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On the Temperature Dependence of Enzyme-Catalyzed Rates

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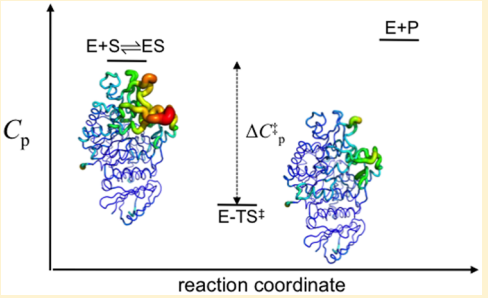
Supporting Information

ABSTRACT: One of the critical variables that determine the rate of any reaction is temperature. For biological systems, the effects of temperature are convoluted with myriad (and often opposing) contributions from enzyme catalysis, protein stability, and temperature-dependent regulation, for example. We have coined the phrase "macromolecular rate theory (MMRT)" to describe the temperature dependence of enzyme-catalyzed rates independent of stability or regulatory processes. Central to MMRT is the observation that enzyme-catalyzed reactions occur with significant values of $\Delta C_p^{\ddagger}$ that are in general negative. That is, the heat capacity (C_p) for the enzyme−substrate complex is generally larger than the C_p for the enzyme-transition state complex. Consistent with a classical description of enzyme catalysis, a negative value for $\Delta C_p^{\ddagger}$ is the result of the enzyme binding relatively weakly to the substrate and very tightly to the transition state. This observation of negative $\Delta C_p^{\ddagger}$ has important implications for the temperature dependence of enzyme-catalyzed rates. Here, we lay out the fundamentals of MMRT. We present a number of hypotheses that arise directly from MMRT including a theoretical justification for the large size of enzymes and the basis for their optimum temperatures. We rationalize the behavior of psychrophilic enzymes and describe a "psychrophilic trap" which places limits on the evolution of enzymes in low temperature environments. One of the defining characteristics of biology is catalysis of chemical reactions by enzymes, and enzymes drive much of metabolism. Therefore, we also expect to see characteristics of MMRT at the level of cells, whole organisms, and even ecosystems.



The rate of any chemical reaction is a function of the temperature (T) and the energy difference between the reactants and the transition state, the so-called activation energy (E_a). Arrhenius was the first to formalize this relationship in the 19th century (based on empirical observations) with his famous eponymous equation $k = A\ \exp(-E_a/RT)$, where k is the rate constant and R is the universal gas constant. Early in the 20th century, the development of transition state theory (TST) by Eyring, Polanyi, and others led to the Eyring equation for rate constants (eq 1 for a first order rate constant, where $\Delta G^{\ddagger}$ is the change in Gibbs free energy between reactants and the transition state, k_B and h are Boltzmann's and Planck's constants respectively, and κ is the transmission coefficient, hereafter assumed to be 1 for simplicity. For a more general definition in terms of partition functions see, e.g., ref 1).[1] This led to an understanding of, and statistical mechanical justification of, the terms in the Arrhenius expression. The Arrhenius and Eyring equations are found in most modern (bio)chemistry textbooks and provide an excellent description of the temperature dependence of a wide array of chemical processes.[1] The Eyring equation in its simplest form is sufficient for our purposes here (eq 1). An assumption often made with respect to eq 1 is that $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are independent of temperature (and hence that $\Delta G^{\ddagger}$ varies with temperature according to the Gibbs equation: $\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$). Indeed, this assumption holds well for many reactions involving small molecules in standard solvents. However, a number of investigators have noted deviations from eq 1 when plotting temperature versus enzyme-catalyzed rates, suggesting that the above assumption is not valid and that there is a more complex temperature dependence for these systems.[1,2]

Enzymes are flexible macromolecules of high molecular weight and with correspondingly high heat capacities (C_p). For example, the heat capacity for folded proteins is estimated to be ~45 J mol^{-1}·K^{-1} per amino acid,[3] and thus a typical enzyme of molecular mass 65 kDa will have a heat capacity in water of ~25 300 J mol^{-1}·K^{-1} (for comparison, liquid water has C_p = 76 J mol^{-1}·K^{-1} at 25 °C).

$$k = \frac{\kappa k_B T}{h} e^{(-\Delta G^{\ddagger}/RT)} = \frac{\kappa k_B T}{h} e^{(\Delta S^{\ddagger}/R)} e^{(-\Delta H^{\ddagger}/RT)} \quad (1)$$

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$$\Delta G^{\ddagger} = [\Delta H^{\ddagger}_{T_0} + \Delta C_p^{\ddagger}(T - T_0)] - T[\Delta S^{\ddagger}_{T_0} + \Delta C_p^{\ddagger}(\ln T - \ln T_0)] \quad (2)$$

$$k = \frac{k_B T}{h} e^{\left[\frac{-\Delta H^{\ddagger}_{T_0} - \Delta C_p^{\ddagger}(T - T_0)}{RT} + \frac{\Delta S^{\ddagger}_{T_0} + \Delta C_p^{\ddagger}(\ln T - \ln T_0)}{R}\right]} \quad (3)$$

$$\ln k = \ln \frac{k_B T}{h} - \left[\frac{\Delta H^{\ddagger}_{T_0} + \Delta C_p^{\ddagger}(T - T_0)}{RT}\right] + \left[\frac{\Delta S^{\ddagger}_{T_0} + \Delta C_p^{\ddagger}(\ln T - \ln T_0)}{R}\right] \quad (4)$$

The C_p of a system is a fundamental thermodynamic parameter that quantifies the temperature dependence of the enthalpy (H) and entropy (S) according to eq 2, and incorporation of a $\Delta C_p^{\ddagger}$ term into the Eyring eq (eq 1) gives eqs 3 and 4. If $\Delta C_p^{\ddagger} = 0$, eq 3 collapses into eq 1. However, for reactions catalyzed by enzymes with high heat capacities, the $\Delta C_p^{\ddagger}$ term may be nonzero, and eqs 3 and 4 should be implemented. Is there a difference in heat capacity between the enzyme−substrate and enzyme−transition state species for enzyme-catalyzed reactions (i.e., is $\Delta C_p^{\ddagger}$ nonzero for enzyme-catalyzed reactions)? If so, what are the consequences for the temperature dependence of enzyme catalyzed rates?

