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Role of Dynamics in Enzyme Catalysis: Substantial versus Semantic Controversies

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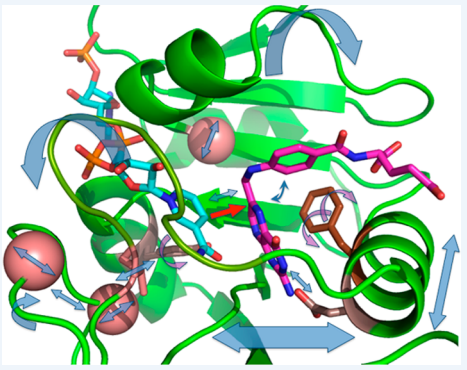
CONSPECTUS: The role of the enzyme's dynamic motions in catalysis is at the center of heated contemporary debates among both theoreticians and experimentalists. Resolving these apparent disputes is of both intellectual and practical importance: incorporation of enzyme dynamics could be critical for any calculation of enzymatic function and may have profound implications for structure-based drug design and the design of biomimetic catalysts.



Analysis of the literature suggests that while part of the dispute may reflect substantial differences between theoretical approaches, much of the debate is semantic. For example, the term "protein dynamics" is often used by some researchers when addressing motions that are in thermal equilibrium with their environment, while other researchers only use this term for nonequilibrium events. The last cases are those in which thermal energy is "stored" in a specific protein mode and "used" for catalysis before it can dissipate to its environment (i.e., "nonstatistical dynamics"). This terminology issue aside, a debate has arisen among theoreticians around the roles of nonstatistical vs statistical dynamics in catalysis. However, the author knows of no experimental findings available today that examined this question in enzyme catalyzed reactions.

Another source of perhaps nonsubstantial argument might stem from the varying time scales of enzymatic motions, which range from seconds to femtoseconds. Motions at different time scales play different roles in the many events along the catalytic cascade (reactant binding, reprotonation of reactants, structural rearrangement toward the transition state, product release, etc.). In several cases, when various experimental tools have been used to probe catalytic events at differing time scales, illusory contradictions seem to have emerged. In this Account, recent attempts to sort the merits of those questions are discussed along with possible future directions.

A possible summary of current studies could be that enzyme, substrate, and solvent dynamics contribute to enzyme catalyzed reactions in several ways: first via mutual "induced-fit" shifting of their conformational ensemble upon binding; then via thermal search of the conformational space toward the reaction's transition-state (TS) and the rare event of the barrier crossing toward products, which is likely to be on faster time scales then the first and following events; and finally via the dynamics associated with products release, which are rate-limiting for many enzymatic reactions. From a chemical perspective, close to the TS, enzymatic systems seem to stiffen, restricting motions orthogonal to the chemical coordinate and enabling dynamics along the reaction coordinate to occur selectively. Studies of how enzymes evolved to support those efficient dynamics at various time scales are still in their infancy, and further experiments and calculations are needed to reveal these phenomena in both enzymes and uncatalyzed reactions.

INTRODUCTION

Enzymes catalyze most chemical reactions in biology by many orders of magnitude (8 to 25) relative to the uncatalyzed reaction in the same aqueous media.[1] While speeding up a specific chemical conversion, an enzyme also inhibits the many other reactions that could have taken place if the same reactant(s) was reacting in solution. Nicotinamide cofactors, for example, in aqueous buffer and at concentrations of the hydride-donor substrate typical of enzymatic reactions, undergo hydrolytic decomposition long before a redox reaction would occur at the nicotinamide ring (Scheme 1). In nicotinamide-dependent enzymes, on the other hand, only the redox reaction occurs to any measurable extent. The protection of bonds that the enzyme has not evolved to cleave (marked red in Scheme 1) is fairly well understood. However, the physical means by which the enzyme catalyzes the reaction of interest are still the focus of intense examination.

The reaction rate is often rationalized by transition state theory (TST)[2] and the many corrections and additions that have been added to it along the years. TST assumes an adiabatic reaction path where the reaction coordinate can be described by a continuous energy landscape, with a dividing line between reactants and products near the saddle point that constitutes the transition state (TS). TST also assumes a dynamic equilibrium between the TS and the ground state

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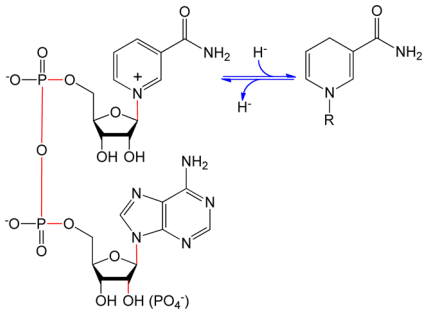
Scheme 1. Nicotinamide Cofactor, NAD(P)$^+$, with Bonds That Are Prone to Hydrolysis Highlighted in Red[a]



[a]The blue arrows indicate the enzyme-catalyzed redox reaction.

