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Entropy and Enzyme Catalysis

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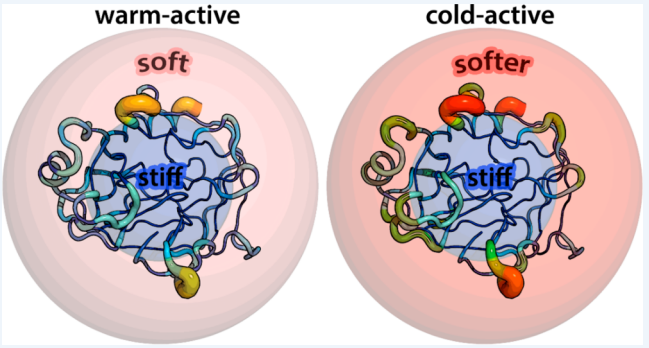
CONSPECTUS: The role played by entropy for the enormous rate enhancement achieved by enzymes has been debated for many decades. There are, for example, several confirmed cases where the activation free energy is reduced by around 10 kcal/mol due to entropic effects, corresponding to a rate enhancement of $\sim 10^7$ compared to the uncatalyzed reaction. However, despite substantial efforts from both the experimental and theoretical side, no real consensus has been reached regarding the origin of such large entropic contributions to enzyme catalysis. Another remarkable instance of entropic effects is found in enzymes that are adapted by evolution to work at low temperatures, near the freezing point of water. These cold-adapted enzymes invariably show a more negative entropy and a lower enthalpy of activation than their mesophilic orthologs, which counteracts the exponential damping of reaction rates at lower temperature. The structural origin of this universal phenomenon has, however, remained elusive. The basic problem with connecting macroscopic thermodynamic quantities, such as activation entropy and enthalpy derived from Arrhenius plots, to the 3D protein structure is that the underlying detailed (microscopic) energetics is essentially inaccessible to experiment. Moreover, attempts to calculate entropy contributions by computer simulations have mostly focused only on substrate entropies, which do not provide the full picture.



We have recently devised a new approach for accessing thermodynamic activation parameters of both enzyme and solution reactions from computer simulations, which turns out to be very successful. This method is analogous to the experimental Arrhenius plots and directly evaluates the temperature dependence of calculated reaction free energy profiles. Hence, by extensive molecular dynamics simulations and calculations of up to thousands of independent free energy profiles, we are able to extract activation parameters with sufficient precision for making direct comparisons to experiment. We show here that the agreement with the measured quantities, for both enzyme catalyzed and spontaneous solution reactions, is quite remarkable. Importantly, we can now address some of the most spectacular entropy effects in enzymes and clarify their detailed microscopic origin. Herein, we discuss as examples the conversion of cytidine to uridine catalyzed by cytidine deaminase and reactions taking place on the ribosome, namely, peptide bond formation and GTP hydrolysis by elongation factor Tu. It turns out that the large entropy contributions to catalysis in these cases can now be rationalized by our computational approach. Finally, we address the problem of cold adaptation of enzyme reaction rates and prove by computational experiments that the universal activation enthalpy–entropy phenomenon originates from mechanical properties of the outer protein surface.

INTRODUCTION

Uncatalyzed chemical reactions in aqueous solution are often found to be associated with sizable negative activation entropies, which may add a considerable penalty ($-T\Delta S^{\ddagger}$) to the overall activation free energy ($\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$).[1] Such an entropy penalty is most often interpreted in terms of a loss of translational and rotational motions of the reactants as they pass through their transition state (TS).[2] That is, with the reactants at a 1 M standard state there is a translational entropy loss associated with bringing them into proximity in the TS. Likewise, in the reactant state the reacting molecules rotate freely, while the TS may involve specific angular requirements for productive barrier crossing. This implies a loss also of rotational entropy as the accessible configurational volume is then bound to decrease in the TS (the same argument may also be applied to internal rotations of the reactants). Hence, bimolecular reactions are considered to be intrinsically associated with more negative activation entropies than unimolecular ones, disregarding possible solvent contributions. However, the solvent entropy contribution must also be kept in mind, particularly for processes involving charge transfer and separation, where it may dominate the entropy change.[3]

Because enzymes snugly bind their substrates, it seems reasonable to expect that the translational and rotational entropy penalties may already be paid. This is also at the heart of Jencks' "Circe effect" hypothesis,[4] which postulates that the enzyme spends part of the binding free energy on destabilizing the substrates. Although such a destabilization could conceivably involve enthalpic factors (e.g., desolvation, electrostatic

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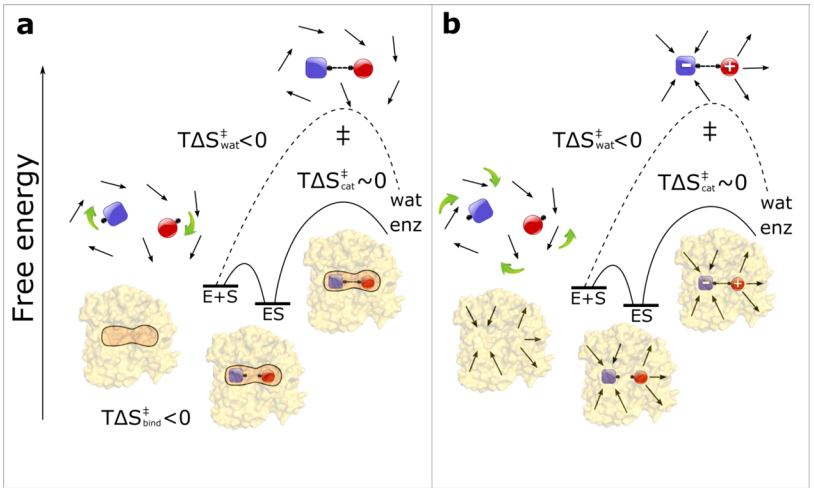

