their model differs from the Equilibrium Model in assuming that the “activity” (which was based on a bio-assay) is independent of temperature and also that both

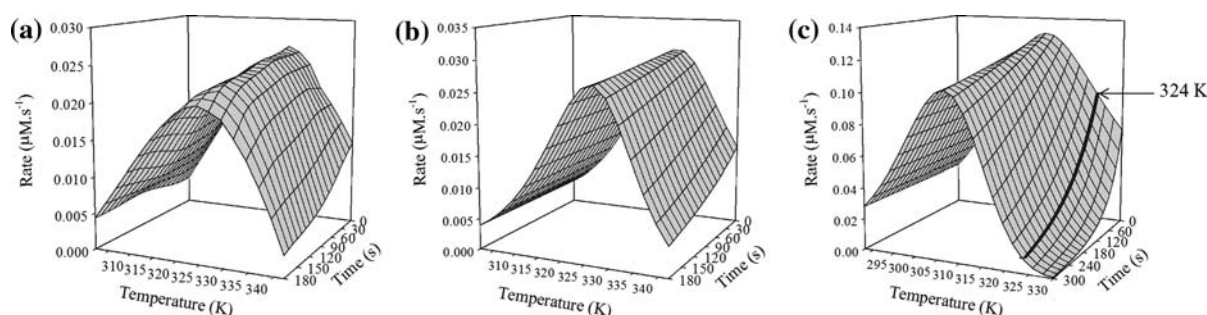


Fig. 3 Experimental verification of the Equilibrium Model and an indication of the timescale of the $E_{\text{act}}/E_{\text{inact}}$ Equilibrium. Phenylalanine ammonia lyase was assayed as described by Lee et al. (2007). The experimental data was transformed using a Loess smoothing technique and the results (a) are plotted here as rate ($\mu\text{M s}^{-1}$) versus temperature (K) versus time (s). The results of fitting the experimental data to the Equilibrium Model are also plotted here (b). Parameter

values derived from this fitting are: $\Delta G_{\text{cat}}^{\ddagger} = 80 \text{ kJ mol}^{-1}$, $\Delta G_{\text{inact}}^{\ddagger} = 97 \text{ kJ mol}^{-1}$, $\Delta H_{\text{eq}} = 181 \text{ kJ mol}^{-1}$ and $T_{\text{eq}} = 330 \text{ K}$. The experimental data itself cannot be fitted to the Classical Model. c For the enzyme aryl acylamidase (Peterson et al. 2004), at 324 K the very rapid loss of activity due to the $E_{\text{act}}/E_{\text{inact}}$ equilibration is $\sim 60\%$. At the same temperature, a similar loss of activity due to denaturation occurs over several minutes

non-denatured forms are active. Lumry and Eyring (1954) pointed out that the first-order behaviour of irreversible protein denaturation processes is often the result of the overall process being limited by a preliminary reversible step (Hardy 1899); for example, unfolding followed by aggregation. In the Equilibrium Model, the description of the conversion of E_{inact} to X is not intended to preclude the presence of multiple steps in this inactivation process.

Determination of the new parameters

The detection of the reversible enzyme inactivation, which forms the basis of the Equilibrium Model, requires careful acquisition and processing of assay data due to the number of conflicting influences that arise when changing the temperature of an enzyme assay (Daniel and Danson 2001). Determination of T_{eq} and ΔH_{eq} has until recently used continuous assays, because this method produces progress curves directly. It has utilised enzymes whose reactions are essentially irreversible (far from reaction equilibrium), do not show any substrate or product inhibition, and remain saturated with substrate throughout the assay. Furthermore, in the absence of information as to the effect of data on accuracy, each determination of T_{eq} and ΔH_{eq} has been carried out using up to 15,000 data points. However, there are a large number of enzymes that do not fit these criteria, or where the assay method makes such extensive data collection prohibitive. Methods are now available for the reliable determination of T_{eq} and ΔH_{eq} under ideal or non-ideal enzyme reaction conditions, and with the minimum data required for accuracy (Peterson et al. 2007). The minimum requirements for accurate results are, firstly, that more than one data point is taken at a temperature above

