Temperature and the catalytic activity of enzymes: A fresh understanding

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The discovery of an additional step in the progression of an enzyme from the active to inactive state under the influence of temperature has led to a better match with experimental data for all enzymes that follow Michaelis–Menten kinetics, and to an increased understanding of the process. The new model of the process, the Equilibrium Model, describes an additional mechanism by which temperature affects the activity of enzymes, with implications for ecological, metabolic, structural, and applied studies of enzymes.

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1. Introduction

Leonor Michaelis and Maud Menten are often regarded as the founders of modern quantitative enzymology. In their studies on invertase [1], they recognised the necessity of assaying the enzyme under defined and controlled conditions, principally with respect to the pH of the reaction, and the need to measure initial rates, thereby avoiding various complicating factors including the known inhibition of the enzyme by the product of the reaction. Their data then fitted what we now know as the Michaelis–Menten equation, the fundamental equation of enzyme kinetics:

\[ V = \frac{V_{\text{max}}[S]}{K_M + [S]} \]  

(1)

Clearly, in addition to a defined pH, kinetic measurements are made with a constant amount of enzyme in the assay, and at a constant temperature that does not result in loss of catalytic activity during the time course of the assay. The choice of assay temperature is extremely important since the way enzymes respond to temperature is fundamental to many areas of biology. Thus, it is this aspect, the effect of temperature on enzyme catalytic activity, that we wish to explore in the current paper, and provide new insights that are of both theoretical and practical importance.

Where the effect of temperature on enzyme activity has been considered, textbooks have said that higher temperatures increase enzyme activity to a certain point, after which the enzyme denatures, losing its activity irreversibly (e.g., [2–4]). In this model, the change in enzyme activity with increasing temperature is simply the combined result of the effect of temperature increasing \( k_{\text{cat}} \) and \( k_{\text{inact}} \) on a simple two step conversion (i.e., \( E_{\text{act}} \rightarrow X \)), then the time-dependent loss of activity, expressed as \( V_{\text{max}} \), is described by the following equation:

\[ V_{\text{max}} = k_{\text{cat}} \cdot [E_0] \cdot e^{-k_{\text{inact}}t} \]  

(2)

where \( V_{\text{max}} \) = maximum velocity of enzyme; \( k_{\text{cat}} \) = enzyme's catalytic constant; \( [E_0] \) = total concentration of enzyme; \( k_{\text{inact}} \) = thermal inactivation rate constant; \( t \) = assay duration.

The variation of the two rate constants in Eq. (2) with temperature is given by:

\[ k_{\text{cat}} = k_B \frac{T}{h} e^{-\left(\frac{D_{G_{Z,\text{cat}}}}{RT}\right)} \]  

(3)

and

\[ k_{\text{inact}} = k_B \frac{T}{h} e^{-\left(\frac{D_{G_{Z,\text{inact}}}}{RT}\right)} \]  

(4)

where \( k_B \) = Boltzmann’s constant; \( R \) = Gas constant; \( T \) = absolute temperature; \( h \) = Planck’s constant; \( D_{G_{Z,\text{cat}}} \) = activation energy of the catalysed reaction; \( D_{G_{Z,\text{inact}}} \) = activation energy of the thermal inactivation process.
Using these equations, and inserting plausible values for $\Delta G_{\text{cat}}^\circ$ and $\Delta G_{\text{inact}}^\circ$, a plot of activity against temperature and time can be constructed (Fig. 1). At zero time there is no denaturation, defined here as the time-dependent, irreversible loss of activity, and initial rates will therefore rise continuously with temperature; the expected reaction progress with time at various temperatures can be seen later in Fig. 5C. However, despite this model being commonly used to describe the effect of temperature on enzyme activity, it is now clear that no enzymes actually exhibit this behaviour; careful measurements show that initial rates decline at high temperatures independent of irreversible inactivation. A re-examination of the effect of temperature on enzyme catalytic activity has therefore led to the proposal of a new model [5,6], the Equilibrium Model, described below.

2. The Equilibrium Model

The new model for the temperature-dependent behaviour of enzymes, the Equilibrium Model, introduces an intermediate inactive (but not denatured) form of the enzyme that is in rapid equilibrium with the active form, and it is the inactive form that undergoes irreversible thermal inactivation to the denatured (irreversibly inactive) state (X):

$$E_{\text{act}} \leftarrow E_{\text{inact}} \leftarrow X$$

where $E_{\text{act}}$ is the active form of the enzyme, which is in equilibrium with the inactive form, $E_{\text{inact}}$. $K_{\text{eq}}$ is the equilibrium constant describing the ratio of $E_{\text{act}}/E_{\text{inact}}$. $K_{\text{eq}}$ is the rate constant for the $E_{\text{inact}} \to X$ reaction; and X is the irreversibly-inactivated form of the enzyme. Within this model, there is no implication that the conversion of $E_{\text{inact}}$ to X occurs in a single step, or that X is a single species, only that they can be considered as such since all species beyond $E_{\text{inact}}$ are irreversibly inactivated.

