A reanalysis of the foundations of the macromolecular rate theory

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Abstract. The macromolecular rate theory (MMRT) has been proposed as a mechanistic scheme to describe the temperature dependence of enzymatic reactions, and has enjoyed quite some popularity recently. MMRT was motivated by assuming that enzyme denaturation is not sufficient to explain the decline of enzyme activity above an optimal temperature, and was derived with two experimental assumptions: (1) the half saturation parameter is independent of temperature; and (2) when the substrate

10 concentration is kept at 10 times of the half saturation parameter at reference temperature, the enzyme assays are substrate saturated under all experimental temperatures. We show that both the motivating and experimental assumptions do not hold under many conditions. Consequently, the MMRT estimated temperature sensitivity of the maximum catalysis rate is inaccurate. It can mischaracterize temperature-related biochemical behaviors, such as inferring the existence of a unique optimal temperature where biochemical rate peaks, and the shift of this optimal temperature as an indicator of thermal

15 acclimation or adaptation. We recommend that chemical kinetics theory is a better candidate for mechanistic modeling of the temperature dependence of biogeochemical rates.

1 Introduction

Recently, the macromolecular rate theory (MMRT) has been proposed to interpret observations of enzyme-catalyzed chemical reactions (Hobbs et al., 2013). These rates often show a pattern that first increases gradually, then plateaus, and

20 finally decreases rapidly with temperature. The authors of MMRT were motivated by asserting that "*denaturation is insufficient to explain the decline in enzymatic rates above T_{opt}*", and proposed that the change in heat capacity associated with enzyme catalysis and its consequent effect on the temperature dependence of the Gibbs free energy of activation can describe the temperature dependence of enzyme activity. Following the success of Hobbs et al. (2013), Schipper et al. (2014) showed that MMRT is able to better than the Arrhenius-like functions for fitting measured relationships between soil

25 biogeochemical rates and temperature, including those for aerobic respiration, methane oxidation, nitrification, and denitrification. Alster et al. (2016) then demonstrated that MMRT was successful at capturing the temperature dependence of extracellular enzyme activities, including those of β-glucosidase, leucine aminopeptidase, and phosphatase. Following these

studies, Liang et al. (2018) recommended that MMRT should be used for improved description of the measured relationship

between plant leaf respiration and temperature. Recently, Alster et al. (2020) advocated that MMRT should be used widely to

30   represent the temperature dependence of many types of soil biogeochemical processes.

The popularity of MMRT is built upon two observations: (1) MMRT is able to match measured relationships of

biochemical rates *versus* temperatures better than the popular Arrhenius-like functions and the Q_{10} function, and (2) MMRT

parameters have more mechanistic meaning by involving thermodynamic definitions than other empirical functions that are of

similarly good descriptive power but with mechanistically less interpretable parameters (e.g., the log-polynomial function

35   (e.g., O'Sullivan et al., 2017), the four-parameter square root function (Ratkowsky et al., 1983)). Despite these merits, we show

here limitations arising from the two experimental assumptions used in developing MMRT (Hobbs et al., 2013): (1) the half

saturation parameter is independent of temperature (as implied by their assumption that the ratio of substrate concentration to

half saturation parameter was kept at two while using the same substrate concentrations under all temperatures for enzyme

barnase and its mutant), and (2) the constant value 10 for the ratio of substrate concentration to half saturation parameter at

40   reference temperature ensures that their enzyme assay system is substrate saturated under all experimental temperatures. (We

note that Hobbs et al. (2013) adopted their second experimental assumption from the enzyme assay protocol in (Peterson et

al., 2004)). Instead, we show that by incorporating the reversible thermal denaturation of enzymes into the chemical kinetics,

we can satisfactorily explain the non-monotonic temperature response of enzyme catalysis rate.

In the following, we first present our analysis of the assumptions involved in the development of MMRT. Then we

45   describe an alternative interpretation of the observed non-monotonic relationship between biochemical rates and temperatures

that is more consistent with protein physics and the theory of chemical kinetics. Finally, we discuss how our alternative

formulation will lead to mechanistically more accurate representations of the temperature dependence of biogeochemical

reaction rates.

