Dynamically Achieved Active Site Precision in Enzyme Catalysis

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CONSPECTUS: The grand challenge in enzymology is to define and understand all of the parameters that contribute to enzymes' enormous rate accelerations. The property of hydrogen tunneling in enzyme reactions has moved the focus of research away from an exclusive focus on transition state stabilization toward the importance of the motions of the heavy atoms of the protein, a role for reduced barrier width in catalysis, and the sampling of a protein conformational landscape to achieve a family of protein substates that optimize enzyme−substrate interactions and beyond.

This Account focuses on a thermophilic alcohol dehydrogenase for which the chemical step of hydride transfer is rate determining across a wide range of experimental conditions. The properties of the chemical coordinate have been probed using kinetic isotope effects, indicating a transition in behavior below 30 °C that distinguishes nonoptimal from optimal C−H activation. Further, the introduction of single site mutants has the impact of either enhancing or eliminating the temperature dependent transition in catalysis. Biophysical probes, which include time dependent hydrogen/deuterium exchange and fluorescent lifetimes and Stokes shifts, have also been pursued. These studies allow the correlation of spatially resolved transitions in protein motions with catalysis. It is now possible to define a long-range network of protein motions in ht-ADH that extends from a dimer interface to the substrate binding domain across to the cofactor binding domain, over a distance of ca. 30 Å. The ongoing challenge to obtaining spatial and temporal resolution of catalysis-linked protein motions is discussed.
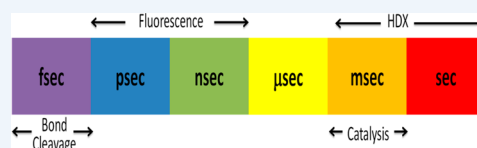
INTRODUCTION

Protein motions are increasingly discussed as a "missing link" in our search for a generalized theory of the rate accelerations achieved by enzymes. While changes in protein conformation, generally between two dominant forms, are universally recognized as contributing to enzyme function, the motions discussed within this Account are more subtle and not readily detected via X-ray crystallography. An emergent concept, addressed herein and in other Accounts in this special issue, is the presence of a manifold of protein structures that are achieved only transiently and differ from the dominant ground state protein structure. Linking such "alternate" structures to catalysis has emerged at the cutting edge of our efforts to understand the physical bases for enzyme function.

THE MAJOR WORKING PREMISE

Since the 1960s, an enormous array of X-ray structures has been available to guide us in our interpretation of enzyme function. These beautiful structures generate a biological art gallery, revealing the identity and positions of protein residues within the active site. Perhaps the most significant feature to emerge from such structures is the very large number of functional groups that are assembled near the substrate(s) binding site(s), with a capacity to participate in acid/base, H-bonding, dipolar, and hydrophobic interactions, etc. This property, which distinguishes an enzyme reaction from the comparable reaction of a small molecule in solution, has been framed in the context of the entropic barrier to catalysis.[1] For a

solution reaction, the entropic barrier to arranging such a large number of functional groups in proximity to the substrate would be insurmountable; by contrast, the enzyme has resolved such a dilemma via covalent linkage of the functional groups at the stage of protein synthesis.[2]

With this property in mind, we can ask the question: Why has it been so difficult, if not impossible, to synthesize models of enzyme active sites that can reproduce the enormous rate accelerations of enzyme? Using site specific mutagenesis and our ability to alter single amino acids anywhere in the protein, it has become clear that residues far from the active site can also play important roles in enzyme function. During the past several years, research in the areas of protein design and enzyme evolution has confirmed the need to introduce multiple modifications throughout an entire protein to bring about high rates of turnover.[3,4] While the role for remote residues in catalytic efficiency begins to offer an explanation for why the overall size of enzymes greatly exceeds the active site itself, the next level of questioning is whether remote effects can be explained largely by long-range, static electrostatic interactions propagated through the protein interior to the active site.[5]

An alternate premise is that the intrinsic link between a large protein structure and its catalytic efficiency lies in the flexibility of proteins that tunes catalysis at each stage along the reaction trajectory. Flexibility is a clear-cut advantage in the binding of

Scheme 1. A Physical Picture That Can Describe Changing Patterns for the Temperature Dependence of KIEs in Enzyme Reactions under a Variety of Reaction Conditions[7][a]
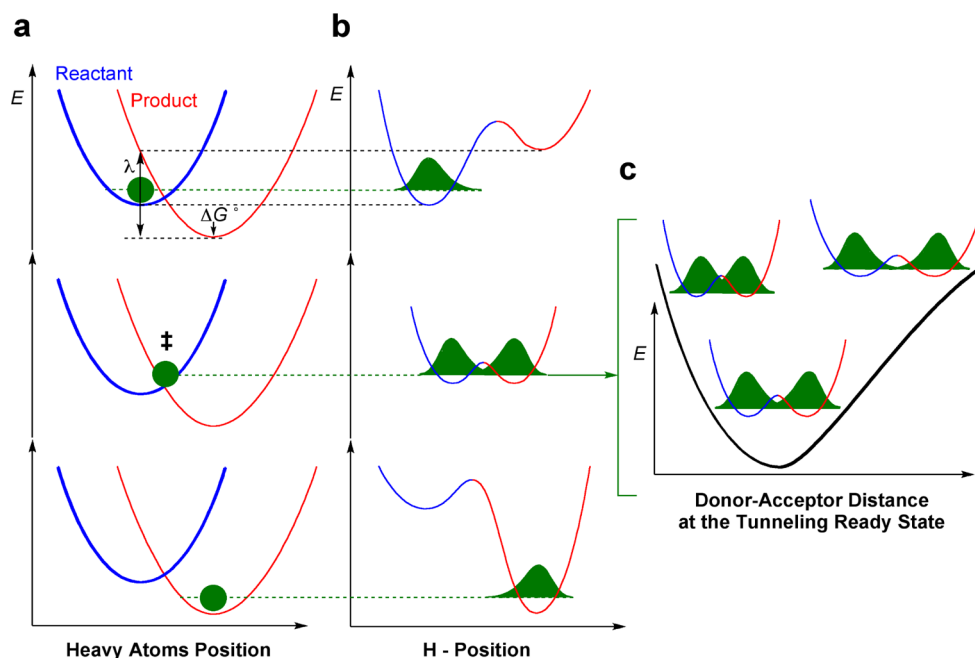
[a]The reaction is controlled by three coordinates that contain (a) the temperature dependent heavy atom environmental reorganization that transiently achieves energetic degeneracy between reactant and product, (b) the mass, frequency, and distance dependence of hydrogenic wave function overlap between the donor and acceptor wells, and (c) the mass and temperature dependence for the sampling of a range of donor–acceptor distances. This last feature is the source of temperature dependent KIEs. When the donor–acceptor distance is optimal, there is little requirement for coordinate c, and KIEs become temperature independent.