Using the Equilibrium Model, the variation of enzyme activity with temperature can be expressed by:

$$V_{\text{max}} = \frac{k_{\text{cat}}E_0 e^{-\frac{\Delta G_{\text{cat}}}{RT}}}{1 + K_{\text{eq}}}$$

where

$$K_{\text{eq}} = e^{\frac{\Delta G_{\text{eq}}}{RT}}$$

$T_{\text{eq}}$ is the temperature at which the $E_{\text{act}}/E_{\text{inact}}$ equilibrium is at its mid-point ($K_{\text{eq}} = 1$, $\Delta G_{\text{eq}} = 0$, and therefore $T_{\text{eq}} = \Delta H_{\text{eq}}/\Delta S_{\text{eq}}$), where $\Delta H_{\text{eq}}$ is the change in enthalpy for the $E_{\text{act}}/E_{\text{inact}}$ transition. In this case, a plot of rate vs temperature vs time (Fig. 2) does have an optimum for initial rates (i.e., at zero time) because the $E_{\text{act}}/E_{\text{inact}}$ equilibration is rapid. This is known from the experimental observation that enzyme initial rates do in fact decline at high temperatures (see Fig. 6 for an example), and at all temperatures the $E_{\text{act}}/E_{\text{inact}}$ equilibration is faster than the time needed to start the reaction in a stirred spectrophotometer cuvette (of the order of 1–3 s), and the line of product vs time extrapolates back to zero [7–10].

So far, all enzymes we have studied that obey Michaelis–Menten kinetics follow this Model [7–9], irrespective of mechanism or structure (e.g., Table 1), and all have a temperature optimum for initial rates, as expected from the Model. Nevertheless, many enzymes do not follow ideal kinetics, when the enzyme is substrate or product inhibited for example, and the Model must be regarded as describing an ideal, and a degree of departure may occur after significant reaction progress, as is the case for all models of enzyme behaviour.

3. Mechanism of the Equilibrium Model

The discovery of a new and apparently universal mechanism by which enzymes lose activity as the temperature rises is of considerable interest in itself, and has a number of interesting implications. Although the Equilibrium Model does not of itself offer an explanation of the molecular basis of the $E_{\text{act}}/E_{\text{inact}}$ transition, this interconversion can be clearly differentiated from denaturation. Compared with denaturation, it operates over much shorter time-scales [6,7,9,11,12], structural changes are imperceptible [9], and the associated $\Delta H_{\text{eq}}$ is an order of magnitude smaller than $\Delta H_{\text{denat}}$ [9,13–15]. Moreover, changes of $T_{\text{eq}}$ can be independent of changes in stability [9]. Thus, the evidence indicates that the $E_{\text{act}}/E_{\text{inact}}$ transition involves only a small conformational change and there is...
additional evidence that the mechanism is located at the active site. That is, \( T_{eq} \) and \( D_{H_{eq}} \) are substrate dependent, in that they vary in the same enzyme if different substrates are used, \( K_m \) changes often coincide with the \( E_{act}/E_{inact} \) transition, and point mutations at the active site can change \( T_{eq} \) and \( D_{H_{eq}} \) without significant changes in \( D_{G_{z inact}} ^c \) [9,16]. The active-site localisation of the transition is not unexpected, given that conformational flexibility is often inherent in an enzyme’s catalytic mechanism, and thus the active site might be one of the most susceptible parts of an enzyme to temperature-induced conformational changes.

While there are a number of possible molecular bases for the \( E_{inact}/E_{act} \) interconversion, the effect of temperature on charged amino acid residues is a likely factor in the observed effects [3,17]. The \( pK_a \) values of amino acid side-chains are temperature sensitive. In particular, basic residues such as histidine and lysine, which are common components of active sites and their surroundings, and the N-terminal amino group, exhibit relatively large changes in charge with temperature, with shifts of up to a \( \Delta pH \) unit with a 30°C temperature change. Since the ionisable residues of amino acid side chains interact with the charges on adjacent ionised residues, and with neighbouring peptide di- poles, polar residues and bound water, temperature changes have the potential to change the charge and charge distribution at the active site. Many enzyme mechanisms have a close dependence on the charge of specific catalytic residues. On this basis alone, a temperature-driven \( pK_a \) shift can have a significant direct effect on catalysis in many, if not all, enzymes. Equally important, changes in \( pK_a \) can cause conformational changes. For example, if a conformation at or near the active site is retained by an ionic bond (e.g., Asp-Lys) then, if the \( pK_a \) of Lys is shifted down by a temperature increase, its positive charge will decrease and the ionic bond weakened, potentially leading to a conformational change. The overall effect of charge changes on enzyme activity are evidenced by their pH dependence, where large changes in enzyme activity can be caused by changes of less than a single \( \Delta pH \) unit of the solvent.