We have previously demonstrated that enzymatically catalyzed rates proceed with negative values of $\Delta C_p^{\ddagger}$ ranging from −1 to −12 kJ mol^{-1}·K^{-1} (independent of denaturation),[4] and we have coined the phrase macromolecular rate theory (MMRT) to reflect this unusual thermodynamic property of biochemical reactions.[5,6] MMRT has significant implications for the temperature dependence of enzyme-catalyzed rates and for the rates of biologically driven processes in general, such as microbial growth, respiration, and photosynthesis. MMRT unifies a number of disparate observations with respect to thermophilic, mesophilic, and psychrophilic enzymes and also presents some surprising hypotheses. Here, we lay out the basis for MMRT and present new hypotheses based on MMRT that warrant further experimental verification. We also use MMRT to provide a theoretical argument for relative size of enzymes according to the chemistry that they catalyze.

MACROMOLECULAR HEAT CAPACITY

The internal energy of a system is partitioned between translational, rotational, vibrational, and electronic modes. The heat capacity C is formally the change in internal energy with a change in temperature and is a measure of the capacity for the translational, rotational, vibrational, and electronic modes to absorb energy. For systems in water at biologically relevant temperatures (−20 < T < 100 °C), electronic modes above the ground state are generally inaccessible, and thus electronic modes do not contribute to heat capacity in this context. It has been shown experimentally that the greatest contribution to the heat capacity of a folded protein in water is the number of accessible vibrational modes.[3] Molecular dynamics simulations over long time-scales paint a vivid picture of the low-frequency modes for proteins for example.[7] These numerous modes contribute an estimated 82% of the C_p term for globular proteins with a further 15% contribution from the interaction between the protein and water.[3,8] Indeed, this is the origin of the increase in C_p per amino acid described above—the addition of each amino acid to a protein chain adds an additional number of vibrational modes to the molecule (increasing C_p by ∼45 J mol^{-1}·K^{-1} per amino acid). The change in heat capacity, ΔC_p, for a macromolecular system in equilibrium between two states is then most significantly due to the change in the frequencies of the vibrational modes of the molecule between the two states. The simplest example of macromolecular ΔC_p can be seen in protein−ligand binding:

$$P_{apo} + L \rightleftharpoons PL$$

The apo form of the protein (P_{apo}) is often more flexible (i.e., more low frequency vibrational modes) than the ligand-bound form (which has increased rigidity and fewer low frequency modes). There is also a desolvation term that contributes to ΔC_p and is the result of displacement of bound water molecules by the ligand.[9] If we consider the contributions from the protein alone, ΔC_p for such an interaction is generally negative. Protein crystallographers have been exploiting this phenomenon for decades: proteins are more likely to crystallize in the presence of a bound ligand because the protein becomes more "ordered" or more rigid in the ligand-bound state. In general, any process that limits the number of low frequency vibrational modes for macromolecules will be accompanied by a corresponding negative ΔC_p for that process. ΔC_p has been experimentally determined for many protein−ligand interactions. For example, the interaction between the enzyme methylthioadenosine phosphorylase (MTAP) and a tight binding inhibitor has ΔC_p = −2.5 kJ mol^{-1}·K^{-1}.[10] The binding of transcription factors to DNA are characterized by ΔC_p values ranging from −1.4 to −3.4 kJ mol^{-1}·K^{-1}.[11] In this latter case, the role of changing solvation upon binding is thought to make a significant contribution to ΔC_p.[11] In these and other examples where ΔC_p has been measured directly, it has been found to be largely independent of temperature (i.e., linear ΔH versus T plots), and we assume this to be the case for MMRT.

The classical description of enzyme catalysis invokes relatively weak binding of the substrate to the enzyme (K_M) and tight binding of the transition state to the enzyme. The tight binding of the transition state significantly lowers $\Delta G^{\ddagger}$ for the reaction, leading to the extraordinary rate enhancements we see for enzyme catalyzed reactions.[12] By analogy with the description for ligand binding at equilibrium above, we would expect a negative value for $\Delta C_p^{\ddagger}$ for enzyme catalysis—very tight binding of the transition state will reduce the number of low frequency vibrational modes for this state when compared to the relatively weakly bound enzyme−substrate complex.[4]

It must be acknowledged that the classical description for enzyme catalysis, based on tight binding of the transition state, is hotly debated, and the precise origin of enzyme catalysis is currently the subject of controversy in the literature, in particular, the role of protein dynamics in catalysis.[13−16] Various arguments for and against the role of protein dynamics in enzyme catalysis have been presented based on statistical thermodynamics,[1] molecular dynamics,[17] transition state barrier crossing,[14] Marcus theory and preorganization,[18] promoting vibrations,[19] and quantum tunnelling,[20] among others. Notwithstanding this controversy, the sign and magnitude of $\Delta C_p^{\ddagger}$ for enzyme catalysis are a statistical thermodynamic property for the reaction that describes the difference in heat capacity between the ensemble in the ground state and that at the transition state. This property does not report on enzyme dynamics with respect to catalysis. $\Delta C_p^{\ddagger}$ can be mathematically defined in terms of statistical thermodynamics according to eq