Figure 1. Two ways that an enzyme could diminish the $T\Delta S^{\ddagger}$ penalty. (a) If the entropy loss in water is dominated by ordering the reactants, the enzyme pays this penalty upon substrate binding, and the reaction can proceed without further entropy loss.[4] (b) If $T\Delta S^{\ddagger}$ in water is dominated by solvent reorganization, the enzyme eliminates the penalty by active site preorganization. The binding entropy need not be correlated with the activation entropy in this case.

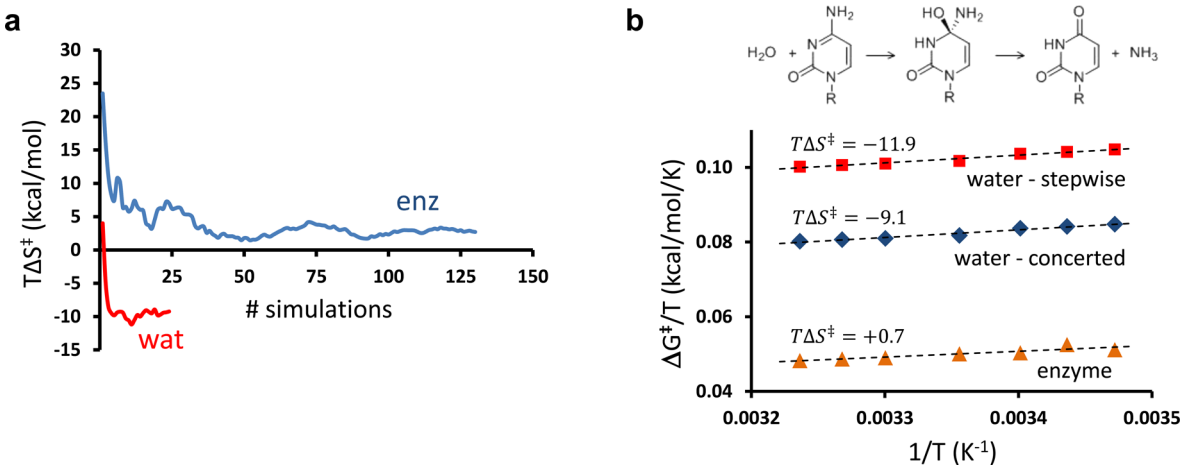

Figure 2. (a) Calculated $T\Delta S^{\ddagger}$ versus number of MD/EVB simulations (5 ns) at each temperature (water, red; enzyme, blue). (b) Computed Arrhenius plots for stepwise (red) and concerted (blue) spontaneous cytidine deamination and for cytidine deaminase (orange). Entropy contributions are given at 298 K (the concerted water plot is shifted downward for clarity).

or conformational strain), the most common interpretation invokes substrate entropy. That is, if the entropy penalties have already been paid then the activation barrier could be climbed without any entropy loss (Figure 1), which would yield a major acceleration of the enzyme reaction rate.[4] While there is clearly a loss of translational and rotational entropy involved in substrate binding, the debatable issue is the magnitude of its catalytic contribution and whether it is overshadowed by other factors. Jencks proposed that it is the dominant contribution to lowering the activation barrier (up to 11 kcal/mol).[5] He also pointed out that this intrinsic entropy change of the reactants cannot be experimentally disentangled from contributions from the surrounding medium, but apparently considered activation free energy effects from the latter to be of lesser importance and subject to enthalpy−entropy compensation.[5] Wolfenden has shown that enzymes, in general, rather reduce $\Delta H^{\ddagger}$ than the $-T\Delta S^{\ddagger}$ penalty.[6] The conclusion that enthalpy and not entropy is the driving force for the high fraction of "near-attack conformations" in enzymes has also been reached by Bruice,[7] although the increased population of such conformations may simply reflect TS stabilization.[8] Computational analysis examining free energy contributions due to restraining the reactants in enzymes and solution have further concluded that the effect is smaller than anticipated, primarily since motions both in the reactant and in transition states are suppressed in the enzyme.[8,9] Hence, the assumption of a complete loss of translational and rotational entropy upon binding, with no further entropy change toward the TS,[4] appears over-simplified.[8,9] However, to make direct comparisons with experiment, it is necessary to calculate the overall activation entropies both in enzymes and in solution, including the contributions from both solvent and protein, rather than just the substrate entropies.