T_{opt} that gives a rate less than that at T_{opt} at zero time; this ensures that the Equilibrium Model is the appropriate way to treat the data (although no exceptions have been found to date), and secondly, that data are gathered at more than eight temperature points. Major errors in enzyme quantification affect only the determination of $\Delta G_{\text{cat}}^{\ddagger}$. Although continuous assays will give the most accurate results, $\Delta G_{\text{cat}}^{\ddagger}$, $\Delta G_{\text{inact}}^{\ddagger}$, ΔH_{eq} , and T_{eq} can be determined with good accuracy using discontinuous assays. Among other things, this will allow the determination of the parameters of enzymes from extreme thermophiles, since T_{opt} for these enzymes may be above 100°C and, since few continuous assay methods are practical at these temperatures, most such assays will have to be discontinuous (Daniel and Danson 2001). One of the most likely sources of error for the determination of the Equilibrium Model parameters will be insufficient substrate present at zero time to saturate the enzyme, either because of solubility limitations, for example, or as a result of increases in K_{M} (Hudson et al. 1993; Thomas and Scopes 1998; Daniel and Danson 2001; Liang et al. 2004) as the temperature is raised. Even here, it may be possible, if the K_{M} is known at each temperature, to obtain reasonable approximations of the initial rates at saturation by calculating the degree of saturation using the relationship $v/V_{\text{max}} = [\text{S}]/(K_{\text{M}} + [\text{S}])$, and applying the appropriate corrections.

Application of the Equilibrium Model to “non-ideal” enzyme reactions can usually be achieved by restricting assays to the initial rate of reaction. Setting $t = 0$ in Eq. 6 gives Eq. 9 below. Using this, it is possible to fit the experimental data for “zero time” (i.e. initial rates) to the Equilibrium Model to determine $\Delta G_{\text{cat}}^{\ddagger}$, ΔH_{eq} , and T_{eq} , although the time-dependent thermal denaturation parameter, $\Delta G_{\text{inact}}^{\ddagger}$, cannot be determined.

At $t = 0$,

$$V_{\max} = \frac{k_B T \cdot e^{\left(-\frac{\Delta G_{\text{cat}}^\ddagger}{RT}\right)} \cdot E_0}{h \left(1 + e^{\left(\frac{\Delta H_{\text{eq}} \left(\frac{1}{T_{\text{eq}}} - \frac{1}{T}\right)}{R}\right)} \right)} \quad (9)$$

A stand-alone Matlab® application (Version 7.1.0.246 [R14] Service Pack 3, The Mathworks Inc., Natick, MA, USA), enabling the facile derivation of the Equilibrium Model parameters from a Microsoft® Office Excel file of experimental progress curves (product concentration versus time), is available on CD from the corresponding author. This application is suitable for computers running Microsoft Windows XP, and is for non-commercial research purposes only.

The molecular basis of the equilibrium model

It should be noted that the Equilibrium Model itself makes no predictions as to the physical nature of the E_{inact} state or the basis for its equilibrium with E_{act} . We do know that the $E_{\text{act}}/E_{\text{inact}}$ equilibration is rapid compared with denaturation. The experimental determination of the parameters is carried out using time course reactions at a succession of fixed temperatures. Each reaction is started by the rapid addition and mixing of a few microlitres of enzyme at 0°C into 1.5–3 ml of temperature-equilibrated buffer (to enable a fast start to the reaction); the rate is sampled several times per second, with the first reliable data from the spectrophotometer being obtained after a few seconds. In all cases, the rates for the first 10 s show an origin at zero, and bearing in mind that at T_{eq} half of the enzyme activity has already been lost, it is clear that after a few seconds at most, at temperatures above T_{eq} the majority of the activity has been lost as a result of the reversible active–inactive equilibration by the time the first reliable data point is determined; conversely, irreversible activity loss (denaturation), determined in the same experiment and under the same conditions from examination of the activity–time plots at each temperature, takes minutes at these temperatures (Fig. 3c). So, the $E_{\text{act}}/E_{\text{inact}}$ equilibration occurs over a period of a few seconds, even at relatively low temperatures compared with those causing significant denaturation, and at least two orders of magnitude faster than denaturation under the same conditions. Indeed, the onset of the active to inactive transition can occur near 0°C for mesophilic enzymes with a low ΔH_{eq} (see below). Major protein unfolding is