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5.[9] For any particular state on the reaction coordinate, the heat capacity is proportional to the mean squared fluctuation of the enthalpy divided by kT^2. Thus, $\Delta C_p^{\ddagger}$ is the difference in the mean squared fluctuation in the enthalpy at the transition state compared to the ground state.

$$\Delta C_p^{\ddagger} = \frac{\Delta\langle \partial H^2 \rangle^{\ddagger}}{kT^2} \quad (5)$$

IMPLICATIONS OF NEGATIVE $\Delta C_P^{\ddagger}$ VALUES FOR ENZYME CATALYSIS

We have previously determined $\Delta C_p^{\ddagger}$ values for several enzymes.[4] The $\Delta C_p^{\ddagger}$ values range from −1 to −12 kJ mol^{-1}·K^{-1}. When the MMRT function is fitted to data collected by others, the data are also well described and $\Delta C_p^{\ddagger}$ values also lie in this range (see Figure S2). This has significant implications for the temperature dependence of enzyme-catalyzed rates. First, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are steeply dependent on temperature according to eq 2 (Figure 1A). $\Delta G^{\ddagger}$ is curved as a result and the curvature is determined by the magnitude of $\Delta C_p^{\ddagger}$ (to illustrate, we have set $\Delta C_p^{\ddagger}$= −3.0 kJ mol^{-1}·K^{-1} in Figure 1A–C). In this context, it makes little sense to discuss in general terms the relative contributions of enthalpy and/or entropy to enzyme catalysis without precisely defining the temperature. For example, the scheme presented in Figure 1A shows that the entropic contribution ($-T\Delta S^{\ddagger}$) to $\Delta G^{\ddagger}$ is precisely zero at 289 K. In contrast, at 312 K, the enthalpic term is zero and $T\Delta S^{\ddagger}$ = −70.5 kJ mol^{-1}. The steep temperature dependence of these terms may account for the disparate observations in the literature regarding the relative contributions of enthalpy and entropy to ligand binding events (though for binding, solvation changes will make a large contribution),[10] and the relative importance of entropy and enthalpy to enzyme catalysis.[21] Suffice to say that at low temperatures the $\Delta G^{\ddagger}$ barrier for the reaction is predominantly enthalpic, and at high temperatures, the $\Delta G^{\ddagger}$ barrier is predominantly entropic. $\Delta G^{\ddagger}$ is a minimum when $\Delta S^{\ddagger}$ = 0. Empirical examples of the temperature dependence of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ have been observed and discussed previously.[16]

It follows that for $\Delta C_p^{\ddagger} < 0$, the rate of an enzyme catalyzed reaction initially rises with temperature and then reaches an optimum temperature (T_{opt}) after which the rate falls again, in contrast to simple Arrhenius and Eyring kinetics (Figure 1B,C). The increase in rate at temperatures up to T_{opt} is driven by the enthalpic term in eq 4 ($-\Delta H^{\ddagger}/RT$). However, this term is slowly overcome by the entropic term ($\Delta S^{\ddagger}/R$) at temperatures above T_{opt}, leading to a reduction in the reaction rate (Figure 1C).

The steep temperature dependence of $-\Delta H^{\ddagger}/RT$ and $\Delta S^{\ddagger}/R$ also makes it difficult to describe a general mechanism regarding how enzymes reduce the activation barrier for the uncatalyzed reaction. What can be said is that at temperatures close to the optimum temperature for the enzyme catalyzed rate (T_{opt}) $\Delta H^{\ddagger}$ for the enzyme catalyzed reaction is near zero (Figure 1C).

Curvature in a rate-versus-temperature plot is thus a generic property of enzyme-catalyzed rates where the heat capacity of the enzyme–substrate complex is greater than the heat capacity of the enzyme-transition-state species (i.e., $\Delta C_p^{\ddagger} < 0$). This curvature has previously been attributed to Arrhenius-like behavior below the optimum temperature (T_{opt}) and denaturation above T_{opt}. Of course, the process of denaturation