substrate and release of product, via the ability to move between open and closed protein forms that both provides access to substrate/product and facilitates loop closures over the active site once the substrate is in place (e.g, ref 6) The role of flexibility in tuning the chemical step(s) once the enzyme–substrate complex is formed is more subtle yet appears equally compelling (e.g., ref 7). It is now increasingly accepted that a dynamical sampling of enzyme–substrate complexes is an important component of catalysis, *enabling a search for the subset of configurational states in which the plethora of catalytically relevant ground state protein–substrate interactions can be simultaneously and transiently optimized.* This sampling performs the dual roles of fine-tuning ground state electrostatic potentials and reducing internuclear distances, converting modest protein catalysts into molecules capable of rate enhancements in the reported upper ranges of 10^{26}-fold.[8]

A family of prokaryotic alcohol dehydrogenases, comprised of three related variants from thermophilic, mesophilic, and psychrophilic sources, has emerged as a paradigmatic system for understanding the role of protein conformational sampling in enzymes.[9] In particular, the thermophilic enzyme (designated ht-ADH) has turned out to be a remarkable model for understanding how long-range protein motions can affect catalysis. Herein, recent experimental advances are summarized that link protein flexibility to the efficiency of substrate oxidation in a thermophilic alcohol dehydrogenase (ht-ADH). The principal findings are a dynamic and long-range network of protein connectivity that impacts catalysis in a temperature dependent manner that can be either enhanced or eliminated via alteration of single protein side chains.

■ THE PROPERTIES OF THE CHEMICAL COORDINATE CATALYZED BY HT-ADH

There are kinetic features of ht-ADH that make it an ideal system in which to study the detailed properties of substrate oxidation; these include random binding of the NAD$^+$ cofactor and benzyl alcohol substrate to enzyme and a rate limiting hydride transfer step to form NADH and benzaldehyde under a wide range of experimental conditions: The initial surprising property of ht-ADT was the appearance of a cooperative break in kinetic behavior for both k_{cat} and k_{cat}/K_m at 30 °C (see Figure 2B,C).[10] While such a break could have indicated a change in rate-determining step, this appears not to be the case. Labeling of substrate with isotopes of hydrogen at the primary reactive position of the alcohol substrate shows a pattern for wild-type (WT) enzyme in which a primary kinetic isotope effect (KIE) of ca. 3 is present and invariant above 30 °C (the physiologically relevant temperature range), becoming larger and more temperature dependent below 30 °C. This pattern of temperature independent KIEs under conditions of optimal enzyme catalysis that segues into temperature dependent KIEs when the system is perturbed has become a dominant one for enzymes that catalyze C–H cleavage, that is, encompassing proton and hydrogen atom transfer as well as the classic hydride transfer mechanism (see ref 7).

A second and equally compelling property of the ht-ADH concerns the rule of the geometric mean, which states that the magnitude of the KIE arising from labeling of the non-transferred position of substrate (representing a secondary KIE) will be independent of the isotope transferred at the primary position.[11] The breakdown from this rule of the geometric mean in the WT ht-ADH reaction is seen to be
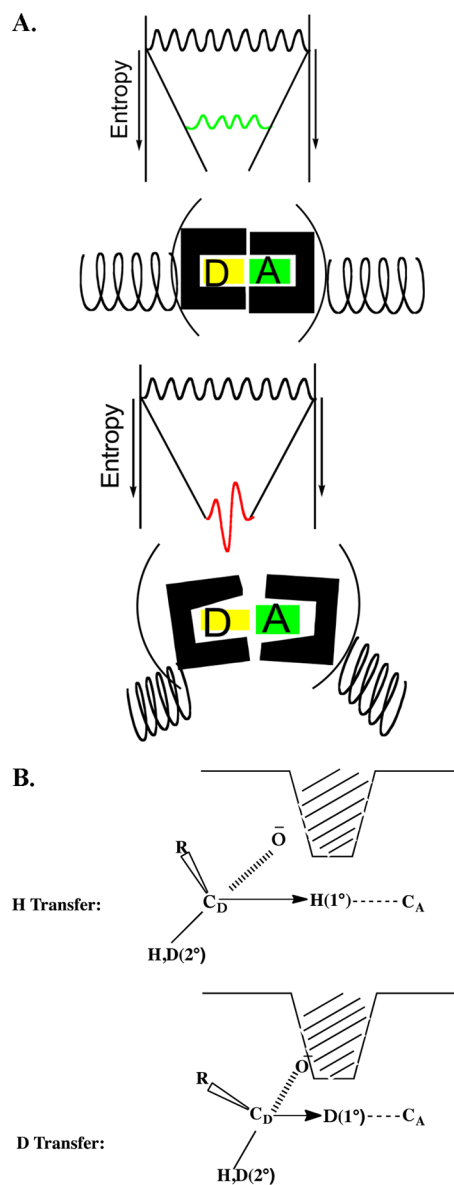
extreme above 30 °C and to undergo a progressive decrease toward the semiclassical limit as the temperature approaches 273 K.[10] The behavior of the secondary KIE, together with the above-mentioned trends in the temperature dependence of the primary KIE, was initially interpreted as resulting from changes in the contributions of tunneling (i.e., the size of a tunneling correction) to the reaction coordinate. With the emergence of a large body of experimental data implicating a role for tunneling in a range of different enzyme systems, together with models that can rationalize this behavior,[7,12] the trends in the primary and secondary KIEs of ht-ADH are now conceptualized in the context of active site geometries that facilitate wave function overlap. A multidimensional physical picture, frequently adopted to explain the properties of C–H activation in enzymes[13,14] is reproduced in Scheme 1. A full mathematical description of this model is available for reactions that fall into the regime of vibronically[15] and electronically[16] nonadiabatic, while alternate analytical solutions for the adiabatic counterparts that encompass hydride transfer reactions will require further development. Perhaps most importantly from a historical perspective, these models deviate from an exclusive focus on isotopic differences in ground state vibrational frequencies as the origin of KIEs. The movement of hydrogen is effected via wave function overlap from donor to acceptor (Scheme 1b), with the magnitude of the primary KIE representing the degree of reaction adiabaticity, the distance between the hydrogen donor and acceptor, and the impact of the mass of the transferred particle on wave function overlap.