(GS). The Boltzmann distribution of populations between the GS and TS gives an exponential relationship between the rate constant (k) and the free energy difference between those populations ($\Delta G^{\ddagger}$):

$$k = A_{(T)}\, e^{-\Delta G^{\ddagger}/(RT)} \quad (1)$$

where T is the absolute temperature and R is the gas constant. The function A includes all the preexponential terms, such as the transmission coefficient (κ), friction factors, recrossing events, and more. In the framework of TST, the enzyme needs to reduce the energy barrier for the reaction via electrostatic pre- or reorganization of the active site, moving toward stabilization of the reaction's TS. Since the contribution of the barrier height to the reaction rate is exponential, it is considered to be more important to the reaction's rate than pre-exponential terms. Thus, much of the catalytic effect of the enzyme is the reduction of the free energy barrier (i.e., $\Delta G^{\ddagger}_{\text{catalyzed}} \ll \Delta G^{\ddagger}_{\text{uncatalyzed}}$). From the point of view of design of biomimetic catalysts, the many orders of magnitude between the rates of catalyzed and uncatalyzed reactions is of interest. However, biology and evolution are more strongly affected by smaller effects on the rate of catalyzed reactions, and most importantly by the organism's ability to fine-tune those small inputs (i.e., regulation). For example, rate differences of just 1 order of magnitude, or even a factor of 3, can be lethal to the organism and impose huge evolutionary pressure on many biological systems. Consequently, effects on the preexponential terms, such as quantum nuclear tunneling and recrossings, are critical to the understanding of enzymatic function.

Probing Motions Affecting the Bond Activation Using Kinetic Isotope Effects (KIEs)

The case studies presented below focus on experiments using the temperature dependence of KIEs because of their ability to illuminate the very fast motions involved in bond activation. In each case presented, the intrinsic KIEs (KIE_{int}) were assessed—that is, the KIE on the bond cleavage *per se*, which is free of kinetic complexity resulting from other kinetic steps such as