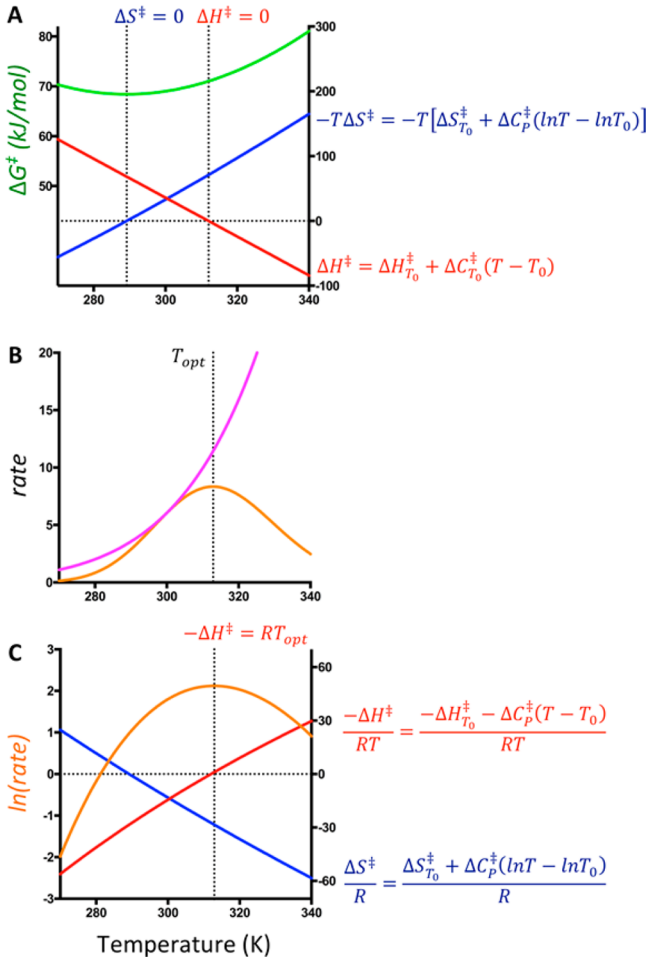

Figure 1. The temperature dependence of rates where $\Delta C_p^{\ddagger}$ is nonzero. (A) The temperature dependence of $\Delta G^{\ddagger}$ (left-hand *y*-axis) is shown in green. Individual contributions to $\Delta G^{\ddagger}$ from enthalpy and entropy are shown in red and blue, respectively (right-hand *y*-axis). Vertical dotted lines denote temperatures where $\Delta S^{\ddagger}$ = 0 and $\Delta H^{\ddagger}$ = 0. $\Delta G^{\ddagger}$ is a minimum when $\Delta S^{\ddagger}$ = 0. $\Delta C_p^{\ddagger}$ is set to −3.0 kJ mol^{-1}·K^{-1}. (B) The temperature dependence of the reaction rate according to simple Eyring (purple, eq 1) and MMRT (orange, eq 3, $\Delta C_p^{\ddagger}$ = −3.0 kJ mol^{-1}·K^{-1}) predictions. The optimum temperature for MMRT is shown by a vertical dotted line. (C) The temperature dependence of ln(rate) showing MMRT (orange, left-hand *y*-axis) and the individual contributions to ln(rate) from the enthalpy term ($-\Delta H^{\ddagger}/RT$) and the entropy term ($\Delta S^{\ddagger}/R$), red and blue lines, respectively (right-hand *y*-axis). The vertical dotted line shows T_{opt} and $-\Delta H^{\ddagger} = RT_{opt}$.

impacts on enzyme-catalyzed rates at high temperatures. However, decreases in rate above T_{opt} occur even in the absence of denaturation/unfolding, as a direct consequence of MMRT (when $\Delta C_p^{\ddagger} < 0$). For example, psychrophilic enzymes have long been considered enigmatic as they display decreases in rate above T_{opt} in the absence of denaturation.[22]

OPTIMUM TEMPERATURE FOR ENZYME CATALYSIS

When $\Delta C_p^{\ddagger} < 0$, T_{opt} is mathematically defined when the first derivative of eq 3, with respect to temperature, is equal to 0 (dk/dT = 0, see Figure 1B) Setting dk/dT = 0 for eq 3 gives eq 6. Equations 7 and 8 follow directly (Figure 1C).

$$\Delta H^{\ddagger} = -RT_{opt} \quad (6)$$

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$$\Delta H^{\ddagger}_{T_0} + \Delta C_p^{\ddagger}(T_{opt} - T_0) = -RT_{opt} \quad (7)$$

$$T_{opt} = \frac{\Delta H^{\ddagger}_{T_0} - \Delta C_p^{\ddagger} T_0}{-\Delta C_p^{\ddagger} - R} \approx T_0 - \frac{\Delta H^{\ddagger}_{T_0}}{\Delta C_p^{\ddagger}} \quad (\text{for } |\Delta C_p^{\ddagger}| \gg R) \quad (8)$$

Equation 6 places exacting constraints on the thermodynamics of enzyme-catalyzed reactions at the optimum temperature and limits $\Delta H^{\ddagger}$ to a very narrow range of values at T_{opt}. Enzyme T_{opt} values typically range from ~15 °C up to ~100 °C and this limits $\Delta H^{\ddagger}$ to values between −2.4 and −3.1 kJ mol^{-1} at T_{opt}. In contrast, the entropic term is almost always the dominant term at T_{opt}. It is noteworthy that at temperatures below T_{opt} (where many enzyme kinetic studies are performed), $\Delta S^{\ddagger}/R$ approaches zero and the enthalpic term dominates at these temperatures (Figure 1C). Feller and coworkers have described these trends in the enthalpic and entropic contributions to enzyme catalyzed rates when homologous psychrophilic, mesophilic, and thermophilic enzymes are compared,[23,24] and their observations are a direct consequence of eqs 3 and 6. Åqvist and colleagues have shown lower enthalpies of activation and more negative entropies of activation for psychrophilic enzymes which also follows from eqs 3 and 6 (Figure 1).[25,26]