generally two-state for single domain proteins (Creighton 1993), and unfolding takes place over timescales of 10^{-3} – 10^6 s (Fulton et al. 2005). However, of the proteins for which unfolding rates are available, more than two-thirds take longer than 3 s to unfold. Since all the enzymes examined so far show the rapid reversible equilibration between E_{act} and E_{inact} , it is unlikely that E_{inact} is significantly unfolded.

The molecular basis of the active–inactive transition described by the Equilibrium Model is most likely to be a reversible conformational change. The differing effects of temperature on the various weak forces stabilising and destabilising protein structure offer many opportunities for a shift in structure with temperature, leading to a change in activity. The strength of hydrophobic interactions, for example, tends to increase with temperature, while conformational entropy will also increase. That such changes can occur at the active site is evident from the sudden and dramatic changes in K_M that take place in some enzymes as temperature is increased (Hudson et al. 1993; Thomas and Scopes 1998).

Preliminary experimental data suggest that T_{eq} and ΔH_{eq} involve local and not global events, and that the associated conformational changes are at the active site, since stabilising and destabilising agents affecting stability do not necessarily affect T_{eq} or ΔH_{eq} , while changing the substrate or cofactor changes T_{eq} and ΔH_{eq} but does not necessarily affect stability (Eisenthal et al. 2006; M.E. Peterson, C. Monk, C.K. Lee, and R.M. Daniel, unpublished results). All this leads us to propose that the active-to-inactive transition described by the Equilibrium Model is caused by subtle, enzyme-specific, changes in the structure (and the associated dynamics) at, but not necessarily restricted to, the active site, arising from small changes in the network of interactions stabilizing and destabilizing the enzyme as the temperature is raised. Such small conformational changes can be expected to occur over millisecond or shorter timescales, may affect K_M or k_{cat} or both, and can have large effects on activity.

More data, especially direct data on the structural changes involved, are required to test this proposal.

Potential environmental and evolutionary implications

The Equilibrium Model provides a description of enzyme temperature-linked behaviour under assay conditions, so that the derived constants such as T_{eq} and ΔH_{eq} are potentially physiologically based. Since enzyme temperature adaptation is a major component of the adaptation of organisms to different temperatures and temperature regimes, the Equilibrium Model has the potential to be useful in such studies.

The application of the Model to 22 enzymes has allowed statistical analysis to make a preliminary identification of any associations of the Equilibrium Model parameters with each other or with an organism's growth temperature optimum (Table 1). All enzymes, including those from extremophiles, were found to display the zero-time activity optima with temperature that is characteristic of the Equilibrium Model. The parameters of all enzymes were derived by fitting assay data to the Equilibrium Model and thus relate to active enzymes in the presence of substrate and cofactor. The exception is the growth temperature optimum of the source organism, which is cited from various sources (Lee et al. 2007).