■ COMPUTATION OF ARRHENIUS PLOTS

The approach that we have followed for obtaining thermodynamic activation parameters is analogous to the experimental construction of Arrhenius plots of the rate logarithm versus inverse temperature. The main difference is that computer simulations do not yet allow direct evaluation of reaction rates

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by counting rare barrier crossing events, so we instead calculate free energy profiles and use transition state theory to obtain the rates.[3,10] With an accurate estimate of the free energy profile $\Delta G(X)$ along the reaction path X, the activation free energy, $\Delta G^{\ddagger}$, and its temperature dependence can be obtained by extensive molecular dynamics (MD) simulations at a series of different temperatures. The activation parameters are then extracted by linear regression from

$$\Delta G^{\ddagger}/T = \Delta H^{\ddagger}/T - \Delta S^{\ddagger} \quad (1)$$

Calculations of $\Delta G(X)$ require very extensive MD simulations to minimize the convergence errors associated with $\Delta G^{\ddagger}(T)$, and the typical precision required is about 0.2 kcal/mol.[3] This is the primary reason why presently only the empirical valence bond (EVB) method appears viable for this purpose. Since EVB describes the reaction surface in terms of a mixture of analytical valence bond force fields,[11,12] energies for a huge number of configurations can be obtained at a moderate computational cost, which is hardly possible with other QM/MM methods.

In practice, $\Delta G(X)$ profiles are calculated using an umbrella sampling procedure,[13] and typically, between 15 and 150 independent profiles are calculated per temperature.[3,14] We have generally observed that fewer calculations are needed for solution compared to enzyme reactions for a given precision. This is presumably due to the more homogeneous environment in water, whereas local protein conformational transitions tend to slow down convergence in the enzyme. Figure 2a compares the activation entropy convergence for a single chemical step corresponding to the concerted hydrolytic cytidine deamination in solution and the rate-limiting step for the same reaction catalyzed by cytidine deaminase.[15] In the latter case, the overall reaction involves five distinct steps. With 130 independent profiles for each of these at seven different temperatures, we end up with a very large number of free energy calculations (~10 μs of MD simulation).

■ ACCURACY OF THERMODYNAMIC ACTIVATION PARAMETERS FOR SOLUTION REACTIONS

To examine whether the catalytic effect of an enzyme is due to reduction of the activation entropy penalty, it is necessary to reliably obtain this quantity also for the uncatalyzed reaction in water, which further serves as an important benchmark. For instance, cytidine deaminase appears to be a perfect example of the Circe effect as the enzyme reaction proceeds with a near-zero activation entropy while the uncatalyzed reaction in water has a $T\Delta S^{\ddagger}$ term of about −8 kcal/mol at 25 °C.[16] Moreover, the entropy contribution to substrate binding ($T\Delta S^{0}_{bind}$) closely matches $T\Delta S^{\ddagger}$ in water in accordance with the hypothesis. We recently explored the spontaneous hydrolytic cytidine deamination reaction in water, yielding uridine, using a combination of density functional theory (DFT) calculations and MD/EVB simulations.[3] Here, both stepwise and concerted mechanisms were explored for cytidine and its saturated variant 5,6-dihydrocytidine by DFT in a continuum solvent, with different numbers of explicitly treated water molecules. EVB models were parametrized against the DFT results and used for free energy simulations to obtain thermodynamic activation parameters as described above. The results demonstrated the dependence of enthalpic strain on the number of water molecules engaged in the TS and showed that with three TS waters, the stepwise and concerted mechanisms had very similar free energy barriers in excellent agreement with experimental kinetics for both substrates.[3] Interestingly, although the free energy barriers for stepwise and concerted mechanisms were almost the same, the two reaction pathways were found to have significantly different enthalpy and entropy contributions (Figure 2b). It became clear that only the concerted mechanism agrees with experimental values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, while the stepwise variant is characterized by a more negative activation entropy and smaller enthalpy. This effect was found to originate from a distinct zwitterionic intermediate along the stepwise path, resulting from proton transfer from water to cytidine. Formation of this intermediate is associated with a large negative $T\Delta S^{0}$ = −16.5 kcal/mol, which dominates over the subsequent $T\Delta S^{\ddagger}$ = +4.6 kcal/mol for attack of hydroxide on the protonated substrate.[3] Encouragingly, this example shows that computational Arrhenius plots may be used to distinguish between different possible reaction mechanisms.

As a reference reaction for GTP hydrolysis on the ribosome, we also carried out simulations of the spontaneous hydrolysis of $Mg^{2+}{\cdot}GTP^{4-}$ in water.[17] Quantum chemical calculations are notoriously difficult in this case, due to the highly charged reactant, and solvation effects are of major importance. In order to avoid possible methodological bias associated with different DFT and solvation models, we chose here to parametrize associative and dissociative EVB activation barriers, which are expected to be similar in energy,[18] using the same experimentally derived free energy barrier.[17] The resulting Arrhenius plots gave similar near-zero activation entropies for both types of transition states, in very good agreement with experiment[19] (Table 1). Here, the slightly more positive $T\Delta S^{\ddagger}$ for the dissociative TS may reflect its more unimolecular character, although non-negligible bond order to both nucleophile and leaving group is still predicted.[18] Finally, we have examined the spontaneous attack of hydroxide ion on CO_2 in water as an additional benchmark (Table 1), with an earlier EVB calibration used to study carbonic anhydrase.[20] Here again, we obtain very reasonable agreement with experimental data,[21] with calculated and observed $T\Delta S^{\ddagger}$ values of +4.5 and +3.6 kcal/mol, respectively, at 25 °C. As with OH^- attack on protonated cytidine, we find a significantly positive $T\Delta S^{\ddagger}$, due to delocalization of hydroxide charge in the TS, thus reflecting an increase in water entropy accompanying a reduced polarization. This effect appears to be a hallmark of OH^- attack as discussed below.