Equation 8 is significant for the evolutionary adaptation of enzymes to thermophilic or psychrophilic environments. In order to evolve to higher or lower T_{opt} values, $\Delta C_p^{\ddagger}$ for the enzyme-catalyzed reaction must increase or decrease, respectively. We have previously demonstrated the correlation between T_{opt} and $\Delta C_p^{\ddagger}$ for a range of enzymes and their mutants.[4] Let us take as an example the evolution of a mesophilic enzyme to a psychrophilic enzyme requiring a shift of T_{opt} downward. Equation 8 implies that $\Delta C_p^{\ddagger}$ must become more negative to shift T_{opt} downward ($\Delta H^{\ddagger}$ is fixed at T_{opt}). In molecular terms, decreasing $\Delta C_p^{\ddagger}$ can be achieved by increasing the C_p of the enzyme−substrate complex (more low frequency vibrational modes), and/or decreasing the C_p of the enzyme-transition-state species (fewer low frequency modes, i.e. making it more rigid). The effect of both of these strategies is evident in the increased K_M and k_{cat} values for psychrophilic enzymes compared to their mesophilic counterparts (at low temperatures),[27] indicative of decreased binding affinity for the substrate and increased binding affinity for the transition state. Åqvist has described this as "protein surface softness" for psychrophilic enzymes.[26] An important consequence of an increasingly negative $\Delta C_p^{\ddagger}$ (with decreasing T_{opt}) is the increased curvature in the ln(rate)-versus-temperature plot (Figure 2). Figure 2A shows ln(k) versus temperature for homologous isopropylmalate dehydrogenase (IPMDH) enzymes from thermophilic, mesophilic, and psychrophilic species of *Bacillus*.[28] Over evolutionary time, the migration of T_{opt} values has tracked with $\Delta C_p^{\ddagger}$ in a manner consistent with eq 8. The same trend is seen for mutants of the *Bacillus subtilis* enzyme MalL (Figure 2B) where single point mutations simultaneously change T_{opt} of the enzyme and $\Delta C_p^{\ddagger}$ of the enzyme-catalyzed reaction.[4] The enzyme rate for each of these MalL variants is almost exactly the same at 37 °C, and it is the temperature dependence of the rates that has been altered (i.e., an altered $\Delta C_p^{\ddagger}$). Combining the temperature-rate data for 10 IPMDH enzymes and seven MalL enzymes from *Bacillus* clearly demonstrates the relationship between T_{opt} and $\Delta C_p^{\ddagger}$ (Figure 2C).

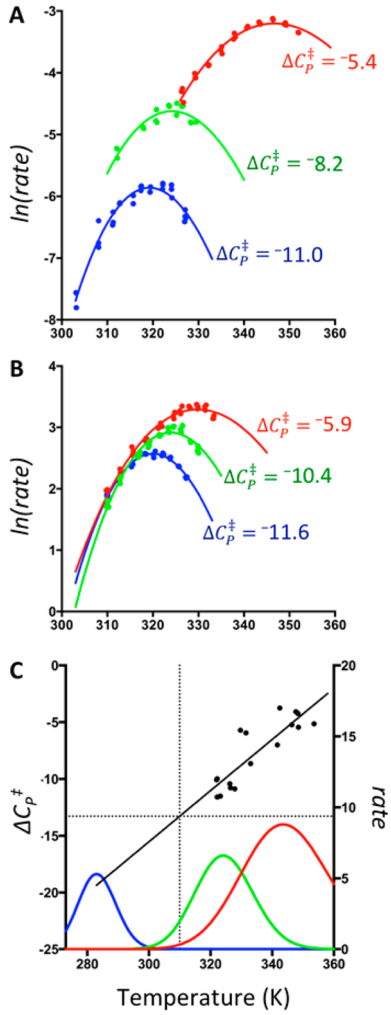

Figure 2. $\Delta C_p^{\ddagger}$, T_{opt}, and the psychrophilic trap. (A) The temperature dependence of IPMDH enzymes from thermophilic (red), mesophilic (green), and psychrophilic *Bacillus* species (blue) illustrating the increasing curvature in the ln(rate) versus temperature plot as T_{opt} decreases. (B) The temperature dependence of MalL enzymes from *Bacillus subtilis*. The wild type enzyme is shown in blue, the mutant V200T is shown in green, and the mutant V200S is shown in red. (C) The correlation between $\Delta C_p^{\ddagger}$ (left-hand y-axis) and T_{opt} (x-axis) for 10 IPMDH and 7 MalL Bacillus enzymes (black dots). A linear correlation is also shown. Colored curves are plots of rate (right-hand y-axis) versus temperature with $\Delta C_p^{\ddagger}$ values that are indicative of those falling on the line. This illustrates the extreme curvature for an enzyme whose T_{opt} lies at 10 °C for which the line predicts $\Delta C_p^{\ddagger} \approx -18.0$ kJ mol^{-1}·K^{-1}.

A PSYCHROPHILIC TRAP

The increasing curvature for the rate-versus-temperature plot as T_{opt} falls results in a "psychrophilic trap" whereby extreme curvature will render enzymes unfit for environments where the temperature varies even by a few degrees (Figure 2C). This explains the rarity of genuine psychrophiles whose T_{opt} matches the mean temperature for the environment for low temperatures (−10 < T < 10 °C). Indeed, IPMDH from *Bacillus psychrosaccarolyticus* (with a T_{opt} of 47 °C) points toward this organism being psychrotolerant as opposed to a genuine psychrophile (Figure 2A).

The large, negative values of $\Delta C_p^{\ddagger}$ required to set T_{opt} at, say, 10 °C (blue line in Figure 2C), creates large curvature in a rate-versus-temperature plot so as to make the enzyme rates

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highly—and probably prohibitively—sensitive to small changes in temperature. For example, a change in temperature of 10 °C away from T_{opt} for the hypothetical "psychrophile" in Figure 2C (blue line) causes a > 70% reduction in rate, whereas for the hypothetical mesophile and thermophile (green and red lines in Figure 2C) rates fall by 39% and 17% over the same temperature range. To avoid the psychrophilic trap, there appears to be a natural minimum T_{opt} of ∼20 °C.[23] The only context where this restriction can be overcome and true psychrophiles may evolve is in environments where the ambient temperature is low and almost invariant over both short and long (evolutionary) time scales. Studying enzymes from deep-sea organisms where temperatures are very stable (and relatively low) may be the best way to test this hypothesis.