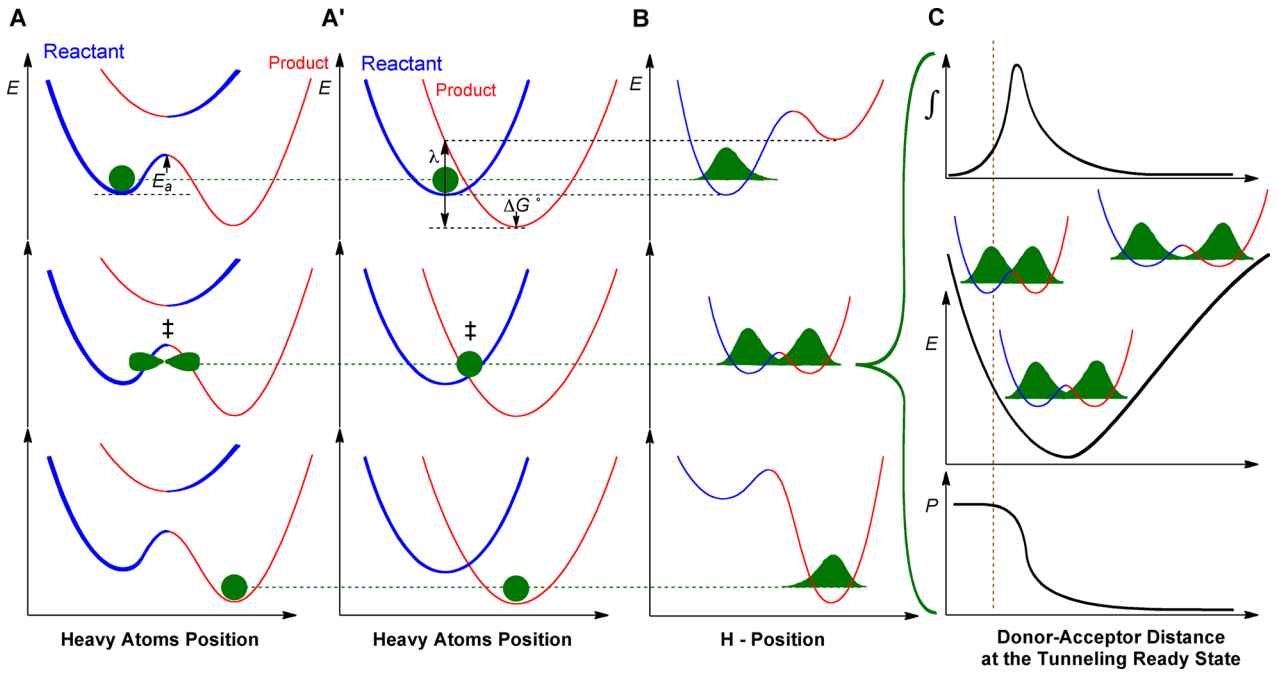


Figure 1. Graphic illustration of eqs 2, 3, and 4. Four slices of the potential energy surface along components of the collective reaction coordinate show the effect of heavy-atom motions on the zero point energy (ZPE) in reactant (blue) and product (red) potential wells. The green structures indicate the probability to find the particle along the different coordinates presented. Panels A and A′ present the heavy atom coordinate. Panel A illustrates eq 2 for TST (adiabatic), with the electronic-GS potential at bottom and the first electronic-excited-state on top. Panel A′ illustrates eq 3 (Marcus parabola). Panel B shows the H atom position. In the top panels of A, A′, and B, the hydrogen is localized in the reactant well, and the ZPE of the product state is higher than that of the reactant state. Heavy atom activation or reorganization brings the system to the tunneling ready state (TRS, middle panels of A, A′, and B), where the ZPE in the reactant and product wells are degenerate and the hydrogen can tunnel between the wells. Further heavy atom relaxation or reorganization breaks the transient degeneracy, trapping the hydrogen in the product state (bottom panels). Panel C shows the effect of DAD sampling on the wave function overlap at the TRS (middle panel). The transmission probability (P) is presented as a function of DAD (bottom panel C). The top panel C presents the contribution to H-transfer rate at each DAD as a function of the P and the population at that DAD (i.e., the integrated terms in eqs 2, 3, and 4). Please note that for DADs shorter than the vertical line in panel C, the ZPE is above the barrier; thus the reaction is practically over-the-barrier.

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substrate binding, product release, etc. Enzymatic reactions involve both millisecond dynamics of reactant binding (which do not directly affect the bond activation), and sub-nanosecond motions at the time scale of bond cleavage, some of which may be coupled to bond activation. KIEs and their temperature dependence can probe the latter, if the isotopically substituted atom is sensitive only to the bond cleavage step. The history of how the theory of KIEs and their temperature-dependence evolved has been covered elsewhere.[3] Here we present an approach that summarizes numerous published procedures and seems to be able to explain all current experimental findings.

The case studies examine enzyme-catalyzed redox reactions involving C−H bond activation. More specifically, they investigated the role of the protein dynamics in C−H → C hydride transfer. In such reactions, the particle moves to the product side of the potential without surmounting the peak of the energy barrier (the TS). Because of the light mass of the hydrogen nucleus, quantum mechanical (QM) tunneling can occur once the wave function of the particle in the reactant's state overlaps with that of the product state (see illustration in Figures 1A,B, middle panel). This phenomenon is very sensitive to the width and height of the barrier; that is, it can happen well below the barrier's maximum if the barrier is sufficiently narrow, but will occur close to the peak of a broad barrier. It is also very sensitive to the mass and contributes more to electron-transfer, less to hydrogen-transfer, and little to transfer of heavier particles.

The region along the reaction coordinate in which the particle is capable of tunneling is called the tunneling-ready-state (TRS) and can be thought of as a QM-delocalized TS. A TST equation where $A_{(T)}$ from eq 1 is divided into $C_{(T)}$ and an integral that sums all the TRSs by their tunneling probability (P) is presented in eq 2.

$$k = C_{(T)}\, e^{-\Delta G^{\ddagger}/(RT)} \int_0^{\infty} P_{(m,DAD)}\, e^{-(E_{(DAD)}/(k_B T))}\, dDAD \tag{2}$$

The terms before the integral represent heavy atom motions, which carry little or no isotope effect and are dropped when dividing the reaction-rate with one isotope by the rate with the other (e.g., k_H/k_D). The integral, on the other hand, is isotopically sensitive and measures the probability of hydrogen transfer once the system reaches a TRS. The first factor inside the integral, P, reflects the probability of tunneling as a function of mass (m) and the donor-acceptor distance (DAD), and the second factor is the Boltzmann factor giving the distribution of DADs. Graphical illustration of eq 2 is presented in Figure 1A.

TST assumes strong electronic coupling between the reactant and product states, yielding a continuous potential surface (see electronic GS potential in Figure 1A). For systems where that assumption is not reasonable (e.g., electron transfer reactions), nonadiabatic models have been developed (e.g., Marcus theory[4] using the "Golden rule" limit where the reactant and product potentials are weakly coupled to each other), and using the same integral term as in eq 2 allows for it to account for the DAD fluctuations at the TRS by eq 3.[5−9]

$$k = C_{(T)}\, e^{(-\Delta G^{\circ}+\lambda)^2/(4\lambda k_B T)} \int_0^{\infty} P_{(m,DAD)}\, e^{-(E_{(DAD)}/(k_B T))}\, dDAD \tag{3}$$

The factors in front of the integral give the rate of reaching a TRS based on the fraction of reactive complexes, the electronic coupling between reactant and product (C), the reorganization energy (λ), and the driving force of the reaction (ΔG°). This nonadiabatic approach is presented in Figure 1A′.