Table 1. Calculated and Observed Activation Parameters for Reactions in Water[a]

reaction	$\Delta H^{\ddagger}$ calcd	$T\Delta S^{\ddagger}$ calcd	$\Delta H^{\ddagger}$ expt	$T\Delta S^{\ddagger}$ expt
cytidine + H_2O	21.4	−9.1	22.1	−8.3
5,6-dihydrocytidine + H_2O	12.7	−10.9	13.4	−10.1
$Mg^{2+}{\cdot}GTP^{4-}$ + H_2O (associative)	26.0	−1.0	27.1	−0.8
$Mg^{2+}{\cdot}GTP^{4-}$ + H_2O (dissociative)	28.2	+1.2	27.1	−0.8
CO_2 + OH^-	13.9	+4.5	13.2	+3.6

[a]Energies in kcal/mol at 298 K. Experimental data from refs 16, 19, and 21.

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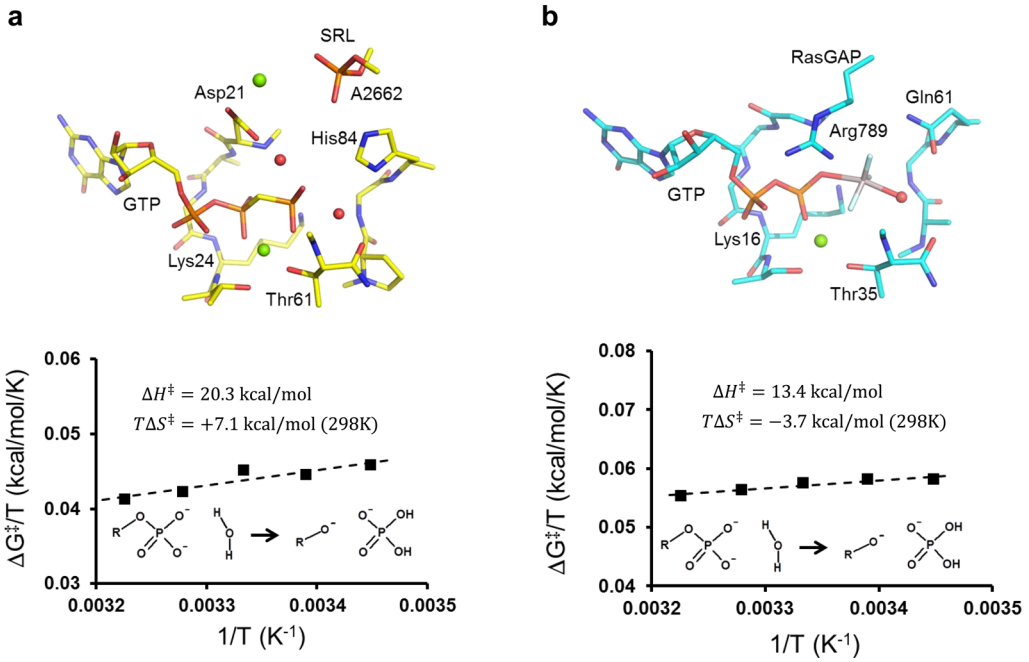

Figure 3. Active site structures of the trGTPases (a) and of the Ras−RasGAP complex (b) and their calculated Arrhenius plots for GTP hydrolysis. Water molecules and Mg^{2+} ions are shown as red and green spheres, respectively.

■ ORIGINS OF FAVORABLE ENZYME ACTIVATION ENTROPIES

Peptide Bond Formation on the Ribosome

A notable example where the enzyme activation entropy is extraordinarily favorable compared to the uncatalyzed reaction is the ribosome. That is, for its primary reaction, peptide bond formation, Wolfenden and co-workers showed that the near-zero activation entropy for k_{cat} with the puromycin substrate is at least 13 kcal/mol more favorable than for the corresponding reaction in water.[22] Subsequently, Ehrenberg and co-workers showed very similar activation parameters with full-length tRNA substrates.[23] Interestingly, both studies showed a near-zero entropy associated with K_M, which at least in the case of the small puromycin substrate should cleanly reflect the binding entropy. Hence, there is no indication that a significant negative binding entropy contribution could be used to facilitate catalysis of the peptidyl transfer reaction, as prescribed by the Circe effect. Moreover, the large negative $T\Delta S^{\ddagger}$ for uncatalyzed ester aminolysis likely originates from solvation of a zwitterionic TS, involving significant water reorientation, rather than from alignment of the substrates. This was also the conclusion from EVB simulations of peptide bond formation on the ribosome, where the favorable activation entropy was explained in terms of a preorganized hydrogen bond network that reduces the cost of solvent reorganization (Figure 1).[24] Warshel and co-workers further showed that proton shuttling via the 2′-OH group of the P-site substrate does not confer any entropic advantage in solution compared to proton transfer via a water molecule.[25] The entropy evaluation in ref 24 was, however, based on calculation of the total potential energies at a single temperature, which is associated with convergence problems. Further benchmark simulations[26,27] showed that direct calculation of the temperature dependence of free energies is a more promising route for obtaining enthalpic and entropic components.