T_{eq} correlates well with growth temperature, to the extent that T_{eq} is statistically a better predictor of growth temperature than stability ($\Delta G_{inact}^\ddagger$). In general, T_{eq} of an enzyme tends to be about 20°C higher than the growth temperature of its source organism; this is not surprising, since at T_{eq} half of the enzyme activity is unavailable, while at 20°C below T_{eq} most of the enzyme will be in the active form (depending on ΔH_{eq} ; see below). Thermophiles seem to be an exception, with T_{eq} fairly close to the growth

temperature. A study using k_{cat} as a measure of temperature optimum also suggested that enzymes tend to have optimal activity at temperatures higher than its host's living temperature, and this gap narrows as the growth temperature of the organism rises (Georlette et al. 2004). There are several possible explanations for this proximity of T_{eq} to growth temperature in thermophiles. There may be a poor match between the environmental temperature of thermophiles and their laboratory growth temperature optima; thermophiles are often isolated from sources that are appreciably hotter than their determined optimal growth temperatures. Alternatively, it might be argued that the origin of current mesophilic microbial life is thermophilic, i.e. that microbial evolution has proceeded down-temperature from a thermophilic last common ancestor (Schwartzman and Lineweaver 2004; Pace 1997; Wachtershauser 1998), and that the evolution of T_{eq} lags behind optimal growth temperature. Depending on how crucial their roles may be in the cell, different enzymes might evolve at different speeds to adapt their T_{eq} to the environment, thereby resulting in the variation in T_{eq} among enzymes from one organism (e.g. for *Bos taurus*). Finally, it may be an artefact arising

Table 1 Thermodynamic parameters for 22 enzymes fitted to the Equilibrium Model

Organism	Enzyme	T_{growth} (°C)	ΔG_{cat}^\ddagger (kJ mol ⁻¹)	$\Delta G_{inact}^\ddagger$ (kJ mol ⁻¹)	ΔH_{eq} (kJ mol ⁻¹)	T_{eq} (°C)	T_{opt} (°C)	HWHM (degrees)	$T_{eq} - T_{growth}$	$T_{eq} - T_{opt}$
<i>Thermus sp.</i> RT41a	AKP	75	72	99	305	90	86	9	15	4
<i>T. aquaticus</i>	α -GLU	70	88	103	149	74.4	77	22	4.4	-2.6
<i>B. caldovelox</i>	IPMDH	70	76	101	573	63.9	61	4.5	-6.1	2.9
<i>C. saccharolyticus</i>	β -GLU	70	81	98	154	73.9	75	18.5	3.9	-1.1
<i>G. stearothermophilus</i>	α -GLU	55	64	96	225	67.8	64	10.5	12.8	3.8
<i>G. stearothermophilus</i>	DHFR	55	67	97	96	53.9	63	40.5	-1.1	-9.1
<i>E. coli</i>	MDH	40	55	96	619	70.7	67	4.5	30.7	3.7
<i>B. taurus</i>	MDH	39	53	85	826	67.4	64.5	4.5	28.4	2.9
<i>B. taurus</i>	AKP	39	57	97	86	59.6	69	44	20.6	-9.4
<i>S. scrofa</i>	FUM	39	60	92	378	59.1	55	6.5	20.1	4.1
<i>B. taurus</i>	GGTP	39	63	98	109	52	55.5	26	13	-3.5
<i>C. utilis</i>	GDH	31	57	94	409	59.5	55.5	6	28.5	4
<i>B. subtilis</i>	IPMDH	30	73	94	255	52.8	50	8.5	22.8	2.8
<i>B. cereus</i>	DHFR	30	66	90	261	58.5	55	8.5	28.5	3.5
<i>S. cerevisiae</i>	α -GLU	28	71	90	272	39.4	36.5	7.5	11.4	2.9
<i>P. fluorescens</i>	AAA	25	74	92	115	35.8	41	24	10.8	-5.2
<i>R. glutinis</i>	PAL	24	80	97	181	56.5	56	13	32.5	0.5
<i>Wheat germ</i>	ACP	20	79	95	142	63.9	66	20	43.9	-2.1
<i>P. dulcis</i>	β -GLU	20	63	95	100	55.9	61.5	33.5	35.9	-5.6
<i>B. psychrophilus</i>	IPMDH	20	72	100	123	56.7	60	24	36.7	-3.3
ASB HK47	AKP	15	55	90	149	17.5	15	12	2.5	2.5
<i>M. profunda</i>	DHFR	2	67	93	104	54.6	60.5	32	52.6	-5.9

The parameters of all enzymes are derived by fitting assay data to the Equilibrium Model and thus relate to active enzymes in the presence of substrate and cofactor. The exceptions are the growth temperature optimum (T_{growth}) of the source organism, which is cited from various sources (Lee et al. 2007), and T_{opt} and HWHM, which are both graphically determined from the zero-time temperature versus activity plots

from too small a selection of thermophiles in the experiment, although statistically this is unlikely. It is likely that an understanding of the molecular basis of T_{eq} will assist in resolving this finding.