Since it is the integral in eqs 2 and 3 that is mostly sensitive to the mass of the transferred particle (i.e., the KIE), the KIE expression is the same for either TST (electronically adiabatic) or Marcus-like (electronically nonadiabatic) models:

$$KIE = \frac{\int_0^{\infty} P^{l}_{(DAD)}\, e^{-(E_{(DAD)}/(k_B T))}\, dDAD}{\int_0^{\infty} P^{h}_{(DAD)}\, e^{-(E_{(DAD)}/(k_B T)))}\, dDAD} \tag{4}$$

where $P^{l}_{(DAD)}$ and $P^{h}_{(DAD)}$ are the transfer probabilities for the light and heavy isotopes, respectively. Those extensions of TST and Marcus theory have been addressed by multiple names, such as "environmentally coupled tunneling",[10] "thermally activated tunnelling",[11] "Marcus-like models",[3,12] and others; however, no terminology is yet broadly accepted by the scientific community. It is important to note that in contrast to some misinterpretation in the literature, these models do not assume or require any nonstatistical dynamics (all states are presumed to be in thermal equilibrium, Boltzmann distribution), though those models can accommodate nonstatistical dynamics by also integrating the process over time. Importantly, when studying KIEs, as in all cases presented under Case Studies, we used eq 4, which could reflect either adiabatic or nonadiabatic cases.

In the framework of the models illustrated (eq 4 and Figure 1), the temperature-dependence of KIEs is a function of the temperature-dependence of the distribution of DADs. That is, temperature independent KIEs result from a very narrow distribution of DADs at the TRS that does not change significantly with temperature. Temperature dependent KIEs, on the other hand, result from a loose active site where the TRS can attain a wide range of DADs at thermal equilibrium, and the distribution of DADs is thus temperature sensitive.

While useful in preliminary analysis of experimental data, the phenomenological models discussed above do not account for the complexity of the energy landscape and its molecular basis. The pseudothermodynamic knowledge (represented by parameters like $\Delta G^{\ddagger}$, or ΔG° and λ, or even the DAD distribution concluded from eq 4) does not indicate how enzymes evolve to bind substrates in a certain order, undergo the associated conformational changes (known as induced-fit), change the pK_a's of many functional groups, or undergo conformational changes that stabilize the TS and effect barrier crossing. Only a combination of interactive studies between theoreticians and experimentalists can address both molecular and phenomenological levels of understanding. Applying physical understanding to enzymes (or any other complex system) is not trivial, because the calculations must be directly related to the experimental data. This may seem obvious, but, for example, many calculations examine only the *bond activation step* in the complex enzymatic cascade and then compare the findings to rates on quite complex rate constants like k_{cat}, which often represents the *product release*. In the interest of clarity, then, here we will focus only on calculations and experiments where we believe the same phenomenon has been examined by both calculations and experiments. The cases presented below examined the relations between enzyme dynamics and the chemical event of C−H bond cleavage.

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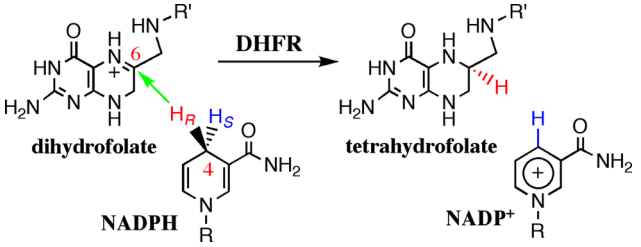
CASE STUDIES

Dihydrofolate Reductase (DHFR)

DHFR from *Escherichia coli* (ecDHFR) catalyzes the reaction depicted in Scheme 2. ecDHFR is one of the paradigmatic systems for examining the link between protein motions at various time scales and the catalyzed C−H bond activation. To explore the relations between the DAD distribution and the temperature dependence of intrinsic KIEs (KIE_{int}), a series of active-site mutants was constructed focusing on Ile14 (Figure 2), which holds the H-donor close to the H-acceptor. This residue was gradually reduced to valine, alanine, and glycine so that the DAD became progressively longer and more broadly distributed.[13] Examination of the H-transfer rates, the temperature dependence of KIE_{int}, and MD calculations revealed that lengthening the average DAD and broadening its distribution leads to a gradual increase in the temperature dependence of $KIEs_{int}$ (Figure 2, inset).

This elucidation of relations between the temperature-dependence of KIE_{int} and DAD distributions allows the examination of the role of enzyme dynamics across the protein on bond activation. Three examples studying DHFRs are as follows: (i) single and double mutants remote from the active site revealed a network of coupled motions, predicted by computer simulation to be associated with the C−H → C hydride transfer[14,15] and further confirmed by new calculations;[16] (ii) comparison of the natural enzyme to an isotopically labeled one confirmed that at physiological temperature, the fast enzyme vibrations are not electronically coupled to the bond activation, as predicted from QM/MM simulation[17] (although under 20 °C, however, such coupling seems to dominate); and (iii) studies of "humanized ecDHFR mutants" indicated that insertions not selected by evolution (i.e., N23PP, see Figure 2) disturbed the rigid and short DAD of the wild type enzyme, but insertions that occurred in evolution from *E. coli* to human DHFR (i.e., N23PP/G51PENK) preserved the dynamic pattern found in the WT.[18] This last finding emphasized the evolutionary pressure on the DAD distribution, despite the fact that the bond activation is far from being rate-limiting.