### Table 1
Thermodynamic parameters for eight enzymes fitted to the Equilibrium Model.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Enzyme</th>
<th>( T_{growth} ) (°C)</th>
<th>( \Delta G_{act} ^c ) (kJ mol(^{-1}))</th>
<th>( \Delta G_{inact} ^c ) (kJ mol(^{-1}))</th>
<th>( \Delta H_{eq} ) (kJ mol(^{-1}))</th>
<th>( T_{eq} ) (°C)</th>
<th>Number of subunits</th>
<th>Relative molecular mass</th>
<th>Reaction class and EC number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Thermus sp. RT41a</em></td>
<td>Alkaline phosphatase</td>
<td>75</td>
<td>72</td>
<td>99</td>
<td>305</td>
<td>90</td>
<td>2</td>
<td>97000</td>
<td>Hydrolyase EC 3.1.3.1</td>
</tr>
<tr>
<td><em>Caldocellulosiruptor saccharolyticus</em></td>
<td>( \beta )-Glucosidase</td>
<td>70</td>
<td>88</td>
<td>103</td>
<td>149</td>
<td>74</td>
<td>1</td>
<td>54000</td>
<td>Hydrolyase EC 3.2.1.2</td>
</tr>
<tr>
<td><em>Thermoplasma acidophilum</em></td>
<td>Citrate synthase</td>
<td>60</td>
<td>77</td>
<td>103</td>
<td>164</td>
<td>88</td>
<td>2</td>
<td>86000</td>
<td>Transferase EC 2.3.3.1</td>
</tr>
<tr>
<td><em>Bos taurus</em></td>
<td>Glutamate dehydrogenase</td>
<td>39</td>
<td>63</td>
<td>93</td>
<td>264</td>
<td>53</td>
<td>6</td>
<td>330000</td>
<td>Oxidoreductase EC 1.4.1.2</td>
</tr>
<tr>
<td><em>Bos taurus</em></td>
<td>( \gamma )-Glutamyl transferase</td>
<td>39</td>
<td>63</td>
<td>98</td>
<td>109</td>
<td>52</td>
<td>2</td>
<td>88000</td>
<td>Transferase EC 2.3.2.2</td>
</tr>
<tr>
<td><em>Sus scrofa</em></td>
<td>Fumarase</td>
<td>39</td>
<td>60</td>
<td>92</td>
<td>378</td>
<td>59</td>
<td>4</td>
<td>194000</td>
<td>Lyase EC 4.2.1.2</td>
</tr>
<tr>
<td><em>Wheat germ</em></td>
<td>Acid phosphatase</td>
<td>20</td>
<td>79</td>
<td>95</td>
<td>142</td>
<td>64</td>
<td>1</td>
<td>55000</td>
<td>Hydrolase EC 3.1.3.2</td>
</tr>
<tr>
<td><em>Moritella profunda</em></td>
<td>Dihydrofolate reductase</td>
<td>2</td>
<td>67</td>
<td>93</td>
<td>104</td>
<td>55</td>
<td>1</td>
<td>18000</td>
<td>Oxidoreductase EC 1.5.1.3</td>
</tr>
</tbody>
</table>

The parameters of all enzymes were derived by fitting assay data to the Equilibrium Model (http://hdl.handle.net/10289/3791) and thus relate to active enzymes in the presence of substrate and cofactor [8,9]. The exceptions are the growth temperature optima (\( T_{growth} \)) of the source organism, which are cited from various sources.
For most enzymes, the E$_{\text{act}}$/E$_{\text{inact}}$ transition described by the Equilibrium Model has an $\Delta H_{\text{eq}}$ in the range 100–300 kJ/mol [8,9], so that a 10–20 °C decrease in temperature below that at which 50% of the enzyme is in the inactive form will lead to a shift in the E$_{\text{act}}$/E$_{\text{inact}}$ transition to a point where 90% of the enzyme is in the inactive form (Fig. 3). This is not inconsistent with the changes at an active site that might arise from a change in pK$_a$ over this temperature change [3,17].

4. Implications of the Equilibrium Model

Measurement of the Equilibrium Model parameters (see: http://hdl.handle.net/10289/3791) has provided a fuller and more accurate explanation of the effect of temperature on enzymes, and enabled the identification of a number of potential implications of the Model.