In the context of hot environments, $\Delta C_p^{\ddagger}$ will approach zero for increasingly thermophilic enzymes (as T_{opt} approaches 100 °C). Indeed, the linear correlation shown in Figure 2C crosses the x-axis at 369 (±13) K, very close to 100 °C. As such, the temperature dependence of thermophilic enzymes will approach Arrhenius behavior and the enthalpic and entropic contributions to the rate are much less temperature sensitive. Only under these circumstances ($\Delta C_p^{\ddagger} \approx 0$, extreme thermophiles) may it be possible to tease out the relative contributions of enthalpy and entropy to catalysis (as $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ will be constant over a relatively wide temperature range).

ENZYME MASS AND REACTION CHEMISTRY

Protein synthesis is one of the most energetically costly processes in the cell. It has been estimated that approximately 80% of cellular ATP is given over to protein synthesis.[29] Given the acute selection pressures that drive energy efficiency in cellular metabolism, this implies that enzymes are a minimum size to carry out their cellular function(s). Further, it implies that there is an important functional purpose for the large mass of an enzyme.

A number of concepts have previously been suggested in an effort to rationalize enzyme size, and these have included the possibility of the enzyme surface acting to concentrate substrate molecules near the active site.[31] Other researchers have suggested that the enzyme acts to channel thermal energy to the active site,[32] or that the enzyme/substrate mass ratio is linked to the level of stabilization of the transition state,[30] or that there is a requirement for additional transition state-specific interactions in order to stabilize this state relative to the unbound transition state.[42] The general need for a preorganized active site (which may place catalytic groups in unfavorable environments) has been suggested to impose a size requirement to give the requisite folding energy.[43,44]

MMRT and its consideration of the role of $\Delta C_p^{\ddagger}$ for enzymes can provide some insight into the relationship between enzyme mass and enzyme catalysis as $\Delta C_p^{\ddagger}$ reports directly on the change in the frequencies of the vibrational modes between the enzyme−substrate complex and the enzyme-transition state complex.

The internal energy of an enzyme in water is partitioned across kinetic, vibrational, and rotational modes (ignoring the electronic modes for the purposes of this discussion). The potential energy residing in the vibrational modes represents a significant energy reservoir. The observation of $\Delta C_p^{\ddagger} = -11.6$ kJ mol^{-1}·K^{-1} for MalL,[4] for example, suggests that this energy reservoir changes on progression from the enzyme−substrate complex to the enzyme-transition state complex (at a given temperature). Let us make the conjecture that this change in energy has contributed toward the stabilization of the transition state. It follows that should there be a requirement for a greater stabilization of the transition state to either increase the reaction rate or to catalyze more "difficult" chemistry, we would expect to see an increase in the mass of the enzyme as this is a mechanism to increase the molecular heat capacity (the potential energy reservoir).

To illustrate this idea, let us adopt Wolfenden's classic formalism[12] and use the hydrolysis of oligosaccarides, DNA, and RNA as examples. In water at 25 °C oligosaccarides are very stable. For example, the half-life ($t_{1/2}$) for the hydrolysis of maltose at 25 °C is ∼11 million years. DNA is less stable to hydrolysis with $t_{1/2} \approx 140\,000$ years and RNA hydrolysis has $t_{1/2} \approx 4$ years.[12] Thus, the uncatalyzed rates of hydrolysis of these compounds vary by 7 orders of magnitude. In contrast, the rate constants for all three enzyme-catalyzed reactions vary by just 2 orders of magnitude and are dramatically larger: $k_{cat} \approx 0.01-1.0$ s^{-1}. If a contribution to catalysis were to originate from the molecular heat capacity reservoir, we would expect to see enzymes increase in size from RNases to DNases to glycosidases. This is what we observe (Figure 3). We have surveyed a range of enzymes that catalyze pseudo-first order reactions (hydrolases, esterases, decarboxylases, and isomerases), and we find a significant correlation (p = 0.0004) between enzyme molecular weight and the log(ratio) of enzyme-catalyzed to noncatalyzed rate constants, $\ln(k_{cat}/k_{non})$. This indicates that larger enzymes are required to catalyze increasingly difficult chemistries. For this data set, we deliberately chose pseudo-first order reactions, and we computed the molecular weight for isolated monomeric catalytic domains. Thus, the data are not confounded by oligomeric states or by entropic considerations for bimolecular and termolecular reactions. We have also added two ribozymes to the data set (in orange diamonds in Figure 3), and these are also consistent with the correlation.

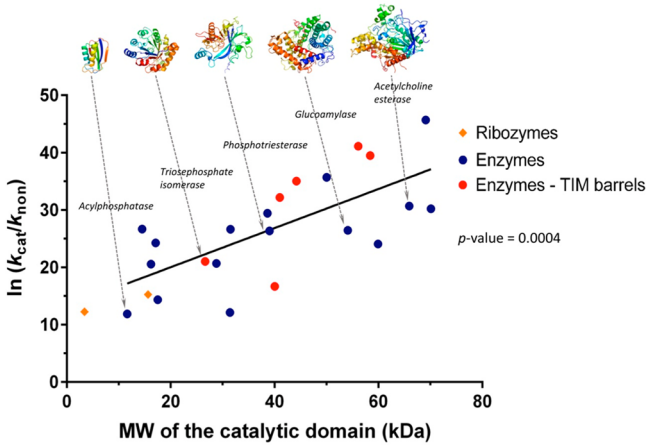

Figure 3. Molecular weight of the catalytic domain of an enzyme versus the observed rate enhancement, $\ln(k_{cat}/k_{uncat})$. Selected structures for the catalytic domains are illustrated across the top of the graph and labeled (arrows point to the corresponding point on the graph). Ribozymes are shown with orange diamonds and enzymes are shown with filled circles. The TIM-domain enzymes are shown in red. A linear regression is also shown ($y = 0.34x + 13.3$, $R^2 = 0.48$). Values are taken from references detailed in Table S1.