There is a very strong negative correlation between ΔH_{eq} and HWHM (the upper temperature half width of the zero-time temperature/activity peak at 50% of maximum activity) (Fig. 4). The upper temperature half peak has been used because the lower temperature half of this peak is dominated by ΔG_{cat}^\ddagger . A large ΔH_{eq} leads to a sharp decline in activity with increasing temperature above T_{opt} , and thus a small HWHM. There are wide variations in both HWHM (from 4.5 to 44°C) and ΔH_{eq} (from 86 to 826 kJ mol⁻¹), and thus considerable variations in the sensitivity of enzyme activity to temperature above T_{eq} (and T_{opt}). ΔH_{eq} will also influence the difference between T_{eq} and T_{opt} (Eq. 8). ΔH_{eq} is the enthalpic change associated with the reversible, temperature-driven inter-conversion of an enzyme between its active and inactive state. It can be considered as a measure of the sensitivity of an enzyme's catalytic activity to temperature. ΔH_{eq} will therefore influence the broadness of zero-time activity

versus temperature plots; a small ΔH_{eq} will lead to a broader zero-time activity versus temperature peak, thus giving rise to the strong negative correlation between ΔH_{eq} and HWHM. ΔH_{eq} can thus be considered to be a quantitative measure of an enzyme's ability to function over a temperature range, a small ΔH_{eq} indicating that the enzyme will function at relatively high activity over a wide range of temperatures, i.e. behave in a eurythermal manner. Conversely, a large ΔH_{eq} indicates stenothermal behaviour. ΔH_{eq} correlates relatively weakly with other thermal properties; we speculate that it is more likely to be determined by the temperature range or temperature variability of the environment(s), although this hypothesis has not been tested.

In Fig. 5, the alkaline phosphatase from isolate ASB HK47 gives an illustration of the relative effects of the four parameters on enzyme activity over a range of temperatures. For many enzymes (e.g. *Bacillus cereus* dihydrofolate reductase), the denaturation rate is such that it will play the major role at temperatures above T_{eq} in controlling enzyme activity over the longer timescales important to the organism. However, for the ASB HK47

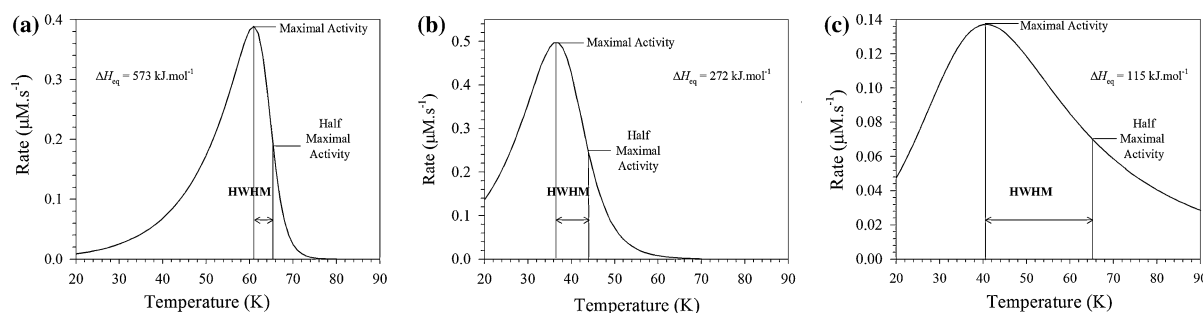


Fig. 4 Zero-time activity plot of enzymes with different eurythermal properties. Plots of zero-time activity ($\mu\text{M s}^{-1}$) versus temperature ($^{\circ}\text{C}$) that illustrate the influence ΔH_{eq} has on the broadness of zero-

time activity for three enzymes: **a** *Bacillus caldovelox* isopropylmalate dehydrogenase; **b** *Sacchromyces cerevisiae* α -glucosidase; **c** *Pseudomonas fluorescens* aryl acylamidase