Scheme 2. C−H → C Hydride-Transfer from C4 of NADPH to C6 of Dihydrofolate Catalyzed by ecDHFR[a]



[a]R = adenine dinucleotide 2′ phosphate and R′ = (*p*-aminobenzoyl) glutamate. It was shown that for ecDHFR the protonation of the N5 position of dihydrofolate occurs prior to hydride transfer.[19−21]

Thymidylate Synthase (TSase)

TSase catalyzes the reductive methylation of 2′-deoxyuridine-5′-monophosphate (dUMP) to form 2′-deoxythymidine-5′-monophosphate (dTMP), using the cofactor N^5,N^{10}-methylene-5,6,7,8-tetrahydrofolate (CH_2H_4F) as both methylene and hydride donor (leading to formation of 7,8-dihydrofolate (H_2F), see Scheme 3). The findings most relevant to this Account include (i) the different DAD distributions and dynamics associated with the two C−H bonds activated (steps 4 and 6 in Scheme 2), (ii) the global effect of mutation on the protein functional dynamics, and (iii) the rigidifying effect of Mg^{2+} binding on activity.

Regarding finding (i), the C−H → C hydride transfer in step 6 is the rate-limiting step of the overall reaction, and no relevant uncatalyzed reaction has been observed, suggesting that this is not a trivial step to catalyze. The proton abstraction from C5 of dUMP (step 4), on the other hand, is very fast, and numerous uncatalyzed equivalent reactions are known. Interestingly, the temperature-dependence of KIE_{int} for these two steps revealed that step 6 (Scheme 3) requires a well-defined and narrowly distributed ensemble of DADs (Figure 3, blue),[25] while step 4 has a longer and much broader DAD distribution (Figure 3, red).[26] The rationale could be that for the more difficult reaction the enzyme had to evolve a very accurate DAD ensemble and well-defined TRS, while the second, easier reaction is fast enough even without careful orientation of the donor and acceptor.

Regarding finding (ii), mutations at the dUMP binding site of ecTSase, residue Y209, result in no observed structural effect, even at 1.3 Å resolution, but the reactions catalyzed by those mutants were much slower than the WT.[27] The only effect observed was that the anisotropic B-factors were all in the same direction for most loops in the WT (suggesting a rigid-body motion at the fast time scale) but had very different

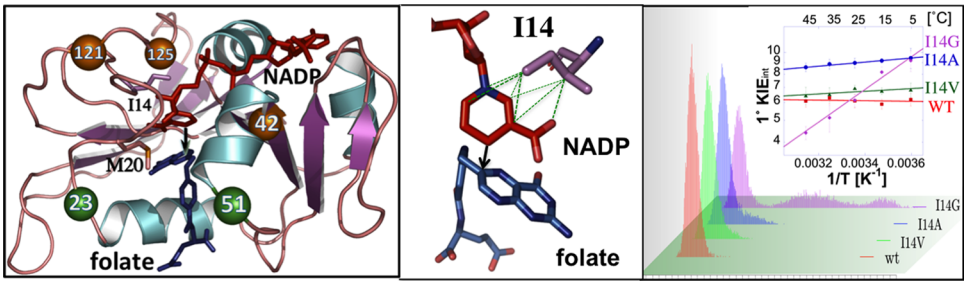


Figure 2. (left) Structure of WT-DHFR (PDB code 1RX2), with folate in blue and NADP in red. Residues that participate in the dynamic network are orange spheres; the sites of insertion in higher organisms are green spheres (α-carbons). M20 and I14, discussed in the text, are sticks. An arrow marks the hydride's path from C4 of the nicotinamide to C6 of the folate. (middle) The position of I14 relative to the reactants. (right) The DADs' distribution from MD calculations; inset, an Arrhenius plot of intrinsic H/T KIEs (on a log scale) for WT ecDHFR (red), I14V (green), I14A (blue), and I14G (purple).[13] The lines represent the nonlinear regression to eq 4.[5] Adapted with permission from ref 13. Copyright 2012 American Chemical Society.

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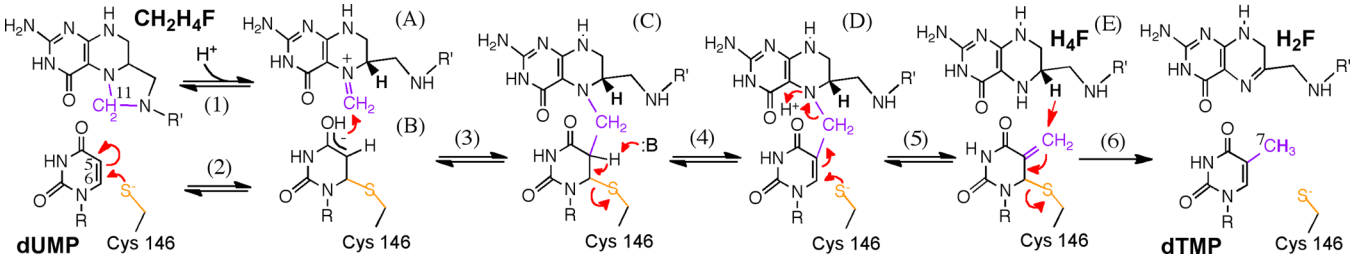
Scheme 3. Reductive Methylation Catalyzed by TSase[a]



[a]The transferred methylene group is purple, and the nucleophilic cysteine is yellow. Recent QM/MM calculations suggested a new intermediate (D),[22] and both calculations[23] and experiments[24] indicated that step 5 is concerted (in contrast to the traditional two-step product formation). R = 2′-deoxyribose-5′-phosphate; R′ = *p*-aminobenzoyl-glutamate.