An intriguing observation from these data is that enzymes with the same fold increase in mass to catalyze more difficult

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chemistries. For example, there are six TIM barrel enzymes in the data set (in red circles in Figure 3), and their increasing masses are consistent with the correlation.

The relationship between enzyme mass and rate enhancement in Figure 3 suggests an approximate lower bound on enzyme catalysis where the linear correlation crosses the *y*-axis at $\ln(k_{cat}/k_{non}) = 13.3$, $(k_{cat}/k_{non}) \approx 6.0 \times 10^5$. Rate enhancements that lie below this figure are certainly accessible via intramolecular small molecule catalysis at ambient temperatures, for example, and thus the *y*-axis intercept suggests an approximate transition between small molecule catalysis and macromolecular catalysis.

The correlation shown in Figure 3 does not prove causality, and further experiments are required to verify the relationship between the macromolecular heat capacity potential energy reservoir and enzyme catalysis. If this behavior is borne out and found to be generally important then it may have implications for enzyme design.

The position of the ribozymes at the left-hand end of the scatter of points may say something about the suitability of ribozymes for catalysis of increasingly difficult chemistry (i.e., very slow chemical reactions). Ribozymes are typically poor enzymes, and even given large mass increases ribozymes do not realize the significant rate enhancements of enzymes. RNase P for example, with a mass of 120 kDa, only achieves a comparatively paltry rate enhancement of $\ln(k_{cat}/k_{non}) = 15$, which is comparable to the two small ribozymes included in Figure 3. This suggests that increasing ribozyme mass has very little effect on catalysis and will not bring slow chemical reactions into the correct time frame for life. This may point to a possible explanation for the supplanting of ribozymes by proteins as biological catalysts over the course of evolutionary history.

A NOTE ON ENZYME DENATURATION

The significance of ΔC_p for the temperature dependence of *protein stability* at equilibrium has been understood for 40 years.[33,34] The role of $\Delta C_p^{\ddagger}$ in the temperature dependence of protein folding kinetics has also been well described.[35] For protein folding, $\Delta C_p^{\ddagger}$ is generally negative as the unfolded state is very dynamic and hydrophobic side chains have a characteristic solvation shell in this state, whereas the transition state for protein folding is generally relatively compact and more closely resembles the native state.[36] For folding, desolvation of hydrophobic residues on the folding pathway is thought to make a significant contribution to $\Delta C_p^{\ddagger}$.[37] In contrast, the similarity between the folded state and the transition state for folding leads to small positive values of $\Delta C_p^{\ddagger}$ for protein unfolding and almost no curvature in the temperature dependence of unfolding rates.[35]

MMRT describes the relationship between temperature and the rate of enzyme catalysis in the absence of denaturation. For a complete description of the temperature dependence of enzyme catalysis, denaturation must be included. As unfolding occurs with small positive values of $\Delta C_p^{\ddagger}$ and shows little curvature over a wide temperature range,[38] it is relatively straightforward in many cases to apply a simple correction term for unfolding to the observed enzyme catalyzed rate. In the case of MalL, the unfolding rate (k_u) is 3 orders of magnitude slower than (k_{cat}), and this allows accurate deconvolution of the temperature dependence of k_{cat} and k_u.[4] Indeed, we would advocate that enzymologists routinely measure unfolding rates at the temperature(s) of interest.

DISCUSSION

The importance of $\Delta C_p^{\ddagger}$ for the temperature dependence of enzyme kinetics was first described by Hobbs et al. 2013.[4] The $\Delta C_p^{\ddagger}$ term for enzyme kinetics has been hidden in plain sight as enzyme denaturation has always been thought to be the cause of decreasing rates for enzymes above their optimum temperature of activity. In this context, psychrophilic enzymes were seen as enigmatic insofar as they display a curved temperature dependence of their catalytic rates despite relatively high unfolding temperatures.[27] MMRT now resolves this enigma by making explicit the difference in heat capacity between the enzyme substrate complex and the enzyme transition state species: this difference dictates curvature in the temperature-ln(rate) plot for enzymes independent of denaturation.

The extent to which MMRT is generalizable to all enzymes remains to be seen and in many cases relies on the deconvolution of the temperature dependence of enzymatic rates from denaturation rates (which is not common practice). Nevertheless, here we review and analyze data for 6 enzymes and 22 mutants that conform to MMRT kinetics. Further, Daniel and Danson[2] have shown curved temperature-rate plots at "zero time" (i.e., independent of denaturation) for 21 enzymes (see Figure S2 for three examples), and the psychrophilic enzymes (as reviewed by Feller and Gerday[22]) also show this same curvature. In a separate paper we have recently analyzed the temperature dependent behavior of three model enzymes in the context of MMRT.[6] On the other side of the coin, there are many historical examples of linear Arrhenius plots for enzymes, and this suggests that in many cases, $\Delta C^{\ddagger}_p$ is close to zero (collapsing eq 3 into eq 1). There are pragmatic reasons why, in some cases, curvature may be obscured. For example, where data are collected over a narrow temperature range or where there are too few data points to assess the presence or absence of curvature. The generalizability of MMRT will be tested by the careful collection of data over a sufficient temperature range (to calculate accurate k_{cat} values) and deconvolution of these rates from denaturation rates.

The origin of a negative value of $\Delta C_p^{\ddagger}$ for enzyme kinetics is a shift in the frequencies of the vibrational modes that leads to reduced fluctuation in energies at the transition state (eq 5). In a classical description of enzyme kinetics, negative $\Delta C_p^{\ddagger}$ values are the result of relatively weak binding of the enzyme to its substrate and tight binding to the transition state. We have described this phenomenon as "macromolecular rate theory" as $\Delta C_p^{\ddagger}$ is enabled by the high intrinsic heat capacity of macromolecules, in contrast to catalysis by small molecules. The high heat capacity for proteins in particular (in contrast to ribozymes for example, see Figure 3) appears to be significant in the context of enzyme catalysis.