The model that has emerged for ht-ADH is as follows: Under the physiological regime of elevated temperature (>30 °C), ht-ADH is concluded to access highly compacted ground state structures that achieve a close approach between the hydrogen donor and acceptor atoms, Scheme 2A, top. These structures arise from transient motions that involve heavy atoms of the protein (represented by springs) and are most generally independent of the isotopic labeling of substrate, producing activation energies that are weakly dependent or independent of the isotopic label at the transferred position. The close approach between donor and acceptor greatly reduces the contribution of a second temperature and isotope dependent donor–acceptor distance sampling mode (Scheme 1c) that frequently becomes necessary when an enzyme has been perturbed from its optimal configuration via mutagenesis, heavy atom labeling of protein, use of alternate substrates, or the introduction of nonphysiological reaction conditions,[7] Scheme 2A, bottom. The 2-fold greater mass of deuterium, relative to protium, is accompanied by a decrease in wavelength and leads to the additional requirement of an even shorter internuclear distance to achieve efficient wave function overlap. The transfer of deuterium can, thus, occur from a distinctive and even more crowded active site than protium, restricting the degree of rehybridization at the reactive, hydride donating carbon center and reducing the magnitude of secondary KIEs,[12] Scheme 2B. This behavior of *isotopically dependent transition state structures* was introduced quite early by Truhlar and co-workers in the context of variational transition-state theory.[17]

■ LINKING THE CHEMICAL COORDINATE TO PROTEIN DYNAMICAL EFFECTS IN HT-ADH

The ability to interrogate a thermophilic enzyme across a temperature range of abruptly changing kinetic properties offers a unique opportunity to correlate motions within the protein coordinate to the properties of the chemical coordinate. With

Scheme 2. Interplay between Protein Motions and High Catalytic Rate Constants[a]

[a](A) Relationship between the protein conformational landscape and the transient creation of the hydrogen donor–acceptor distances (adapted from ref 32). (top) Native flexibility within an optimized catalyst (represented by the springs) leads to a subset of conformers (green) that bring the donor and acceptor into close proximity. (bottom) Under perturbed conditions, the protein experiences a distinctly different conformational landscape (represented by red) that precludes the close approach of donor and acceptor within the active site. The latter can be compensated by a donor–acceptor distance sampling mode, leading to highly temperature dependent KIEs. (B) The smaller wavelength for D tunneling requires a closer donor–acceptor distance. This can lead to steric clash that prevents or limits rehybridization at the reacting bond, producing primary isotope effects on secondary KIEs.

attention focused on the protein coordinate, some of the major questions that can be addressed are (i) the time scales and positions of these protein motions, (ii) the appropriate biophysical methods available for their detection, and (iii) the ability (or not) to correlate detected protein motions with the efficiency of the H-transfer. For hydrogen tunneling reactions,

the wave function overlap of the reacting hydrogen is expected to be very rapid, essentially instantaneous relative to heavy atom motions (see ref 18). In this context, the proteins' motions will all occur more slowly and as such can be represented by Boltzmann distributions that bracket the time range from milliseconds (the observed time scale for enzyme function) to femtoseconds (the minimal time scale for the tunneling event).[7] In the case of ht-ADH, two types of biophysical measurements have been conducted thus far to examine the protein dynamics: the time course for hydrogen–deuterium exchange into the protein backbone amides (HDX)[19] and time-resolved fluorescent measurements that include fluorescent lifetime and Stokes shift measurements.[20,21]