Cytidine Deaminase

This enzyme appears to be a perfect example of the Circe effect. Substrate binding is associated with a significant entropy loss that closely matches the $T\Delta S^{\ddagger}$ penalty for the uncatalyzed reaction, while the enzyme reaction proceeds with $T\Delta S^{\ddagger}$ close to zero.[16] However, our simulations of the solution reaction showed that the $T\Delta S^{\ddagger}$ penalty of ∼9 kcal/mol has more to do with solvent ordering than motions of the cytidine reactant,[3] indicating that the Circe interpretation is problematic. Indeed, subsequent simulations of the enzyme reaction[15] showed a near-zero activation entropy (Figure 2b), in quantitative agreement with the experimental data.[16] However, this reduction of the $T\Delta S^{\ddagger}$ penalty was found to originate from a change of reaction mechanism, rather than from substrate "freezing". That is, the enzyme site is preorganized such that the reactant resting state has a zinc-bound hydroxide ion, which attacks the substrate with an intrinsically favorable activation entropy. In fact, OH^- attack on the protonated substrate was found to have a positive $T\Delta S^{\ddagger}$ value also in solution, although it is not the operational mechanism in that case.[3] The agreement with experimental data for the cytidine deaminase reaction is rather remarkable. It also shows the usefulness of parametrizing EVB potentials with DFT calculations on enzyme cluster models,[28] for enabling extensive configurational sampling. Overall, the cytidine deaminase calculations showed that the Circe effect is not at play and suggest that the similarity between substrate binding entropy and the $T\Delta S^{\ddagger}$ penalty in solution may rather be coincidental.

GTP Hydrolysis by EF-Tu on the Ribosome and the Ras GTPase

A third example of an enzyme reaction with an extraordinarily favorable activation entropy is that of elongation factor Tu (EF-Tu) on the ribosome. EF-Tu is the fastest known GTPase with reaction rates exceeding 500 s^{-1}, and remarkably, the entropic contribution is experimentally found to be $T\Delta S^{\ddagger}$ = +7 kcal/mol.[23,29] Such a positive entropy contribution to the reaction

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rate appears to be larger than for any other enzyme at physiological temperatures.[30,31] We recently explored different possible mechanisms for the catalytic reaction of EF-Tu on the ribosome with MD/EVB simulations.[32,33] The catalytic site common to the translational GTPases (trGTPases) has a charged histidine residue interacting with the γ-phosphate of GTP and the sarcin−ricin loop of the ribosome. This particular arrangement was predicted to promote a mechanism with early proton transfer from the catalytic water molecule to the phosphate, followed by concerted nucleophilic attack and leaving group departure. This mechanism was further analyzed by calculations of computational Arrhenius plots (Figure 3a), which yielded thermodynamic activation parameters for the overall reaction of $\Delta G^{\ddagger}$ = 13.2, $\Delta H^{\ddagger}$ = 20.3, and $T\Delta S^{\ddagger}$ = 7.1 kcal/mol (at 298 K).[17] The predicted entropy contribution is thus extraordinarily large and positive, and the computer simulations essentially give quantitative agreement with the experimental data.[23,29]

Also here it turns out that the positive $T\Delta S^{\ddagger}$ originates from hydroxide attack on the protonated γ-phosphate of GTP and delocalization of the OH^- and γ-phosphate negative charge in the TS. Hence, calculations on the uncatalyzed hydroxide attack on protonated $Mg^{2+}{\cdot}GTP^{3-}$ in water also yielded a positive $T\Delta S^{\ddagger}$ contribution of about 6 kcal/mol,[17] demonstrating this intrinsic feature of the EF-Tu reaction mechanism. A similar entropy contribution was observed experimentally for methylphosphate hydrolysis under highly alkaline conditions, where OH^- attack on the monoanion presumably dominates.[34] In contrast, simulations of the spontaneous GTP hydrolysis reaction in water, where the reaction instead follows either an associative or dissociative pathway with later proton transfer to the γ-phosphate, yielded a near-zero activation entropy.[17] Hence, just as in cytidine deaminase, the origin of the more positive entropy term in the enzyme is that it is able to stabilize a configuration promoting a different mechanism. Therefore, EF-Tu cannot either be considered as an example of the Circe effect but rather illustrates the variant of an electrostatically preorganized active site where an otherwise unstable intermediate state (involving OH^- as in cytidine deaminase) is stabilized. The advantage with this type of preorganization is obvious if the ensuing nucleophilic attack is intrinsically fast and associated with a favorable activation entropy.

An intriguing feature of the translational GTPases is that they have a unique active site composition compared to other members of the GTPase superfamily. Their universally conserved Pro-Gly-His sequence in the so-called G3 motif and, in particular, the critical histidine residue is required for their activation by the ribosome.[35] Other GTPases, such as Ras, activated by its protein partner RasGAP, do not share this motif. Ras has its Gln61 side chain at the histidine position in the trGTPases, and RasGAP inserts its "arginine finger" into the active site where the trGTPases position an aspartate residue. Not surprisingly, this arrangement in the Ras−RasGAP complex completely changes the active site electrostatics. The stabilization of OH^- is now gone, and MD/EVB simulations instead predicted an associative reaction pathway with concerted proton transfer to the γ-phosphate.[36] Such proton transfer could either occur directly or be relayed by an additional water molecule,[37] presumably with similar entropy contributions.[17] Without activation by RasGAP, the reaction was calculated to slow down by a factor of ∼1000, in good agreement with experiment,[19] and the mechanism was instead predicted to become dissociative. Interestingly, however, both the Ras−RasGAP and isolated Ras reactions yielded computational Arrhenius plots that showed $T\Delta S^{\ddagger}$ contributions (298 K) of about −4 kcal/mol, contrary to the +7 kcal/mol found for EF-Tu (Figure 3b).[36] As discussed earlier, the calculated $T\Delta S^{\ddagger}$ values for the two Ras systems are also in very reasonable agreement with experimental data in the relevant temperature range.[36] Hence, the catalytic sequence motif of the fast trGTPases appears to confer a specific reaction mechanism, which is characterized by a uniquely favorable entropic contribution.