MMRT is based on a quasi-two-state model where the kinetics of the rate determining chemical step are determined by the energy difference between the enzyme−substrate complex and the enzyme-transition state complex. Other investigators have postulated quasi-three-state models to account for the curvature in the temperature dependence of some enzyme reaction rates.[1,2] These models postulate an equilibrium between conformations with different activation energies. In an analysis of enzymatic Q_{10} values Elias and Tawfik also find evidence for a three-state model.[39] On the basis of temperature-rate data alone, it is not possible to discriminate between a two-state model incorporating $\Delta C^{\ddagger}_p$

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and three-state models. However, the three-state models assume $\Delta C_p^{\ddagger} = 0$, and this parameter may be measured directly for enzyme kinetics using isothermal titration calorimetry (ITC). These experiments are the subject of ongoing research in our laboratories. Should these experiments reveal a nonzero $\Delta C_p^{\ddagger}$ for enzyme catalyzed rates then MMRT will be sufficient to describe the curvature in temperature-ln(rate) data without recourse to three-state models (Figure 2 and Figure S2).

MMRT uses a greatly simplified scheme (like all models), and there are a number of potentially confounding factors that must be acknowledged. First, we assume that there is no change in the chemical mechanism associated with catalysis, as temperature changes. Possible sources of mechanistic change could arise as a result of changes in pK_a with temperature for example. Second, we assume that the heat capacities of the ground state and transition state ensembles are essentially constant over the biological temperature range. Previous studies on protein folding provide some evidence that justifies this assumption where a very small temperature dependence of $\Delta C_p^{\ddagger}$ for protein unfolding is observed.[35] Linear plots of ΔH versus T for protein−ligand binding also point toward a very small temperature dependence of ΔC_p.[10]

MMRT prescribes a number of traits for the temperature dependence of enzyme catalysis. First, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are steeply temperature dependent and range from large positive values to large negative values across temperatures relevant to biology (0−100 °C). Second, the optimum temperature for activity, T_{opt}, is defined by $\Delta H^{\ddagger}$ (at T_{opt}) and $\Delta C_p^{\ddagger}$ where $\Delta H^{\ddagger}(T_{opt}) = -RT_{opt}$ (eqs 6−8). This dictates that for lower values of T_{opt}, $\Delta C_p^{\ddagger}$ becomes increasingly negative and curvature of the temperature-rate profile becomes extreme. This places a practical limit on T_{opt} for psychrophilic enzymes, and we have called this the psychrophilic trap. In contrast, $\Delta C_p^{\ddagger}$ approaches zero as T_{opt} approaches 100 °C.

The observation of negative $\Delta C_p^{\ddagger}$ values for enzyme catalysis implies that there is a change in the distribution of vibrational modes between the ground state ensemble and the transition state ensemble. Equivalently (according to eq 5) there is a narrowing of the distribution of enthalpies on going from the ground state ensemble to the transition state ensemble. The large number of enzyme vibrational modes and the associated heat capacity may be viewed as an energy reservoir, and the correlation between enzyme mass and k_{uncat} suggests that a larger energy reservoir (in enzymes with greater mass) may be required to help stabilize the transition state. Indeed, this is consistent with the hypothesis of Warshel and colleagues who have suggested the folding energies of enzymes may need to be reduced so that a small reorganization energy and thus a low energy barrier for reaction can be realized. In order to obtain low energy barriers for reaction for difficult chemistries, enzymes of increasing size may be required so that folding energies can be reduced to "pay the price" for low reorganization energies.[40]

Any contribution of vibrational modes to the catalytic effect of enzymes is extremely controversial at present,[16] and no inference concerning the microscopic picture of enzyme catalysis can be drawn from MMRT. Interestingly, however, it was recently observed that the magnitude of $\Delta C_p^{\ddagger}$ appears to be correlated with the presence or absence of vibrational modes that may be coupled to the reaction coordinate for very well-studied, small model enzyme systems[6] (although, when this coupling has been investigated directly, it has been shown to be small).[41] Suffice to say that MMRT incorporates $\Delta C_p^{\ddagger}$ to describe enzyme kinetics, and as such $\Delta C_p^{\ddagger}$ may influence kinetic behavior, but as a statistical thermodynamic property $\Delta C_p^{\ddagger}$ does not inform on any vibrational coupling to the reaction coordinate.

The signature of MMRT (curvature for temperature versus rate in Arrhenius or Eyring plots) has also been described for biological systems at increasing scales—from organism growth rates to ecosystems.[4,5] Extrapolation from the behavior of a few enzymes up to networks of thousands of enzymes or even thousands of organisms is risky—fools rush in where angels fear to tread. However, many schemes that currently describe the temperature dependence of organism growth rates or of ecosystem processes, such as respiration and photosynthesis, use functions based on the Arrhenius relationship. Curvature is observed for these processes, and it remains to be determined whether or not this curvature is a signature of MMRT in these higher order biological systems.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b01094.

Table S1: Numbers and references to accompany Figure 3 (PDF)

Figure S2: Fit of the MMRT function to data taken from papers by Daniel and Danson.[2] (PDF)

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■ ABBREVIATIONS

MMRT, macromolecular rate theory; TIM, triose phosphate isomerase; MTAP, methylthioadenosine phosphorylase; IPMDH, isopropylmalate dehydrogenase

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