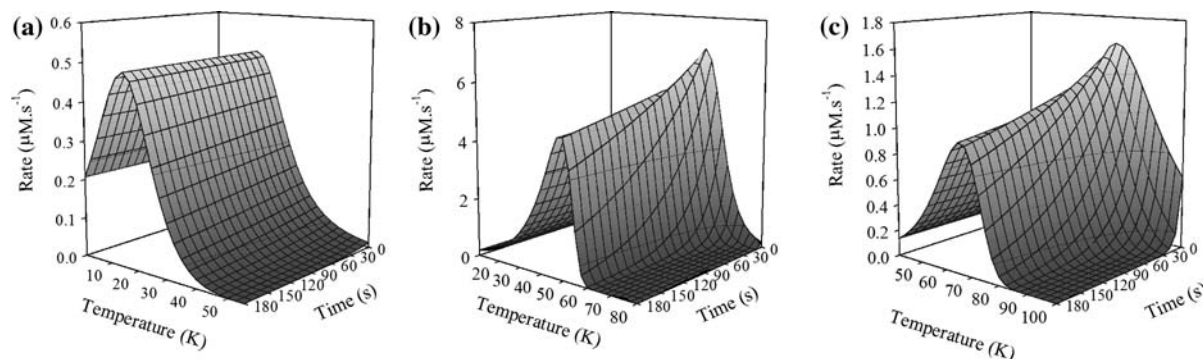


Fig. 5 3D plots of enzymes from different thermal origins. Plots of rate ($\mu\text{M s}^{-1}$) versus temperature (K) versus time (s) for three enzymes from different thermal origins: **a** Antarctic sea bacterium HK47 alkaline phosphatase—psychrotrophic; **b** *Bacillus cereus*

dihydrofolate reductase—mesophilic; **c** *Caldicellulosiruptor saccharolyticus* β -glucosidase—thermophilic. The plots are generated from parameters derived directly from the raw data (see Table 1)

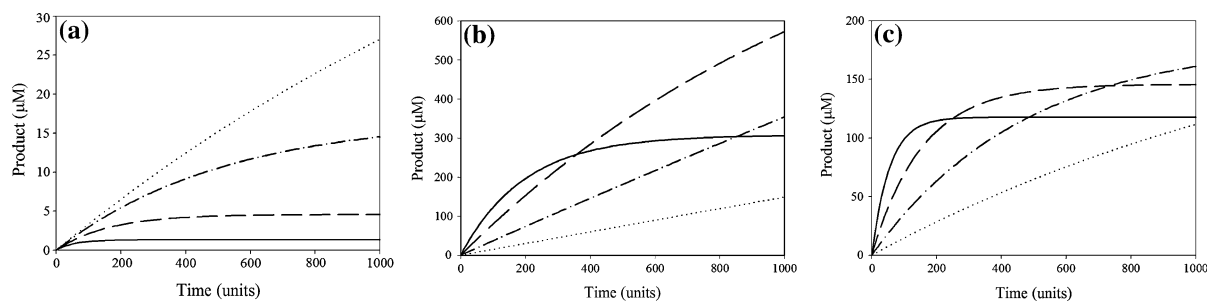


Fig. 6 The effect of T_{eq} on time courses of product concentration simulated at various temperatures. 310 K (dotted line); 320 K (· - · -); 330 K (- - -); 340 K (—). **a** Equilibrium model with $T_{eq} = 300$ K; **b**

Equilibrium model with $T_{eq} = 350$ K; **c** Classical model for comparison. The other parameter values are the same as those used in Fig. 1. Reproduced with permission from Eissenthal et al. (2006)

alkaline phosphatase it is evident that the low rate of denaturation above T_{eq} leads to a situation where activity will be dominated by T_{eq} and ΔH_{eq} over these longer timescales.