Evolutionary Tuning of Activation Parameters and Cold Adaptation

Another interesting aspect of the activation free energy partitioning is that it can actually be used by evolution to tune the temperature dependence of enzymatic reactions. That is, from the transition state theory equation

$$k_{rxn} = \kappa\left(\frac{kT}{h}\right) e^{-\Delta G^{\ddagger}/(RT)} = \kappa\left(\frac{kT}{h}\right) e^{\Delta S^{\ddagger}/R}\, e^{-\Delta H^{\ddagger}/(RT)} \quad (2)$$

it can be seen that the exponential damping of the reaction rate constant (k_{rxn}) with decreasing temperature is due to the enthalpy term (κ is the transmission factor). In fact, it is as if psychrophilic species knew about the Eyring equation, since cold-adapted enzymes invariably have a lower enthalpy and more negative entropy of activation than mesophilic orthologs.[30] That is, they appear to "move" part of the activation free energy from enthalpy to entropy. The different enthalpy−entropy partitioning will give a higher rate at low temperature for the psychrophilic enzyme and, conversely, a higher rate at high temperature for the mesophilic case, while they often have similar rates around room temperature. While this well-documented behavior has recently been questioned, by examination of average so-called Q_{10}-values for many enzymes (fold-increase in rate as a function of temperature interval),[31] these Q_{10}-values per definition only reflect $\Delta H^{\ddagger}$. Statistics based on grouping different types of enzymes together also appears to blur the distinct activation enthalpy differences that emerge from comparison of orthologous enzymes only.[30,38] Moreover, cold-adapted enzymes also generally have a lower melting temperature than their mesophilic orthologs.[30] The key question here is what is the structural origin of the different enthalpy−entropy partitioning? This problem has been analyzed by structural bioinformatics, but the conclusion was that "no structural feature is present in all cold-adapted enzymes, and no structural features always correlate with cold adaptation".[30]

We first analyzed this problem by computer simulations of psychrophilic, mesophilic, and hyperthermophilic citrate synthases, which was also the first attempt to calculate Arrhenius plots from the temperature dependence of free energy profiles.[10] Remarkably, just plugging different PDB structures (psychrophilic and mesophilic) into an EVB model of the catalytic reaction immediately yielded plots with significantly different slopes that obeyed the universally observed phenomenon. Hence, while $\Delta G^{\ddagger}$ at 300 K was very similar, the psychrophilic enzyme had a significantly lower $\Delta H^{\ddagger}$ and a more negative $T\Delta S^{\ddagger}$. Importantly, atomic positional fluctuations of the active site region (including the substrates) were virtually identical in the two enzymes, disproving the hypothesis that different active site mobilities could be causing the effect.[30] Instead, detailed energetic analysis showed that the

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difference in $\Delta H^{\ddagger}$ originated from outside of the active site (Figure 4), although the structural interpretation was not entirely clear.[10]

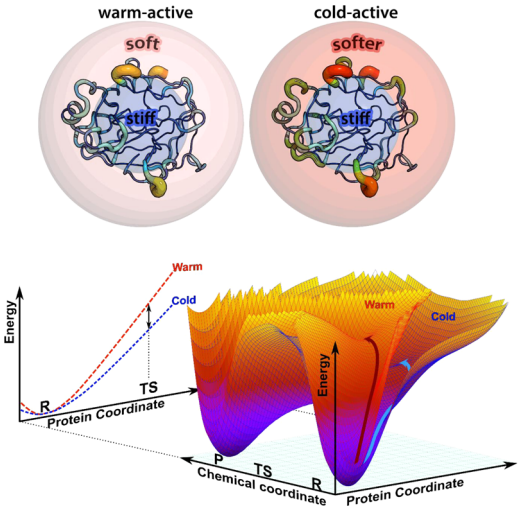

Figure 4. Relationship between surface rigidity and enzyme cold-adaptation. The protein core is rigid with similar mobility in mesophilic and psychrophilic orthologs. Outer parts are softer in the cold-adapted enzyme, and the protein potential energy contribution associated with displacing the system from reactants to transition state is smaller than in the mesophilic enzyme (lower panel).

We subsequently examined peptide hydrolysis by trypsin, where the rate-limiting step was simulated by MD/EVB for cold-adapted salmon trypsin and the bovine ortholog.[14] Again, significantly different Arrhenius plot slopes were calculated, with a marked reduction of both $\Delta H^{\ddagger}$ and $T\Delta S^{\ddagger}$ for the psychrophilic enzyme. As before, no differences in active site mobilities were found, and the differences in energetics originated from the outer regions. These calculations, however, clearly pointed to the protein surface as responsible for the effect, and enrichment of backbone mobility in certain surface regions was seen in the psychrophilic enzyme. A few surface loops were also identified and found to be associated with different conservation patterns among psychrophilic and mesophilic species.[14] Moreover, simulations of some surface point mutations showed that these may indeed change the enthalpy−entropy balance without significantly affecting $\Delta G^{\ddagger}$ at room temperature. Such an effect of single distant mutations has also been observed experimentally.[39] Note that psychrophilic enzymes usually have their rate optimum at around 30−50 °C (above which they start to melt), implying that their evolutionary optimization does not simply move the rate versus temperature curve to lower temperatures but rather corresponds to lifting the low temperature tail by a different enthalpy−entropy partitioning.[30]