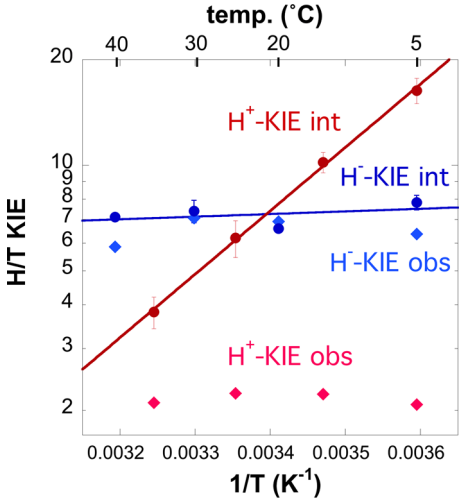


Figure 3. Arrhenius plots of observed H/T KIEs (KIE_{obs}, diamonds) and intrinsic H/T KIEs (KIE_{int}, circles) for the proton abstraction (step 4)[26] and the hydride transfer (step 6)[25] in the ecTSase reaction. The lines are the fit to eq 4. The small difference between KIE_{obs} and KIE_{int} for the hydride transfer (blue) indicate small kinetic complexity (i.e., hydride transfer is mostly rate-limiting), while the large difference between them for the proton transfer (red) indicates that it is far from being rate-limiting.[26]

distributions in the mutant (Figure 4).[28] This observation suggests that the dynamic alteration of the protein reduces the hydride transfer rate. Although the mutation is ca. 10 Å from the hydride donor or acceptor, this dynamically altered mutant results in larger and more temperature dependent KIEs, which, together with other observations, indicates dynamic coupling of motions across the protein affecting this C−H bond activation.

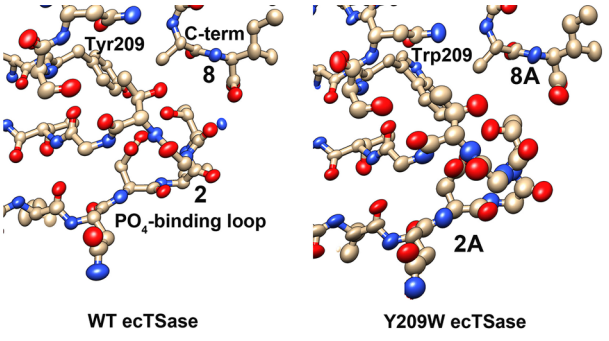


Figure 4. Plot of the thermal ellipsoids of anisotropic B-factors for WT (left) and Y209W ecTSase (right). Reproduced with permission from ref 28. Copyright 2012 American Chemical Society.

Regarding finding (iii), in ecTSase, Mg^{2+} accelerates hydride transfer by an order of magnitude. While the metal ion's binding site appears to be far from the active site, NMR relaxation measurements indicated Mg-induced rigidity throughout the protein.[29] Interestingly, while the hydride transfer was much faster, the DAD distribution was not affected (retaining temperature-independent KIE_{int}). This indicates that Mg^{2+} increases the probability of TRS formation (terms before the integral in eq 2), but once formed, the TRS is unaffected.

Formate Dehydrogenase (FDH)

It is most challenging to assess both the fast fluctuations of the DAD at the TRS and the relevant environmental motions at that time scale (femtosecond to picosecond). A unique opportunity is presented in FDH, which catalyzes a single hydride transfer from formate to NAD^+ (Figure 5). Azide serves as both a TS-analogue and an excellent IR probe. Two-dimensional IR vibrational spectroscopy was used to measure the vibrational relaxation of the enzyme-bound azide to its environment. The quantitative analysis has been presented in ref 30, but here it is sufficient to demonstrate that the center-line slope (CLS, blue line in Figure 5), is quickly reduced (after 5 ps it was close to zero), indicating a very fast relaxation. Such fast relaxation indicates very restricted fluctuations at the femtosecond to picosecond time scale for the ternary complex (FDH−azide−NAD^+), which mimics the TS complex (Figure 5, bottom). The same experiments found broad fluctuations for the binary complex (FDH−azide mimics inactive states with no NAD^+), suggesting that the restricted dynamics are a feature of the TS. Fast relaxation of the TS-mimic suggests a vibrationally rigid TS, which accords well with the temperature-independent KIE_{int} that indicates a narrow DAD distribution, which means fast femtosecond to picosecond fluctuations of the DAD at the TRS.[30]

CONCLUDING REMARKS

The examples presented above are from our laboratory; many other groups have made important contributions using similar analyses of KIEs and their temperature dependence.[32−35] Different researchers working in this area use different theoretical models and experimental probes, and sometimes heated debate has developed over meaning of results. We wish to address two sources of apparent conflicts, experimental and then theoretical.