2. Methods

50   *2.1 The enzymatic reaction problem*

The simplest form of enzymatic reactions as, described by MMRT (introduced in section 2.2) and the alternative

theory (presented in section 2.3), can be formulated as:

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$$E_n + S \underset{k_1^-}{\overset{k_1^+}{\rightleftarrows}} E_n S \overset{v_{max}}{\longrightarrow} E_n + P, \tag{1}$$

where E_n is the concentration of free enzymes whose conformation structure is in the native state (i.e., able to carry out the

catalysis), P is the concentration of product molecules, S is concentration of the substrate, $E_n S$ is concentration of the enzyme-

55 substrate complex, and k_1^+, k_1^-, and v_{max} are temperature (T) dependent kinetics parameters. Although it is not necessary for

the validity of the Michaelis-Menten kinetics (Briggs and Haldane, 1925), for scaling purpose, v_{max} (the maximum enzymatic

catalysis rate) is often assumed to be much greater than k_1^- (Tang and Riley, 2017;Kooijman, 2009;Holling, 1959;Aksnes and

Egge, 1991;Van Slyke and Cullen, 1914). Moreover, throughout this study, we take all variables to be in ISO units.

By applying the law of mass action and the quasi-steady-state-approximation to equation (1), we obtain the Michaelis-

60 Menten equation for the overall reaction rate F:

$$F = v_{max} \frac{E_{nt}S}{K+S}, \tag{2}$$

where $K = v_{max}/k_1^+$ is the half saturation parameter, and E_{nt} is the total concentration of enzymes that are able to form

enzyme-substrate complexes.

We next describe how MMRT and the chemical kinetics theory represent the temperature dependence of F.
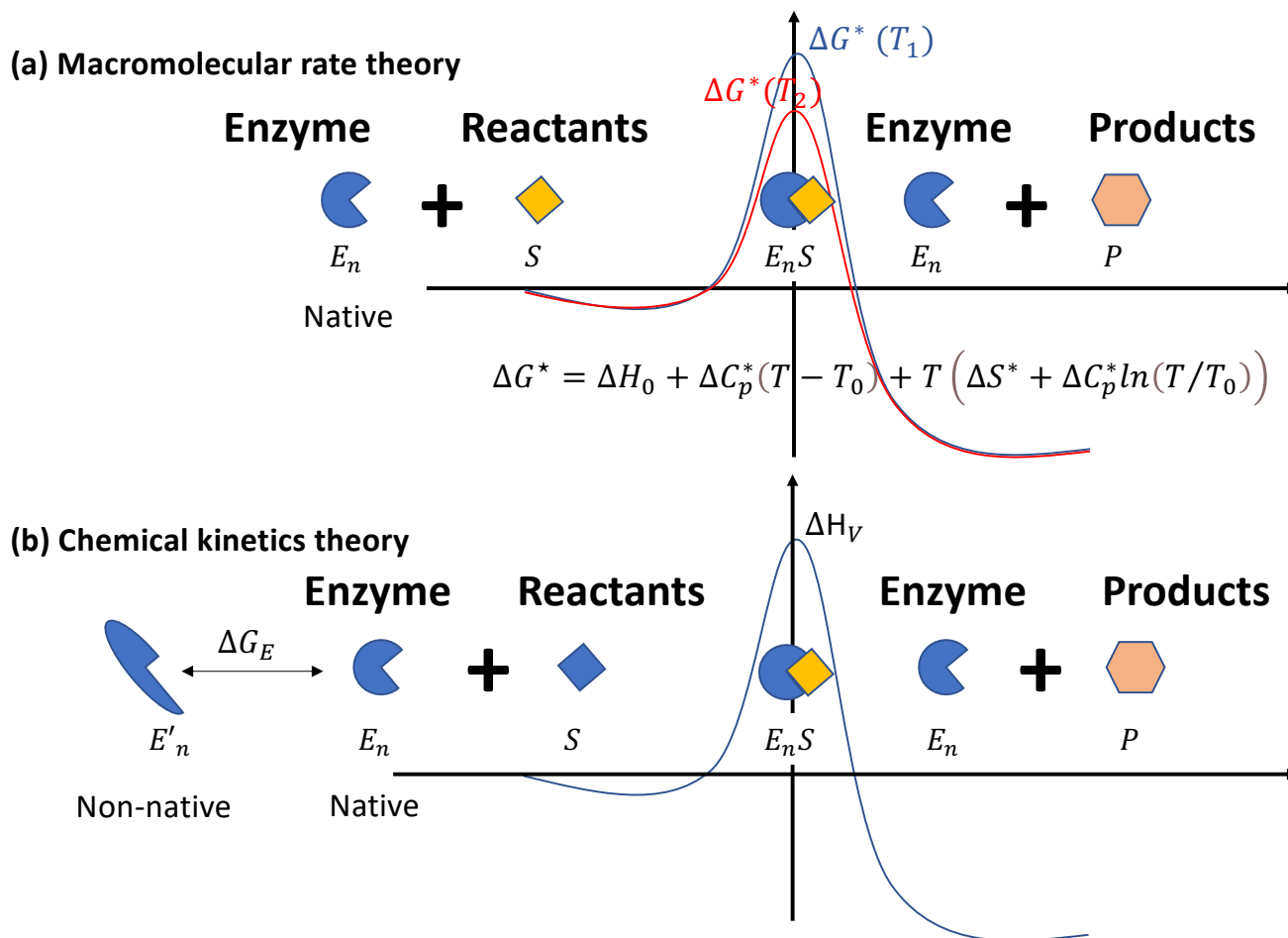
Figure 1: (a) In the macromolecular rate theory (MMRT), the Gibbs free energy of activation ΔG^* is a nonlinear function of temperature, giving rise to the non-monotonic temperature response of catalysis rate; (b) in the chemical kinetics theory, the Gibbs free energy of activation is a linear function of temperature, the enthalpy of activation ΔH_V is constant, and the reversible thermal denaturation leads to the non-monotonic temperature response of catalysis rate. Other variables are defined in the main text.