The structural origin of the enthalpy−entropy effect was further analyzed with help of ultrahigh resolution crystal structures (≤1 Å) of salmon and bovine trypsins.[14] These structures show a large number of surface-bound water molecules making extensive hydrogen bonding networks with surface residues of the protein. In particular, several of these networks act as if to stabilize or rigidify the conformation of surface loops in the mesophilic enzyme, by bridging hydrogen bonds to the protein backbone. Comparison of the mesophilic and psychrophilic structures showed that warm → cold mutations tend to disrupt such networks, consistent with higher mobility of the latter in MD simulations. Mutations that destabilize local hydrophobic surface patches also appear to soften the protein surface.[14] Another interesting example is provided by the differently adapted alcohol dehydrogenases studied by Klinman and co-workers.[38,40] Comparison of the thermodynamic activation parameters for psychrophilic and hyperthermophilic orthologs revealed a striking difference in $\Delta H^{\ddagger}$ (12 kcal/mol) at room temperature, which was almost perfectly compensated by the change in $T\Delta S^{\ddagger}$ (12.5 kcal/mol), again underscoring the general phenomenon. Here, backbone

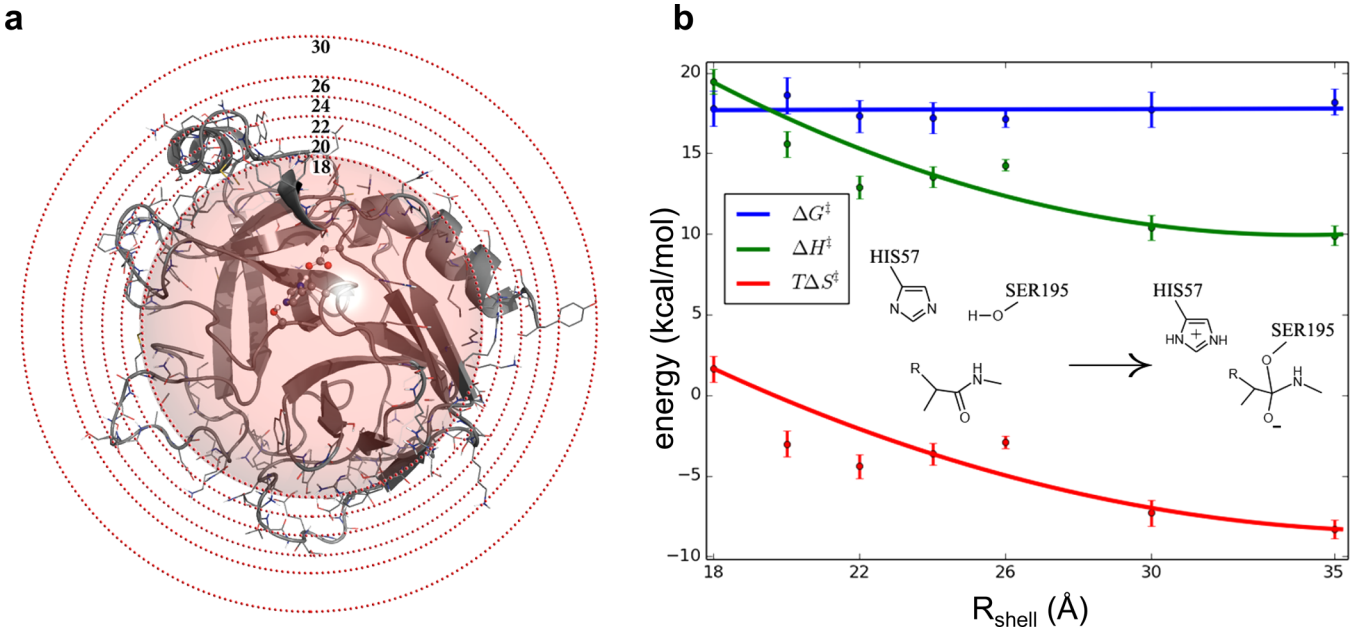

Figure 5. Successive restraining from the surface and inward in spherical shells (a) causes psychrophilic trypsin to acquire the characteristics of the mesophilic enzyme (b).

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amide H/D exchange experiments also point to certain surface regions being more flexible in the cold-adapted enzyme but also those involved in cofactor binding.[40]

Enzyme Surface Rigidity Tunes the Activation Enthalpy–Entropy Balance

The reduced $\Delta H^{\ddagger}$ in a psychrophilic enzyme thus appears due to a softer protein potential outside the rigid active site core. Rare fluctuations leading to reactive events in the active site by necessity also require displacements in the outer protein matrix, away from the reactant state minimum, and these are associated with different effective potentials in the cold- and warm-adapted cases (Figure 4). With a softer protein surface potential (including bound waters) the reactant configurational space will increase, which may lead to enthalpy–entropy compensation and a more negative value of $T\Delta S^{\ddagger}$. A softer effective protein potential is further likely to be intimately connected to thermal stability and melting temperature. The evolutionary principle behind the response to lower temperature thus becomes clearer. Mutations in the active site region will most often destroy enzyme activity if it has been optimized by evolution. However, distant mutations at the protein surface generally have little effect on the rate near the activity optimum but provide a means for shifting the enthalpy–entropy balance that may be beneficial if the working temperature changes.