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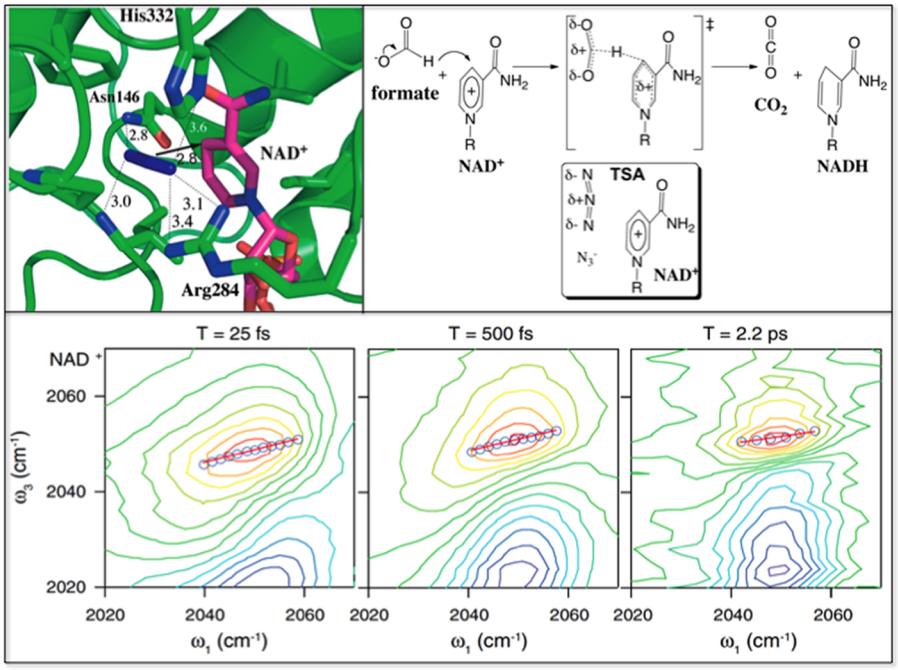

Figure 5. (top left) Active-site structure of FDH (PDB 2NAD), with azide in blue and the NAD^+ in magenta. The arrow indicates the reaction path from the H-donor to acceptor, and the dashed lines represent the hydrogen bonds discussed in the text (distances in Å). (top right) Reaction catalyzed by FDH, with an illustration of the reaction's TS. Below it, the stable complex with azide as TS analogue, bent as observed in the crystal structure.[31] (bottom) Two-dimensional IR spectra of the azide antisymmetric stretch for azide bound in the ternary complexes with NAD^+, for waiting times of T = 25 fs, 500 fs, and 2.2 ps presented from left to right, respectively. The blue circles represent the center lines of the CLS analysis,[30] and the red lines are the linear fits to the center lines. Adapted with permission from ref 30. Copyright 2010 National Academy of Sciences.

Experimental Sources

All the studies of temperature dependence of KIE referenced above used some form of eq 4 or an equivalent model to interpret their data. Furthermore, many of them have made an effort to expose the intrinsic KIEs to a certain extent, such as multiple-KIEs or using pre-steady-state kinetics. This is not a trivial task: in some systems, like DHFR, the rates and KIEs measured via pre-steady-state methods involve at least nine kinetic steps with different temperature and pH dependencies (H_2F binding, consequential protein and solvent rearrangements, protonation of H_2F, the hydride transfer step forward, and the reverse steps of the first three, which mask the forward KIEs on the hydride transfer). This complexity at times impedes communication between groups applying different kinetic methods. Both calculations[19,20] and different triple labeling methods[36,37] assess the intrinsic H/D KIEs to be 3.5 ± 0.1, temperature independent (5−45 °C), and almost pH independent.[18] Pre-steady-state methods, on the other hand, sometimes address the measured rates as "the hydride transfer rates", but report KIEs of <3.0 that are both temperature and pH dependent, strongly indicating that steps other than the hydride transfer affect the observed value. A clear example is ref 34 vs ref 18 with regard to the N23PP mutant of ecDHFR, where the second reproduced the findings of the first and also used a very similar analysis and interpretation of the temperature dependence of KIEs. However, the second also separated intrinsic from observed KIEs, and the conclusion regarding the bond activation step was quite different.

Theoretical Sources

Some disputes over theoretical models and procedures appear to be even larger than the snarls in experimental terminology (above), but we believe that a closer look may shine a different light on some of those too. For the following discussion, it is helpful to revisit the difference between statistical and nonstatistical motions: in statistical motions, at the time scale under study all vibrational modes are in thermal equilibrium (a Boltzmann distribution of populations). In nonstatistical motions, some modes are "hotter" than others; that is, their excess energy dissipates more slowly than it is used to catalyze the reaction of interest. In the context of enzyme catalysis, nonstatistical contribution to catalysis would mean acceleration of rates by motions along the enzyme-catalyzed reaction coordinate that do not equilibrate with their environment (at the time scale of the barrier crossing), while in solution they would.