HDX Measurements

The HDX measurements of ht-ADH were carried out under the EX-2 condition, involving a reversible opening and closing of local regions of protein that allow temporary access of the protein backbone to solvent (D_2O/OD^-) and subsequent exchange of protium by deuterium.[22] The rate expression for HDX under EX-2 conditions is the product of $K_{(open)}$, the equilibrium constant for the transient local unfolding of protein, and $k_{(exch)'}$, the unhindered rate constant for exchange once the peptide bond is exposed to solvent, Scheme 3. Under the EX-2 condition, the observed rate of deuterium exchange can be quite slow, a consequence of small values for $K_{(open)}$. The magnitude of the apparent rate constant for chemical exchange, $k_{(exch)'}$, depends to some extent on the environment of the specific amide bond undergoing exchange and primarily on reaction pH, with pseudo-first-order constants of ca. 1–10
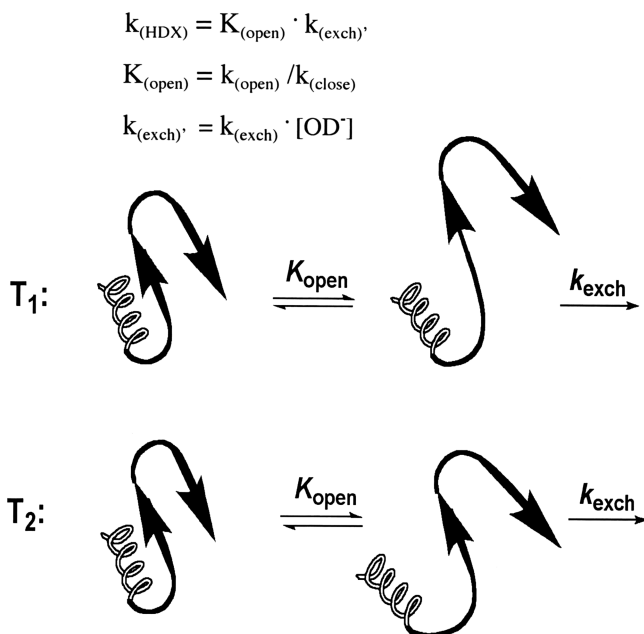
Scheme 3. Illustration of HDX under EX-2 Conditions at Two Different Temperatures $(T_2 > T_1)^a$

$$k_{(HDX)} = K_{(open)} \cdot k_{(exch)'}$$

$$K_{(open)} = k_{(open)} / k_{(close)}$$

$$k_{(exch)'} = k_{(exch)} \cdot [OD^-]$$

^{a}The rate is controlled by the chemical exchange rate, together with the equilibrium constant for achieving a partially unfolded region of protein that permits access of the deuterated solvent to the peptide amide linkages. In fact, as the temperature is elevated, three changes may occur: a rise in $k_{(exch)}$, $K_{(open)}$, and the extent to which the protein becomes more solvent exposed. The latter is illustrated here by the increased distance between the α-helix and its neighboring B-sheet at T_2.

s^{-1} at pH 7. At this pH, $k_{(exch)'}$ is generally slower than the rate of protein closure, with $k_{(close)}$ in the range of $10–10^3$ s^{-1}. Upon elevation of the pH, the increase in $k_{(exch)'}$ relative to $k_{(close)}$ alters the HDX mechanism, allowing access to the EX-1 regime where the rate constant for local protein unfolding limits deuterium incorporation; this condition allows estimates of $k_{(open)}$ as ca. 10 s^{-1} (see ref 22).

The analysis of deuterium incorporation into ht-ADH was carried out at pH 7 as a function of temperature, followed by proteolysis to yield a family of 21 peptides (ca. 15 amino acids on average in length, covering >90% of the protein sequence) and subsequent mass spectrometric analysis for time dependent changes in peptide mass. Proteolysis, carried out under reduced pH, minimizes back exchange of the incorporated deuterium into unlabeled water.[23] As noted above, the measured rate of HDX is dependent on K_{open}, reflecting the ability of protein to sample transiently achieved conformational states that are accessible to OD^-. This is analogous to the conformational sampling model for enzyme catalysis, in which the rate of catalysis depends on the probability of achieving a subset of protein conformers that can promote high rate accelerations. Measurements of HDX are generally on much longer times scales, often carried out to hours. Because of these extended time scales, there is sufficient time for protein to sample a very large family of conformational substates across a range of time scales longer than catalysis; it is to be expected that many of these protein breathing modes will also encompass the subset of protein conformational states affecting catalysis.
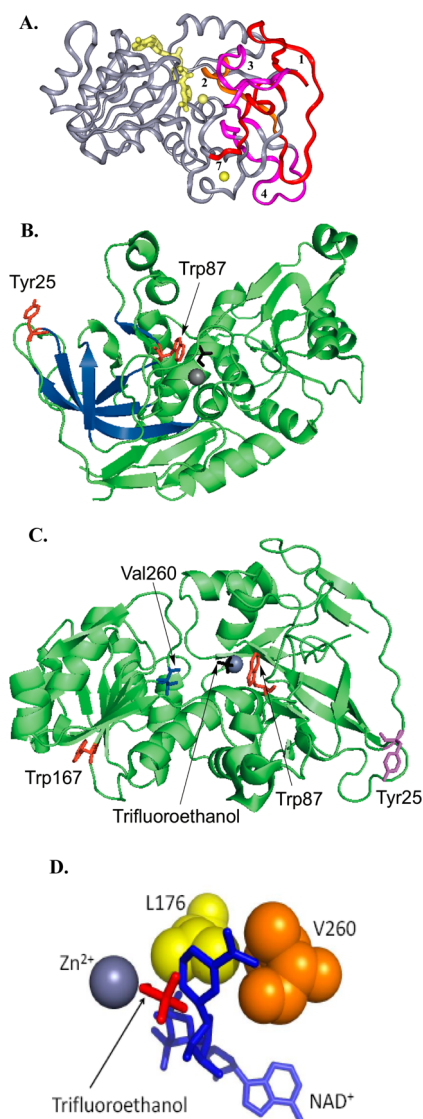
As anticipated, all of the peptides showed a rise in the rate of HDX with increasing temperature, reflecting the anticipated increase in both $K_{(open)}$ and $k_{(exch)}$. Additionally, the total number of amide bonds accessible to solvent at long times can increase, attributed to a temperature dependent fraying or unzipping of local regions of protein, see Scheme 3. A surprising and exciting result was the detection of a family of five peptides that display a break in the temperature dependence of HDX that mirrors the temperature break in catalysis.[19] The observed increase in protein flexibility above 30 °C is localized within a family of β strands that extends from the substrate binding site near the active site Zn^{2+} out to the protein surface, Figure 1A. This pattern of a local and spatially defined increase in ht-ADH flexibility above 30 °C points to a network of protein motions that are capable of generating, in a transient process, the close/compacted donor–acceptor distances that are a prerequisite for efficient H-transfer catalysis (Scheme 2A, top).