2.2 The macromolecular rate theory (MMRT)

MMRT (Figure 1a) applies the transition state theory (Eyring, 1935) to describe the maximum reaction rate v_{max} (called $k(T)$ in the MMRT representation) as

$$k(T) = \frac{k_B T}{h} exp\left(-\frac{\Delta G^\star}{RT}\right), \tag{3}$$

where k_B is the Boltzmann constant, h is the Planck constant, R is the universal gas constant, and $\Delta G^\star$ is the Gibbs free energy of activation. Most importantly, motivated by (La Mer, 1933) (as pointed out by an anonymous expert on MMRT who reviewed a previous version of this manuscript), MMRT assumes $\Delta G^\star$ to be dependent on temperature nonlinearly, such that

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$$\Delta G^{\star} = \Delta H_0 + \Delta C_p^*(T - T_0) + T\left(\Delta S^* + \Delta C_p^* ln(T/T_0)\right), \tag{4}$$

where ΔH_0 and ΔS^* are enthalpy and entropy between the ground and transition states at reference temperature T_0, respectively, and ΔC_p^* is (the change in) heat capacity associated with enzyme catalysis. Without invoking the enzyme denaturation process, MMRT effectively assumes that all enzymes are in their native state and are capable of forming complexes with the substrate molecules, and all enzyme-substrate complexes are active. (Recently, based on molecular dynamics simulations, Aqvist et al. (2020) and Aqvist and Van der Ent (2022) suggested that not all enzyme-substrate complexes are active and inferred ΔC_p^* to be zero.)

Although Hobbs et al. (2013) did not explicitly state that the affinity parameter K in their fitting of MM kinetics is temperature independent, their analysis effectively assumed so by attributing all temperature dependence of F to $k(T)$ and assuming all enzyme assays are substrate saturated. That is, MMRT computes F as

$$F = k(T)\frac{E_t S}{K_0 + S}, \tag{5}$$

where K_0 is K empirically determined at the reference temperature T_0, and E_t is the total concentration of the enzyme.

In the reanalysis using chemical kinetics theory below, we show that the temperature dependence of K is related to that of v_{max}, so that the MMRT-derived temperature dependence of $k(T)$ is a function of substrate concentration. In contrast, in the enzyme assays by Hobbs et al. (2013), it was assumed that a substrate concentration of $\sim 10K$ (with K of value K_0 that is determined at the reference temperature) under all temperatures for five out of seven enzymes is sufficient to infer $k(T)$ being independent of substrate concentration. Although Hobbs et al. (2013) never stated that $k(T)$ fully captures the temperature dependence of F, later applications (e.g., Schipper et al., 2014;Liang et al., 2018;Alster et al., 2016) recommended MMRT to replace the popular Q[10] function or Arrhenius function to represent the temperature sensitivity of biochemical rates. (We highlight that in those applications, what MMRT, the Q[10] and Arrhenius functions represent are emergent temperature response dependent on the substrate and plant or soil conditions used in deriving the empirical data.) Alster et al. (2020) recognized the temperature dependence of K, but still considered the temperature dependence of v_{max} to be captured in $k(T)$. Below we will show that $k(T)$ as determined by MMRT convolves the temperature dependence of v_{max} and K, so that it fails to capture the temperature dependence of F.