First, correlation analysis of the key parameters of the Equilibrium Model ($\Delta G_{\text{cat}}$, $\Delta G_{\text{inact}}$, $\Delta H_{\text{eq}}$, and $T_{\text{eq}}$) of enzymes from organisms with growth temperatures ranging from 2 to 75 °C shows that $T_{\text{eq}}$ correlates with much greater significance to the optimal growth temperature of the source organism than does the $\Delta G_{\text{inact}}$ [8], indicating an evolutionary significance for the Equilibrium Model parameters. Additionally, $\Delta H_{\text{eq}}$ can be considered as a quantitative measure of the temperature range over which an
enzyme is most active. A large $\Delta H_{eq}$ leads to an enzyme with a sharp and relatively narrow temperature optimum, whereas a small $\Delta H_{eq}$ results in an enzyme with a broad temperature optimum, so that activity is relatively less sensitive to changes in temperature (Fig. 3). Therefore, if a particular enzyme has a high $\Delta H_{eq}$, for example, then a shift in temperature away from $T_{eq}$ is much more likely to lead to a significant change in enzyme activity. Of the enzymes for which we have $\Delta H_{eq}$ values, about 50% have $\Delta H_{eq}$ values of less than 150 kJ/mol (i.e., fairly broad temperature optima), and about 15% have values greater than 400 kJ/mol (relatively narrow temperature optima) [8,9]. This effect may imply a more sophisticated and selective control of metabolism by temperature than currently envisaged. However, given that the Model is based on $V_{max}$ data, the extent to which the model applies under physiological conditions may depend upon the degree to which enzymes are substrate-saturated, since the degree to which the Model applies under conditions where substrate is not saturating has not been investigated. The extent of substrate saturation in vivo is uncertain and, although it has been argued that substrate concentrations in vivo should be close to half-saturation [18,19], examples of saturation are known [20].

A second implication concerns the stability of enzymes and attempts to manipulate this. Although enzymes are only marginally stable at the growth temperature of the source, we know they can be stable and functional at very high temperatures [21], and that the addition of a single productive stabilising interaction can greatly increase the half-life [22]. With this in mind, and given the considerable importance of stable enzymes in biotechnology and the substantial efforts in this field (published and unpublished), the attempts to increase the useful temperature of enzyme activity by directed mutagenesis have been, with some notable exceptions, disappointing. Activity at high temperature depends on $T_{eq}$ and $\Delta H_{eq}$ as well as on stability (Fig. 4), offering an explanation for the difficulty of engineering enzymes to act at higher temperatures. That is, most directed mutagenesis has focussed on enhancing $\Delta G^\circ_{\text{inact}}$, but it is clear from Fig. 4 that, while enhancing $\Delta G^\circ_{\text{inact}}$ may reduce the loss of activity with time, unless $T_{eq}$ and/or $\Delta H_{eq}$ are also changed this may have little effect on activity at a higher temperature. This may explain why attempts to improve $\Delta G^\circ_{\text{inact}}$ by directed mutagenesis has been significantly less successful in improving activity at high temperatures than directed evolution of enzymes since these may also lead to productive changes in $T_{eq}$ and/or $\Delta H_{eq}$ (see [23] for a general discussion of engineering strategies).

A third consequence arises when the questions of activity and stability are combined. As pointed out by Eisenthal et al. [24], predictions of the effect of time and temperature on the output of enzyme reactors are different depending on whether the “Classical” or Equilibrium Model is used (Fig. 5). When using an enzyme in a batch reactor for a biocatalytic conversion, intuition would predict that the higher the operating temperature, the faster the catalysed reaction, but also the less stable the enzyme. In fact, experimental data show 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. 5A and B). Typical time courses of product formation at various temperatures predicted by the Classical Model (which will never show the effect seen in Fig. 5A) are shown in Fig. 5C for comparison. Experimental data for an enzyme reactor in which alkaline phosphatase ($T_{eq} = 319$ K) catalyses the hydrolysis of  $p$-nitrophenyl [25] confirm the predictions of the Model, showing that when the reactor working temperature is below $T_{eq}$ then as the temperature rises so does the initial rate (Fig. 6); however, when it is above $T_{eq}$, as the temperature rises the initial rate declines.

5. Concluding remarks

In this paper, we have summarised a new model, the Equilibrium Model, that gives a more complete understanding of the effect of temperature on an enzyme’s catalytic activity. Given the
excellent fit to all enzymes so far tested, the evidence that the Model accurately describes the effect of temperature on enzymes is convincing. The importance of this increased understanding of the effect of temperature on enzyme activity is evident from its occurrence in all enzymes that follow Michaelis–Menten kinetics, irrespective of structure and mechanism. In addition to the implications described above, the Model describes a second way in which temperature affects enzyme activity, probably via the active site, and has implications for structural, metabolic, ecological and applied studies of enzymes.

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