Implications for biotechnology

The Equilibrium Model introduces additional factors to be considered when trying to engineer an enzyme to enhance activity at high temperatures. The model tells us that engineering an increase in stability alone (i.e. an increase in $\Delta G_{inact}^\ddagger$) will not necessarily lead to enhanced activity at high temperatures, unless T_{eq} , the temperature of the equilibrium mid-point between the active and inactive forms, is also shifted to higher temperatures. Also, it will be important in any rational enzyme engineering to screen mutants to determine the effect on T_{eq} as well as on thermostability. Since the molecular basis of the equilibrium described by T_{eq} is probably at the active site, this may be difficult to do without affecting K_M or k_{cat} . Increasing ΔH_{eq} , for example, might be possible through the introduction of stabilising interactions at the active site, since at T_{eq} , $\Delta G_{eq}^\ddagger = 0$, so that $\Delta H_{eq} = T\Delta S_{eq}$. This might reduce the protein's flexibility and thus potentially adversely affect the k_{cat} value of the enzyme.

Enzyme screening or selection for particular thermal properties is normally carried out to obtain good activity and/or resistance to denaturation at a particular temperature. Enzyme thermal stability is indeed correlated (if weakly) with the growth temperature of the source organism. However, the Equilibrium Model shows that reduction of activity as the temperature is raised will not be entirely due to irreversible thermal inactivation—it will depend to a varying degree on T_{eq} , ΔH_{eq} and $\Delta G_{inact}^\ddagger$, as well as on the timescale involved. In short, successful selection from the environment of thermally stable/active enzymes will depend upon making a clear distinction between activity at high temperatures (determined by T_{eq}) and thermal stability.

Maximising the output of enzyme reactors requires a very careful balance of the effects of temperature upon enzyme stability and upon activity. Predictions of the output of product with time and temperature based upon the Classical Model can be quite different from those derived from the Equilibrium Model. When using an enzyme in a batch reactor for chemical synthesis, intuition would predict that the higher the operating temperature, the faster the catalysed reaction, but also the less stable the enzyme. In fact, this is only generally true if T_{eq} exceeds the working temperature of the reactor. If T_{eq} is less than the working temperature, the reverse is true (compare Fig. 6a, b). Typical time courses of product formation at various temperatures predicted by the Classical Model (which will never show the effect seen in Fig. 6a) are shown in Fig. 6c for comparison (Eissenthal et al. 2006)

Conclusions

The Equilibrium Model proposes a new and additional mechanism by which enzymes can (reversibly) lose activity as the temperature rises. The development and verification of the Model have provided additional and quantitative measures of the thermal behaviour of enzymes (Daniel et al. 2001; Peterson et al. 2004) that are essential for describing the effect of temperature on enzyme activity, and useful parameters for measuring the temperature adaptation of enzymes. Additionally, the Equilibrium Model has potential usefulness as a tool to investigate the relationship between enzyme thermal properties and the influence of temperature on the physiology and evolution of the host organism, and in biotechnology. It should be noted that although the Model itself enables an accurate description of the effect of temperature on enzyme activity, it does not purport to describe the molecular basis of this behaviour. Evidence so far (Peterson et al. 2004; M.E. Peterson, C.K. Lee, C. Monk, and R.M. Daniel, unpublished results) suggests that the conformational changes

between the active and inactive forms of the enzyme described by the Model are local rather than global, and possibly quite slight. The challenge now is to uncover the molecular basis of the activity effects described by the Equilibrium Model; it is proposed that site-directed mutagenesis of amino acids in and around the active site and subsequent kinetic comparison of the wild type enzyme and mutants might provide valuable insight in this case.

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