2.3 The chemical kinetics theory

Chemical kinetics theory (Figure 1b) incorporates the observation that a fraction $(1 - f_E(T))$ of the enzymes (E_t) are

in the thermally reversible denatured non-native state (e.g., Finkelstein and Ptitsyn, 2016;Ghosh and Dill, 2009), so that

$$F = \frac{dP}{dt} = \frac{v_{max,f_E(T)}E_t S}{K+S} f_R(T), \qquad (6)$$

100   where

$$v_{max} = v_{max,0} f_v(T), \qquad (7)$$

$$K = \frac{v_{max}}{k_1^+} = K_0 f_K(T), \qquad (8)$$

$$f_R(T) = 1 - exp\left(-\frac{\Delta G_R}{RT}\right), \qquad (9)$$

and $v_{max,0}$ and K_0 are values of v_{max} and K evaluated at temperature T_0, respectively. Moreover, following the definition of

K in equation (2), we adopted the assumption that v_{max} is much greater than k_1^- in equation (8). In equation (6), $f_R(T)$

describes the thermodynamic factor proposed by Jin and Bethke (2003), which is a function of ΔG_R, the Gibbs free energy of

the chemical reaction of converting the reactants into products. Unless there is significant product inhibition, f_R is set to one,

105   which is adopted in the remainder of this paper. We next formulate $f_E(T)$, $f_v(T)$ and $f_K(T)$.

The temperature dependent function $f_E(T)$ is formulated as

$$f_E(T) = \frac{1}{1+exp\left(-\frac{\Delta G_E}{RT}\right)}, \qquad (10)$$

with R being the universal gas constant, and protein-folding Gibbs free energy

$$\Delta G_E = \Delta C_p \left[(T - T_H) - Tln\left(\frac{T}{T_S}\right)\right]. \qquad (11)$$

where ΔC_p is the heat capacity of protein folding, T_H is the temperature at which folding enthalpy is zero, and T_S is the

temperature at which folding entropy is zero, all of which are functions of protein chain length (Ghosh and Dill, 2009). Usually,

110   T_S is greater than T_H.

For v_{max}, applying the transition state theory (Eyring, 1935), we have

$$v_{max} = v_{max,0} f_v(T) = v_{max,0} \left(\frac{T}{T_0}\right) exp\left(-\frac{\Delta H_V}{RT}\left(1 - \frac{T}{T_0}\right)\right), \tag{12}$$

where $v_{max,0}$ is v_{max} evaluated at reference temperature T_0, and ΔH_V is the enthalpy of activation and is temperature independent.

For the temperature dependence of K, applying the diffusion-limited reaction model by von Smoluchowski (1917) indicates that k_1^+ is proportional to diffusivity, which, according to the Stokes-Einstein equation and the temperature dependence of dynamic viscosity of water, will have similar functional form of temperature dependence as v_{max} (Tang et al., 2021), resulting in

$$f_K(T) = exp\left(-\frac{\Delta H_K}{RT}\left(1 - \frac{T}{T_0}\right)\right). \tag{13}$$

Combining equations (6)-(13), we have

$$F = v_{max,0} \frac{f_v(T) f_E(T) E_t S}{K_0 f_K(T) + S}, \tag{14}$$

which describes the temperature dependence of the biochemical reaction rates in the absence of significant product inhibition.

In particular, by assuming that S is much larger compared to $K_0 f_K(T)$, we obtain

$$F_\infty = v_{max,0} E_t f_v(T) f_E(T) = r_0 f_v(T) f_E(T), \tag{15}$$

which is the equation used by Ratkowsky et al. (2005) to describe the temperature-dependent growth of various microorganisms.

2.4 The relationship between chemical kinetics theory and MMRT

By equating equations (4) and (14), we obtain

$$k(T) = v_{max,0} f_v(T) f_E(T) \frac{1 + S/K_0}{f_K(T) + S/K_0}. \tag{16}$$

For the ease of parametric fitting (as will be described in section 2.5), taking the logarithm of equation (16) leads to

$$\ln k(T) = \ln v_{max,0} + \ln(1 + S/K_0) + \ln\left(\frac{f_v(T) f_E(T)}{f_K(T) + S/K_0}\right). \tag{17}$$

Equations (16) and (17) show that the temperature dependence of $k(T)$ is determined by $f_v(T)$, $f_E(T)$, $f_K(T)$, and the normalized substrate availability S/K_0. When enzyme assays are conducted with known values of S/K_0 and reference temperature (where K_0 is defined), the data can be used to derive the parameters ΔH_V, ΔC_p, T_H, T_S, and ΔH_K. In our analysis, since the activation enthalpy for the temperature dependence of the self-diffusion of water is almost a constant at 18 kJ mol^{-1}

130 (Mills, 1973), we set $\Delta H_K = \Delta H_V - 18$, so that only four parameters need to be derived from parametric fitting, which is one parameter more than required by MMRT.