Further insight into a mechanistically relevant region of protein flexibility in ht-ADH has come from a study of a highly homologous psychrophilic ADH (ps-ADH) with 60% sequence identity to ht-ADH. The ps-ADH functions optimally below 30 °C.[24] A comparison of primary and tertiary structures reveals a single π-stacked tyrosine at position 25 of a dimer–dimer interface in ht-ADH that is replaced by alanine in ps-ADH. The subsequent interchange of tyrosine by alanine in ht-ADH produced a complete loss of the 30 °C break in k_{cat} and temperature independent primary KIEs across the full temperature regime,[25] Figure 2A. In the converse experiment, the replacement of alanine by tyrosine in ps-ADH introduced greatly enhanced thermostability together with a kinetic break and impaired catalytic behavior below 20 °C. The identification of a residue at position 25 that controls active site flexibility in a temperature dependent manner is a fortuitous turn of events.[25] From Figure 1B, *Tyr25 in ht-ADH can be seen to reside at the*
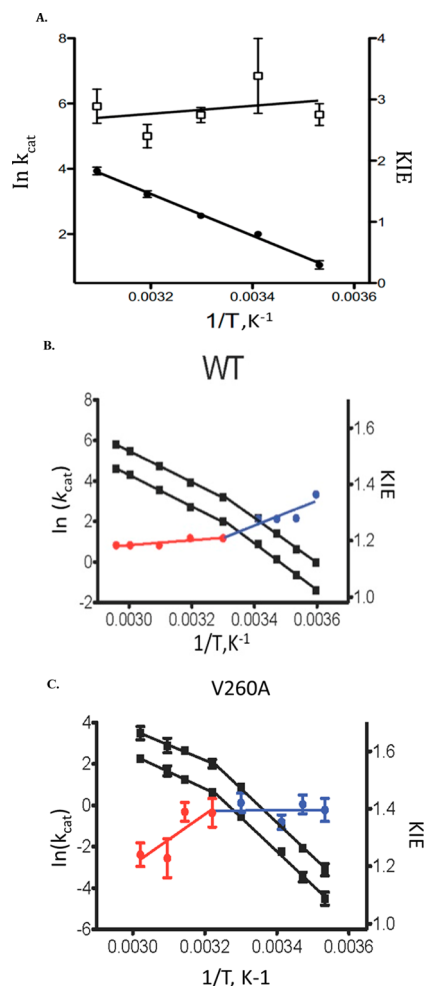
Figure 1. Inter-relationship of side chains in ht-ADH. Zinc ions are shown as spheres. (A) The two zinc ions are yellow, with the active site zinc residing near NAD$^+$, which has been modeled into the site (also yellow). The five peptides that increase in flexibility above 30 °C are colored and labeled 1−4 and 7.[19] (B) The relationship between Tyr25 (red) at the surface of ht-ADH and the active site Trp87 (red) that resides behind the substrate analog (black). The peptides that increase in flexibility above 30 °C are navy blue.[21] (C) The monomeric structure of ht-ADH highlighting key residues in the cofactor binding domain, Val260 (blue), and substrate domain, Trp187 (red).[21] (D) The relationship of Val260 to the nicotinamide of bound cofactor (dark blue).[21]

Figure 2. Temperature dependence of rates and KIEs in ht-ADH (A) The behavior of Y25A, illustrating the temperature independence of the rate (●) and KIE (□).[25] (B The behavior of WT, where the temperature dependence of the KIE (●) increases at low temperature.[27] The transition in behavior occurs at the intersection of the red and blue lines. The individual k_{cat} lines for protio and deuterio substrates are presented by ■. (C) The behavior of V260A showing the opposite behavior to WT: the temperature dependence of the KIE (red and blue) is greater than WT at elevated temperature and less that WT at reduced temperatures.[27] Once again, the k_{cat} lines for protio and deuterio substrates are represented by ■.

outer edge of the β sheet that undergoes a change in flexibility at 30°C (Figure 1A). Below 30 °C, it is likely that interactions at the dimer−dimer interface of WT ht-ADH dampen down protein motions within the substrate binding domain that must be activated for optimal chemical catalysis. Above this temperature, rearrangements involving the Tyr−Tyr π-stack are proposed to initiate a cooperative unlocking of dynamical flexibility that extends >20 Å to the active site Zn^{2+}.[25]