In a notable instance, controversy has erupted over experimentalists' use of the term "dynamics", which has been met with rejection by theoreticians who assumed "dynamics" meant nonstatistical motions, even though the experimentalists using the term "dynamics" obviously meant thermally equilibrated dynamics (as is evident from their use of eqs 3 and 4). Both refs 38 and 39, for example, assume statistical dynamics, but due to different terminologies and because they are focused on different aspects of catalysis, many statements by these researchers appear to contradict one another. One researcher suggests that dynamics contribute to enzyme-catalyzed reaction, while the other claims that nonstatistical dynamics are not significantly different in solution versus enzyme, if they contribute to the rate at all. The first researcher closely examines effects of critical importance to biological systems, like several fold rate enhancement, and the fine-tuning of the system to reach its exquisite specificity and control. The second researcher, on the other hand, mostly focuses on the

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many orders of magnitude difference between catalyzed and uncatalyzed reactions. The second researcher does not commonly pay much attention to ±1 kcal/mol effects on barrier height, which could mean life or death from the perspective of the first one, who does not study uncatalyzed reactions at all. Ultimately, it appears that both researchers actually see the nature of enzyme-catalyzed reactions in a very similar way but, due to different focus and terminology, seem to be in total disagreement if one only reads their titles and statements.

An interesting question is whether DAD fluctuations at the TRS are statistical or not. Most DAD fluctuations calculated by either fitting to phenomenological models[7] or simulation[40,41] are in the 50−200 cm^{-1} range, that is, a time scale of 650−160 fs. While one simulation with ecDHFR has suggested that nonstatistical events decay in less than 200 fs,[42] in most condensed-phase systems, vibrational relaxation takes several picoseconds.[43] It is thus questionable whether the whole system is at thermal equilibrium while the DAD is being sampled. One approach is that the rare event of bond activation is too fast for its environment (solvent and active site) to be at equilibrium during the actual barrier crossing. Reactions coordinates found by transition-path sampling (TPS) yield a statistical collection of nonstatistical trajectories.[44,45] Calculating each trajectory assumes that all environmental motions are much faster than the barrier-crossing event, and includes many enzymatic fast vibrations that are not in statistical/thermal equilibrium during the lifespan of the TS (∼10 fs). Some of these modes are at the same phase and frequency as the barrier-crossing event, and some can even be coupled to it. One method that championed such an approach named those modes "protein promoting vibrations" (PPV).[44]

Interestingly, despite contradictory *ab initio* assumptions, TPS,[44,45] umbrella sampling considering both solvent and solute coordinates,[46] and other theoretical approaches seem to be able to reproduce various experimental findings. A possible rationale for this is that all experiments run on time scales much longer than that of each TPS trajectory and are performed on a large ensemble of molecules. Although the TPS approach involves nonstatistical dynamics per trajectory, sampling the system over a long enough time (e.g., greater than nanoseconds)—or equivalently, sampling Avogadro's number of parallel events at a time slot—always yields a statistical outcome. The rare nonstatistical events at the picosecond to femtosecond time scale have a statistical probability of occurrence at the microsecond to millisecond time scale, and the distribution of those rare barrier-crossing events follows a statistical probability when sampling Avogadro's number of events. This said, the possibility that enzymes evolved to use nonstatistical events such as PPV to catalyze the bond activation event is of great interest from both intellectual and practical point of views, since it may dramatically affect rational biomimetic catalyst design.

Can the statistical and nonstatistical approaches be critically compared? A resolution requires that only one of these approaches will be able to explain an experimental observation, while the other approach cannot. At this time, we are not aware of any experimental data that could directly distinguish between the two models in question. An approach that could distinguish between those themes would be to study a single enzyme molecule using single-turnover kinetics initiated by exciting a single vibrational mode with a very short pulse and followed by time-resolved ultrafast vibrational spectroscopy. Unfortunately, such an experiment is well beyond current technology. A somewhat indirect but readily accessible approach is isotopically labeling the protein (^{13}C, ^{15}N, and ^{2}H for most nonexchanging positions), creating a "Born−Oppenheimer enzyme", which slows the fast vibrations in question with minimal alteration of the electronic potential surface.[47] One can assume that the transformation to a heavy enzyme would affect the barrier crossing only if PPV are coupled to the barrier crossing for the natural enzyme but not the heavy one. Unfortunately, the C−^{2}H bond is a bit shorter than the natural C−^{1}H bond and has a reduced electronic dipole relative to the natural bond. Therefore, in addition to the vibrational effect, the system's electrostatics are also altered, making it hard to clearly separate the effects. Several studies are underway in attempt to resolve those effects and test the different contributions of the "heavy enzyme" to alteration of different kinetic events.

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Notes

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