To directly test whether protein surface rigidity can tune the enthalpy–entropy balance, we carried out computational experiments where surface positional restraints were applied to the cold-adapted enzyme.[41] Hence, we used the simple criterion that if any heavy protein atom in the crystal structure is located further away from the active site center than R_{shell}, the atom is harmonically restrained to its initial position. R_{shell} was then varied from 35 to18 Å in seven discrete steps (Figure 5), where a full Arrhenius plot involving eight different temperatures (275–310 K) was calculated for each value of R_{shell}. With 100 independent free energy profiles calculated at each temperature point this amounts to 9600 separate free energy calculations, corresponding to about 5 μs simulation time. The result of this computational experiment is rather spectacular and shown in Figure 5. First, it can be seen that $\Delta G^{\ddagger}$ (300 K) remains constant as thicker and thicker surface layers are restrained. Second, the remarkable phenomenon that the activation parameters of the cold-adapted trypsin approach those of the mesophilic enzyme is clearly evident, as more and more of the surface is being restrained. At R_{shell} = 18 Å, the psychrophilic enzyme has acquired virtually identical parameters to those of mesophilic trypsin.[14] The dependence of $\Delta H^{\ddagger}$ and $T\Delta S^{\ddagger}$ on the magnitude of the restraints was also examined in another series of MD/EVB simulations at R_{shell} = 18 Å, and it was found that only a relatively weak force constant ≥1 kcal/mol/Å^2 is required to attain the mesophilic characteristics. It should, however, be noted that for smaller values of R_{shell} than 18 Å the active site fluctuations and activation free energies start to become affected.[41] The above calculations thus provide direct computational support for the hypothesis that protein surface rigidity can tune the thermodynamic activation parameters.

CONCLUSIONS

In this Account, we have discussed several aspects of the role of entropy in enzyme catalysis in connection with recent computational studies. First, we have outlined an efficient computational method for calculating thermodynamic activation parameters for both enzyme and solution reactions. This is based on evaluating the temperature dependence of free energy profiles so that the $\Delta H^{\ddagger}$ and $T\Delta S^{\ddagger}$ components can be extracted via regular Arrhenius plots. The results obtained with this approach have been found to yield excellent agreement with the corresponding experimentally measured quantities.

With a reliable method for obtaining $\Delta H^{\ddagger}$ and $T\Delta S^{\ddagger}$ for enzyme reactions, we can examine the origin of the latter term in some specific pertinent cases, where the entropy contribution to k_{cat} is exceptionally large. Here, we have discussed the peptidyl transfer reaction on the ribosome ($T\Delta\Delta S^{\ddagger}$ = 14 kcal/mol), cytidine conversion to uridine catalyzed by cytidine deaminase ($T\Delta\Delta S^{\ddagger}$ = 9 kcal/mol), and GTP hydrolysis by EF-Tu on the ribosome ($T\Delta\Delta S^{\ddagger}$ = 8 kcal/mol), where the $T\Delta\Delta S^{\ddagger}$ values refer to the entropy contribution relative to the uncatalyzed reaction in water (at 298 K). In none of these three cases does the large entropy effect appear to originate from the "freezing" of reactant motions prescribed by the Circe effect.[4,5] Instead, the first example involves elimination of a solvent reorganization penalty rather than a substrate alignment penalty. This is achieved by a preorganized hydrogen bond network in the peptidyl transferase center of the ribosome that involves minimal reorientation during the reaction. The favorable activation entropies in the two latter cases are primarily due to a change of mechanism compared to the solution reaction. Here, hydroxide ion attack is the key feature, which is intrinsically associated with a positive $\Delta S^{\ddagger}$ due to delocalization of the OH^- charge in the transition state.

Finally, we have analyzed the evolutionarily very interesting problem of how enzyme activity in psychrophiles can be adapted to lower temperatures. The universal phenomenon that cold-adapted enzymes display a lower enthalpy and more negative entropy of activation than mesophilic orthologs, remarkably, emerges directly from swapping initial crystal structures in the calculations. From these computer simulations, the origin of the elusive enthalpy–entropy effect has been uncovered and found to be associated with mechanical properties of the protein surface or more precisely the protein–water surface interface. Hence, a softer protein surface is found to yield a lower activation enthalpy for the chemical reaction, which is compensated by larger loss of entropy as the reaction barrier is climbed. The evidence for this behavior comes from (1) analysis of the enthalpic energy components, (2) analysis of protein surface mobility, (3) examination of ultrahigh resolution crystal structures, (4) calculations of the effects of surface mutations, and (5) computational experiments where the surface mobility is successively restrained.

Hence, we can conclude that while entropic effects clearly play a major role in enzyme catalysis, their interpretation is not always straightforward. In particular, it seems that the historical focus on translational, rotational, and conformational entropies of just the substrates is somewhat misdirected. That is, in order to understand the origin of entropic contributions to enzymic rate enhancement, it is necessary to consider all the relevant parts of the measured activation entropies. These include not only the substrates and their immediate active site environment but also the rest of the protein and its surrounding solvent. With efficient methods for computationally analyzing and predicting thermodynamic activation parameters of enzyme reactions, a deeper understanding of the full energetics is within reach and will be useful both for rationalizing enzymological experiments and for enzyme engineering and design.

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