Equations (16) or (17) can be used to analyze the two basic experimental assumptions that underlie MMRT. (The motivating assumption that enzyme denaturation cannot satisfactorily explain the temperature dependence of catalysis rates, and thus all enzymes are in their native state, has been invalidated by many observations (Sizer, 1943;Alexandrov, 1964;Huang

135 and Cabib, 1973;Maier et al., 1955;Weis, 1981), as well as by molecular dynamics simulations (McCully et al., 2008). Nonetheless, irreversible denaturation driven by heat does occur (Perdana et al., 2012), as it is necessary for the cooking of eggs or meat.) First, these two equations clearly show that the enzyme assay-derived $k(T)$ is affected by substrate abundance, through the ratio (S/K_0) of substrate concentration S to K_0. Second, because K is temperature dependent, taking S/K_0 to be a relatively large value (e.g., 10 as adopted by Hobbs et al. (2013) and Peterson et al. (2004), hoping to "*minimize the effect of*

140 *any possible increase in K_M with temperature";* note that they denote K with K_M) does not guarantee that the term $(1 + S/K_0)/(f_K(T) + S/K_0)$ reduces to a value of one (see section 3.1 for more details). Therefore, not only is the validity of their motivating presumption compromised, but also that of their experimental assumptions for deriving MMRT. Instead, as we will show later, a proper incorporation of enzyme denaturation is sufficient to explain the non-monotonic temperature dependence of enzyme catalysis rate, and substrate availability can affect the optimal temperature (T_{opt}) where biochemical

145 reaction rate is maximized.

2.5 Empirical data reanalysis

We extracted the assay data of all seven enzymes from Hobbs et al. (2013) and all five enzymes from Peterson et al. (2004) to evaluate the validity of chemical kinetics theory. (We did not try to analyze data from soils, as that would involve a more comprehensive model, which is beyond the scope of this study.) Since we were not able to extract the reaction rates directly from the figures in these studies, nor obtain the original data, we normalized rates for each enzyme with its own rate

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at a selected reference temperature T_r, based on the criterion that the data point at T_r is crossed by lines of their original numerical fitting (where (Hobbs et al., 2013) used MMRT, while (Peterson et al., 2004) used their equilibrium model). In the logarithm form (i.e., $\ln k(T)$), this normalization ensures that the values of $\ln k(T) - \ln k(T_r)$ used as observations at different temperatures are independent of the value of $\ln v_{max,0} + \ln(1 + S/K_0)$ at the reference temperature T_0 of the enzyme essay.

155 We obtain the best fitting parameters by using the "fminsearch" function from MATLAB R2020b to minimize the summed difference between modeled and measured values of $\ln k(T) - \ln k(T_r)$. Because we were not able to digitally extract meaningful uncertainty of the observations from the figures either in Peterson et al. (2004) or in Hobbs et al. (2013), we do not compute uncertainties of the estimated parameters. (We also tried using finite difference to approximate the Hessian matrix of the cost function at the best parameter estimates obtained by "fminsearch". However, the ill-conditioned approximated

160 Hessian matrix prevents us from estimating the parametric uncertainty meaningfully.)

3. Results

For the data of all twelve enzyme assays, the chemical kinetics theory obtained almost perfect model-data fitting with the "fminsearch" computed best fit parameters (Figure 2). The R^2 values for the linear regression between model predictions and observations are approaching 0.99 or 1.00 for 10 cases, and the lowest value is 0.85 for Barnase (Figure 2f). The best-fit