Time Resolved Fluorescence Measurements

In contrast to the relatively slow and thermodynamically averaged properties of protein flexibility detected using HDX, time dependent fluorescent methods introduce the potential to measure protein motions on a catalytically relevant scale within an enzyme active site. For example, nanosecond and pico-second relaxation rates, which can be accessed via time dependent fluorescence lifetime and Stokes shift measurements, are in the range estimated for the isotopically sensitive donor−acceptor distance sampling modes in H-transfer enzymes (represented by coordinate c in Scheme 1). With time dependent fluorescence measurements in mind, single tryptophan constructs of ht-ADH were created that contain a single tryptophan either at the substrate binding pocket (Trp87) or at a solvent exposed position 25 Å away (Trp167),[20] Figure 1C. Both single tryptophan constructs retain the break in catalytic behavior at 30 °C, making them suitable models for the native ht-ADH. Although donor−acceptor distance sampling is not anticipated to play a major role for native ht-ADH above 30 °C, where the primary KIEs are temperature independent, a fast donor−acceptor distance sampling mode is expected to contribute to the reaction

coordinate at low temperatures where inactive regions of the conformational landscape accumulate and the KIEs become temperature dependent. Contrary to these expectations, only a single transient (τ_1) in the fluorescence lifetime experiments shows a temperature dependent break that correlates with catalysis; this has been attributed to a subtle shift in the position of Trp87 in relation to the active site Zn^{2+} above 30 °C.[20] A number of explanations can be offered for the lack of a more extensive correlation between the fluorescence behavior of native ht-ADH and catalysis. These include (1) a time scale for the protein motions that control conformational sampling that is significantly slower (microsecond or slower) than the rapid motions being interrogated by fluorescence or (2) a lack of correspondence between the motions controlling the single tryptophan fluorescence relaxation properties and those that control the donor−acceptor distance sampling mode along the chemical coordinate.

We also considered the possibility that further perturbation of ht-ADH might be necessary to detect an anticipated impact of temperature on fluorescence relaxation behavior that parallels catalytic behavior. Toward this end, two additional amino acid side chains were mutated (to alanine) within parent single tryptophan protein constructs of ht-ADH. These are Tyr25 at the dimer interface (discussed above) and Val260 located within the cofactor binding domain and situated directly behind the reactive nicotinamide ring of NAD^+,[21] Figure 1D. Of note, the distance between Tyr25 and Val260 is ca. 30 Å. The single site tryptophan proteins containing Y25A or V260A display kinetic features and KIEs similar to native ht-ADH containing Y25A or V260A, once again making them suitable constructs for further spectroscopic exploration. In earlier kinetic studies of V260A, low temperature was shown to generate a large increase in the enthalpy of activation for C−H activation together with a concomitant increase in the Arrhenius prefactor (to 10^{24} s^{-1} contrasting with a semiclassical limit of ca. 10^{13} s^{-1});[26] both of these properties implicated an increased population of protein in conformational substates with little or no activity in the low temperature regime. Further exploration of the temperature dependence of the KIEs indicated that V260A produces opposing effects relative to WT enzyme,[27] Figure 2B,C. As described in the text above and shown in Figure 2B, WT ht-ADH segues from temperature independent KIEs above 30 °C to temperature dependent KIEs in its nonoptimal, low temperature regime. By contrast, V260A generates a temperature dependent KIE above 30 °C that becomes temperature independent at low temperature, Figure 2C. The first property of V260A (>30 °C) is the more general one for mutant forms of protein, attributed to the impact of an active site packing defect that disrupts the catalytic configuration and donor−acceptor distance of native enzyme (Scheme 2A, bottom), introducing a donor−acceptor distance sampling mode that displays isotope and temperature dependence (part c in Scheme 1). The second property (<30 °C) was unexpected and indicates confined/restricted active site geometry for the enzyme ternary complex that, correspondingly, has become impaired in its ability to sample the protein landscape for catalytically competent configurations.

The differing impacts of Tyr25 and Val260 on the low temperature properties of the hydride transfer step of ht-ADH made them excellent candidates for testing the sensitivity of time dependent changes in protein fluorescence at the active site Trp87 to catalytically relevant protein dynamics. In addition, the availability of a remote fluorescence probe,

Trp167, introduced a control for dynamical effects that are specific to the active site. In addition to fluorescence lifetime measurements, the Stokes shift, which measures a time dependent relaxation of protein around the excited state dipole of the chromophore that produces a red shift in the fluorescence emission peak (from $t = 0$ to the steady state)[28] has been particularly revealing. For Y25A, a loss in the temperature dependent break in the fluorescent transient (τ_1) at Trp87 was observed, together with an increased relaxation rate for the Stokes shift. The latter is accompanied by a decreased overall red shift in λ_{max} and temperature dependence for the (picosecond/nanosecond) reorganization of the environment surrounding the excited state dipole. All of these properties are reflective of greater active site dynamical behavior upon mutation at Tyr25 at the dimer interface, exactly as predicted from the kinetic properties of this variant, Figure 2A. In contrast to its impact on Trp87, Y25A is found to retard slightly the Stokes shift relaxation rate at Trp167.[21]

A significantly different pattern of fluorescence emerges for V260A. The most rapid transient from lifetime measurements at Trp87, τ_1, is completely lost, as is the enthalpic barrier for the Stokes shift. Instead V260A shows a Stokes shift lifetime at low temperature that equals that of the parent construct (W87in) at 10 °C, without any further change up to 30 °C. Between 25 and 40 °C, V260A transitions to a new conformational space in which the Stokes shift lifetime has become ca. 2-fold slower, Figure 3A. All of this occurs without any change in the amplitude of the Stokes shift. These data offer some of the strongest spectroscopic evidence for the presence of two distinct forms of ht-ADH above and below 30 °C. This impact
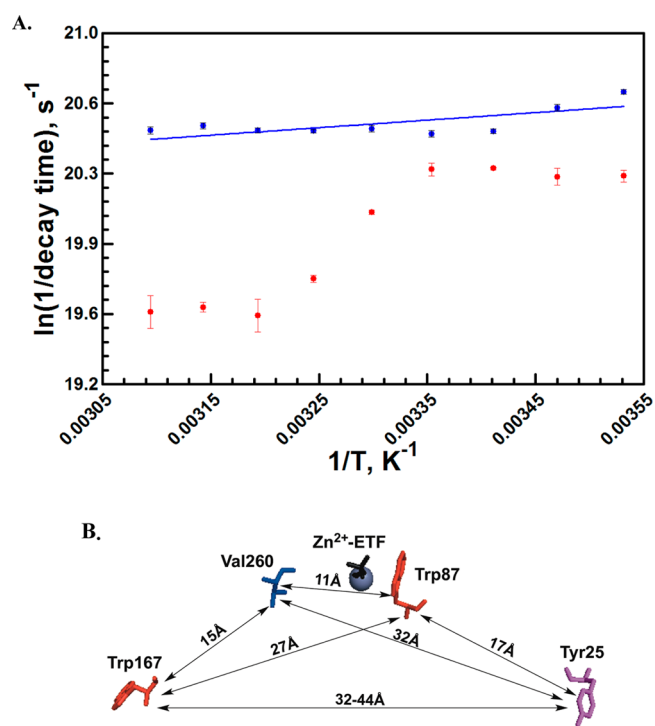