165 heat capacity ΔC_p of the thermodynamically reversible conversion between native and non-native conformations of the enzymes are all positive, varying between 1.34 kJ mol^{-1} K^{-1} (for Adenosine deaminase in Figure 2i) and 22.74 kJ mol^{-1} K^{-1} (for Aryl-acylamidase in Figure 2k), in agreement with the range reported in Figure 2C by Ghosh and Dill (2009).
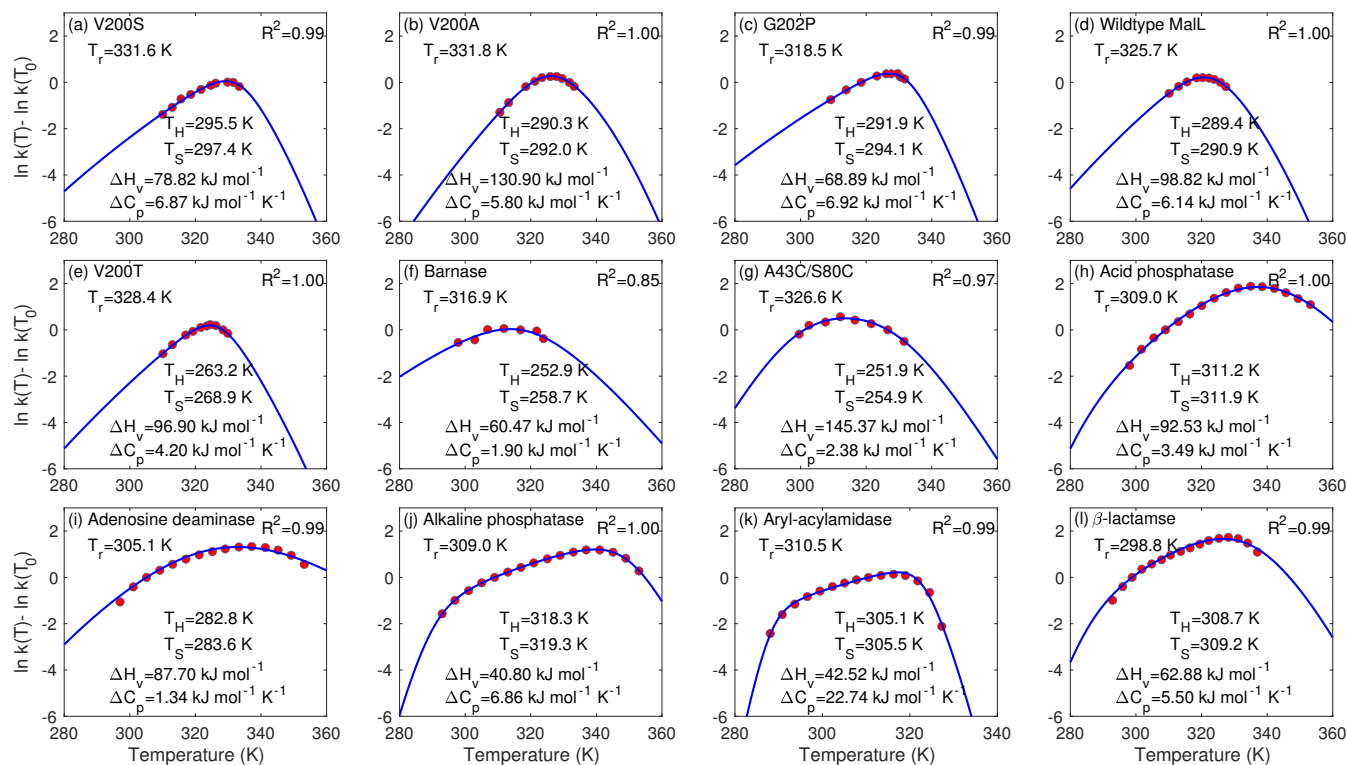
Figure 2. Fitting of the chemical kinetics theory (solid lines) to the enzyme assay data (red filled circles). Panels (a)-(g) are measurements from Hobbs et al. (2013) and panels (h)-(l) are from Peterson et al. (2004). T_r is the reference temperature used in the data extracted from published figures (and it is different from the reference temperature T_0 that was actually involved in the enzyme assay experiments). R^2 is for the linear regression between the model predictions with best-fit parameters (blue lines) and measurements (in red circles). Following their original studies, parametric fitting for panels (f) and (g) used $S/K_0=2$, while others used $S/K_0=10$.
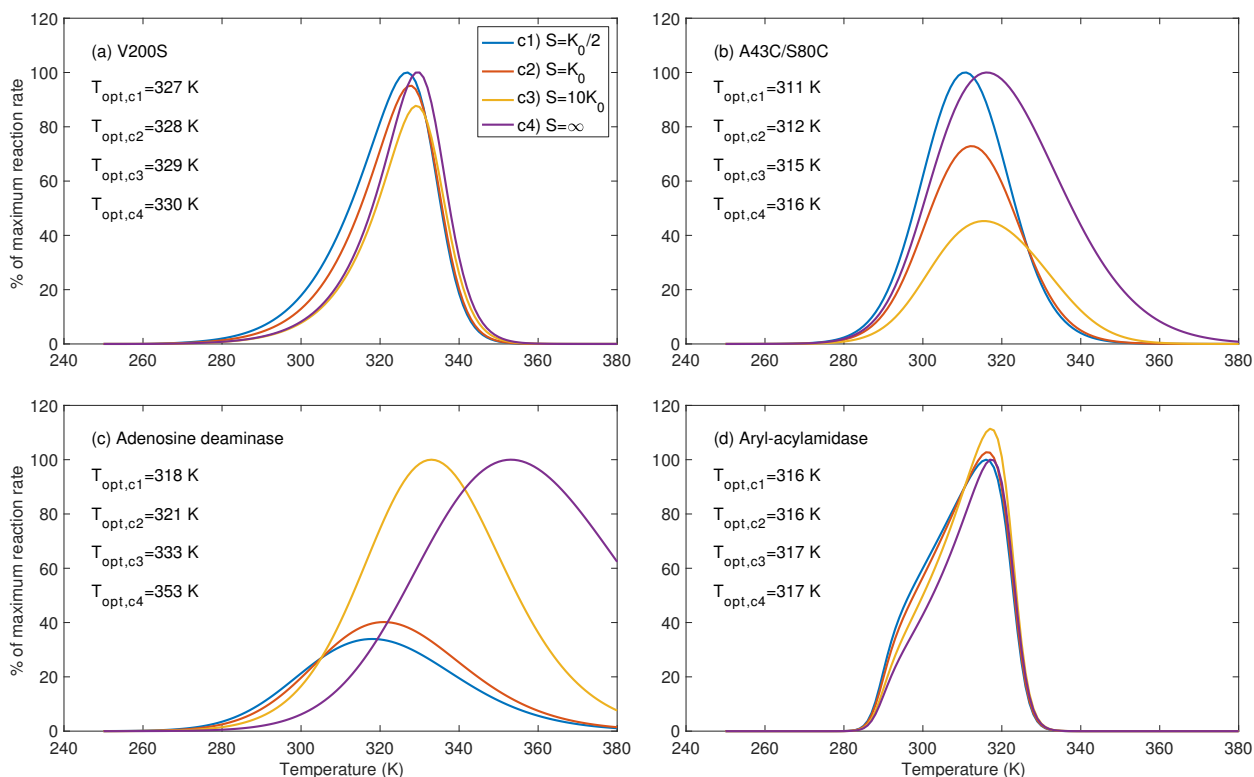
Figure 3. Examples for the influence of substrate availability on the temperature response of enzymic reaction rates. For substrate level c4), the rate curve corresponds to $f_v(T)f_E(T)$ from equation (15).

180   For the four example enzymes picked from the estimated parameters from Figure 2, we found the optimal temperature (i.e., where the reaction rate reaches its maximum) has varying dependence on substrate availability. (We do not show temperature response curves of the other eight enzymes, because they respond similarly to substrate availability, and thus do not change our conclusions here.) All examples show that as substrate availability increases, the optimal temperature increases and the temperature response curve shifts towards higher temperatures. For enzyme aryl-acylamidase, the actual physiological

185   optimal temperature (computed from the curve by $f_v(T)f_E(T)$) equals the emergent optimal temperature at a substrate concentration of $10K_0$, and is 1 K higher than those at substrate concentrations of K_0 and $K_0/2$. For enzymes V200S and A43C/S80C, the optimal temperature at substrate concentration $10K_0$ is 1 K lower than the physiological optimal temperature (Figure 3a, b). However, this difference is 20 K for adenosine deaminase (Figure 3c). These results clearly demonstrate that substrate availability plays a potentially important role in the emergent temperature response of biochemical reaction rates.

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4. Discussion and conclusion

Our theoretical analysis suggests that, even for a single-substrate-single-enzyme reaction, its temperature response involves contributions from at least four processes: (1) the thermodynamically reversible conversion between native and non-native state enzymes; (2) the binding between native enzymes and substrates to form enzyme-substrate complexes; (3) the transition state activation of the enzyme-substrate complex; and (4) the thermodynamic feasibility for the biochemical reaction to generate product molecules. The chemical kinetics theory is able to explicitly account all four processes, and can be extended to include more processes when more complex biochemical reactions are considered (e.g., as discussed in Tang et al. (2021)). In contrast, in spite of its simpler form, MMRT may have misinterpreted the functional relationship between measured biochemical rates and temperatures, in particular, by being unable to account for the modulation of optimal temperature and overall temperature response curve due to substrate availability. One consequence is that MMRT may be misinterpreting the inferred optimal temperature as the true physiologically optimal temperature, and regarding a measured shift of optimal temperature as evolutionary adaption. We find that higher optimal temperatures can be achieved under higher substrate availability. Further, the inferred temperature dependence by MMRT also includes contributions from the temperature sensitivity of affinity parameter. Therefore, if one is using MMRT to represent reaction rate temperature dependencies and also includes a temperature-dependent substrate affinity parameter, the resultant model risks double counting the temperature response.