Figure 3. Key results from nanosecond to picosecond fluorescence studies of ht-ADH.[21] (A) The temperature dependent break in the Stokes shift for Trp87 in V260A (red), together with the contrasting behavior of Trp187 (blue). (B) Dynamical network of communication in ht-ADH extends from Tyr25 to Trp87 and Val260. There is also evidence for some communication between Tyr25 and Trp167.

appears specific to the active site, since analogous to the absence of a large impact of Y25A on fluorescence at Trp167, the insertion of V260A leads to almost unchanged behavior at Trp167. From a structural perspective, the additional observation of a reduced enthalpic barrier for collisional quenching at Trp87 in the presence of V260A suggests a protein configuration that places Trp87 closer to bulk solvent. Perhaps of greatest interest with regard to the impact of mutants on catalysis, the fluorescent data with V260A implicate a temperature-dependent communication from the cofactor binding domain to the fluorescence properties at Trp87 on the substrate domain. That the resulting structural impairment in V260A can be overcome in the presence of cofactor and substrate above 30 °C, where kinetic behavior approximating WT is observed,[26] speaks to the fluidity of this interdomain communication. In the aggregate, these studies have defined an extended dynamical communication network within ht-ADH that extends from one of the dimer interfaces to the substrate binding domain and across the active site pocket to the opposing cofactor binding domain,[21] Figure 3B.

■ IMPLICATIONS FROM THE AVAILABLE MEASUREMENTS ON HT-ADH FOR UNDERSTANDING THE FUNCTIONAL ROLES OF PROTEIN MOTIONS

We now have at our disposal three independent probes of a transition that occurs for ht-ADH at ca. 30 °C. These involve changes in the properties of catalysis, HDX, and fluorescence lifetimes, occurring on widely varying experimental time scales that include seconds to hours (HDX), milliseconds (catalysis), and nanosecond to picosecond (fluorescence), Scheme 4.

Scheme 4. Different Time Frames Examined in ht-ADH
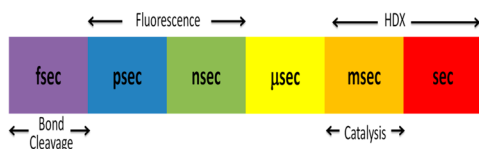
While the presence of substrate and cofactor are expected to affect the conformational landscape of ht-ADT, it has been possible to detect temperature dependent transitions in both apoenzyme (HDX and fluorescence measurements) and in the ternary complex (catalysis). In all three types of measurements, temperature-dependent alterations are sufficiently great to impact the interrogated property.

The trends in rate constants detected for catalysis and HDX are believed to depend on very similar phenomena, that is, the probability of achieving a subset of protein conformers among a very large number of rapidly interconverting conformers that are suited to the measured parameter (H-transfer for catalysis vs exposure of an amide backbone to solvent in the case of HDX). This behavior is distinct from the fluorescence lifetime and Stokes shift measurements. In these latter cases, a direct measure of the relaxation of an excited state dipole is possible, but the rapid time scale of the experiment means that the protein conformational substates are effectively frozen in time. Thus, the changes that are measured upon a reduction in temperature or insertion of a site specific mutant reflect a shift in distribution of protein conformers, which either remain effectively frozen on the time scale of measurements (fluorescence) or are thermodynamically averaged (HDX). In

no instance yet is there a direct measure of the actual rate for conformational interconversions at high vs low temperatures or the cooperative transition that takes place at 30 °C. The major time scale absent from the available data on ht-ADH lies between nanosecond and millisecond, Scheme 4, and future NMR as well as *T*-jump fluorescence studies may allow access to motions in this time regime. It should be noted that while the original X-ray structure of ht-ADH was determined at cryogenic temperatures, it is now possible to examine changes in protein side chain conformations at elevated temperatures using X-ray crystallography.[29] Analogous to HDX, available X-ray methods are too slow to attempt detection of changes in protein structure on the microsecond time scale. Ongoing methodological developments may, however, open up diffraction methods for this purpose as well (see ref 30 and refs therein).

In addition to the question of time scales for protein motions, there is the issue of the degree of protein involvement, that is, to what extent are we able to define and understand the amplitude of motions affecting a measured parameter. Whereas there is a tendency to relate slow rate constants to more global motions and by extension fast rate processes to local motions, care is needed in this regard and it should be considered on a case by case basis. For example, of the measurements discussed herein, relatively high amplitudes of protein motions may be necessary to achieve significant levels of deuterium incorporation into the protein's buried peptide bonds. By contrast, during enzymatic turnover, small amplitude motions may be sufficient to tune the precise electrostatic and distance interactions that control catalytic efficiency. Finally, in the case of the fluorescence lifetime and Stokes shift measurements applied to ht-ADH, the interplay between a transiently induced dipole at the chromophoric tryptophan residues and the protein environment may, indeed, be quite local, given the distance relationships for induced dipole−induced dipole interactions $(1/r^6)$ and induced dipole−dipole interactions $(1/r^6)$.[31] However, without experimental verification, we cannot rule out more long-range effects between *fixed* charge protein side chains and the generated dipole $(1/r^4$ dependence) at the positionally specific tryptophans during picosecond to nanosecond Stokes shift measurements.