One outstanding difference between MMRT and the chemical kinetics theory is that MMRT generally infers a negative heat capacity, while the chemical kinetics theory infers a positive heat capacity (associated with thermally reversible enzyme denaturation). The negative heat capacity of MMRT is associated with the catalysis process, while some other studies have attributed it to the binding process (e.g., Wang et al., 2009;Buczek and Horvath, 2006;Dullweber et al., 2001). However, using molecular dynamics simulations, Aqvist and Van der Ent (2022) inferred the heat capacity to be zero for both catalysis and binding processes. Moreover, Aqvist and Van der Ent (2022) suggested that the non-monotonic relationship between temperature and catalysis rate can be explained by the existence of an equilibrium between active enzyme substrate complex E_nS and inactive enzyme substrate complex (E'_nS). To some extent, the conceptual model by Aqvist and Van der Ent (2022) is equivalent to the chemical kinetics theory, if the latter allows the inactive enzymes to form inactive enzyme-substrate

215 complex. One author of the MMRT theory (who reviewed a previous draft of this manuscript) suggested that they obtained

the idea of non-zero heat capacity from the classical study by La Mer (1933). Our literature review found that although the

study by La Mer (1933) was historically influential, it was later recognized as flawed and inconsistent with the transition state

theory (Laidler and King, 1983). Therefore, we contend that the chemical kinetics theory, which accounts for the observed

reversible transitions of enzymes between their native and non-native states in the absence of substrate molecules (Anfinsen,

220 1973;Finkelstein and Ptitsyn, 2016;Sizer, 1943), and can satisfactorily explain the non-monotonic relationship between

temperature and catalysis rates, is a better mechanistic representation of the temperature dependence of enzyme-catalyzed

biochemical rates than MMRT.

Combining the transition state theory and the protein denaturation model by Lumry and Eyring (1954), Peterson et

al. (2004) proposed an equilibrium model that includes both reversible and irreversible enzyme denaturation to explain their

225 observed non-monotonic relationship between temperature and catalysis rates. However, because they assumed a constant

enthalpy for the reversible enzyme denaturation (thus the corresponding Gibbs free energy is a linear function of temperature

rather than a nonlinear function that is well recognized in protein physics (Ghosh and Dill, 2009;Silverstein, 2020)), their

model involves an explicit temporal dependence in the formulated catalysis rates, which introduces one more parameter (i.e.,

time) than the chemical kinetics model. Thus, the chemical kinetics theory is still be a better choice.

230 Can chemical kinetics theory be upscaled to an organism from the single-substrate-single-enzyme examples

presented here? While it is likely impossible to demonstrate such a scaling analytically, using the Ohm's law analogy from

Tang et al. (2021), where the temperature dependence of the emergent kinetic parameters (i.e., the overall v_{max} and K) for

chains of enzymes are found to follow similar forms as described by the chemical kinetics theory, we can qualitatively assert

that the answer is true. Indeed, some previous studies (e.g., Ratkowsky et al., 2005;Corkrey et al., 2012;Ghosh et al., 2016)

235 have showed that even equation (15) (which excludes substrate dependence) is able to satisfactorily describe temperature

dependent growth of many organisms. Moreover, the success of using the reversible denaturation idea to describe the

temperature-dependent development of poikilotherm by Sharpe and Demichele (1977) (who obtained a mathematically

equivalent representation to equation (15)) suggests that the chemical kinetics theory should have potential applications for

microbes, animals, and plants.

240   Finally, because almost every microbe, animal, and plant is able to respire on multiple substrates (Madigan et al., 2009;Cooper and Hausman, 2007) and the natural availability of those substrates usually fluctuate, the chemical kinetics theory and the equilibrium chemistry approximation kinetics for substrate competition networks (Tang and Riley, 2013) together suggest that a given organism will be unlikely to have either a fixed temperature response curve or optimal temperature even with a fixed proteome distributions. Rather, they both are likely to be dynamic.

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