■ LOOKING TO THE FUTURE

The experimental focus in the area of protein dynamics has now moved beyond efforts to demonstrate a correlation between perturbations in protein motions and alterations in catalytic efficiency. A more exacting challenge involves defining the time constants and amplitudes for motions within the specific regions of a protein that impact the chemical steps of catalysis. While computational work can aid in addressing these key issues, experimental validation, using a range of biophysical tools, remains an essential component of efforts in this area. Until a reasonably high level of both temporal and spatial refinement of catalytically relevant motions can be achieved, efforts at first principle design of highly active protein catalysts is likely to remain a future goal rather than a present day accomplishment.

■ AUTHOR INFORMATION

Notes

The authors declare no competing financial interest.

Biography

Judith Klinman received her undergraduate and graduate degrees at the University of Pennsylvania. She was a postdoctoral associate and then an independent researcher at the Institute for Cancer Research in Philadelphia before moving in 1978 to the University of California, Berkeley, as a Professor of Chemistry. She is currently Professor of the Graduate School in the Departments of Chemistry and Molecular and Cell Biology.

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■ REFERENCES

(1) Page, M. I.; Jencks, W. P. Entropic contributions to rate accelerations in enzymic and intramolecular reactions and the chelate effect. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 1678−1683.

(2) Klinman, J. P. An integrated model for enzyme catalysis emerges from studies of hydrogen tunneling. *Chem. Phys. Lett.* **2009**, *471*, 179−193.

(3) Blomberg, R.; Kries, H.; Pinkas, D. M.; Mittl, P. R.; Grutter, M. G.; Privett, H. K.; Mayo, S. L.; Hilvert, D. Precision is essential for efficient catalysis in an evolved Kemp eliminase. *Nature* **2013**, *503*, 418−421.

(4) McIntosh, J. A.; Farwell, C. C.; Arnold, F. H. Expanding P450 catalytic reaction space through evolution and engineering. *Curr. Opin. Chem. Biol.* **2014**, *19*, 126−134.

(5) Schutz, C. N.; Warshel, A. What are the dielectric "constants" of proteins and how to validate electrostatic models? *Proteins* **2001**, *44*, 400−417.

(6) Nestl, B. M.; Hauer, B. Engineering of flexible loops in enzymes. *ACS Catal.* **2014**, *4*, 3201−3211.

(7) Klinman, J. P.; Kohen, A. Hydrogen tunneling links protein dynamics to enzyme catalysis. *Annu. Rev. Biochem.* **2013**, *82*, 471−496.

(8) Edwards, D. R.; Lohman, D. C.; Wolfenden, R. Catalytic proficiency: The extreme case of S−O cleaving sulfatases. *J. Am. Chem. Soc.* **2012**, *134*, 525−531.

(9) Liang, Z.-X.; Tsigos, I.; Bouriotis, V.; Klinman, J. P. Impact of protein flexibility on hydride-transfer parameters in thermophilic and psychrophilic alcohol dehydrogenases. *J. Am. Chem. Soc.* **2004**, *126*, 9500−9501.

(10) Kohen, A.; Cannio, R.; Bartolucci, S.; Klinman, J. P. Enzyme dynamics and hydrogen tunneling in a thermophilic alcohol dehydrogenase. *Nature* **1999**, *399*, 496−499.

(11) Gold, V. Rule of the geometric mean: Its role in treatment of thermodynamic and kinetic deuterium solvent isotope effects. *Trans. Faraday Soc.* **1968**, *64*, 2770−2779.

(12) Klinman, J. P. Linking protein structure and dynamics to catalysis: The role of hydrogen tunnelling. *Philos. Trans. R. Soc., B* **2006**, *361*, 1323−1331.

(13) Kuznetsov, A. M.; Ulstrup, J. Proton and hydrogen atom tunnelling in hydrolytic and redox enzyme catalysis. *Can. J. Chem.* **1999**, *77*, 1085−1096.

(14) Knapp, M. J.; Rickert, K.; Klinman, J. P. Temperature-dependent isotope effects in soybean lipoxygenase-1: Correlating hydrogen tunneling with protein dynamics. *J. Am. Chem. Soc.* **2002**, *124*, 3865−3874.

(15) Hammes-Schiffer, S.; Soudackov, A. V. Proton-coupled electron transfer in solution, proteins, and electrochemistry. *J. Phys. Chem. B* **2008**, *112*, 14108−14123.

(16) Soudackov, A. V.; Hammes-Schiffer, S. Probing the non-adiabaticity in the proton-coupled electron transfer reaction catalyzed by soybean lipoxygenase. *J. Phys. Chem. Lett.* **2014**, *5*, 3274−3278.

(17) Truhlar, D. G.; Garrett, B. C. Variational transition-state theory. *Acc. Chem. Res.* **1980**, *13*, 440−448.

(18) Herek, J. L.; Pedersen, S.; Banares, L.; Zewail, A. H. Femtosecond real-time probing of reactions. 9. Hydrogen-atom transfer. *J. Chem. Phys.* **1992**, *97*, 9046−9061.

(19) Liang, Z.-X.; Lee, T.; Resing, K. A.; Ahn, N. G.; Klinman, J. P. Thermal-activated protein mobility and its correlation with catalysis in thermophilic alcohol dehydrogenase. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 9556−9561.

(20) Meadows, C. W.; Ou, R.; Klinman, J. P. Picosecond-resolved fluorescent probes at functionally distinct tryptophans within a thermophilic alcohol dehydrogenase: Relationship of temperature-dependent changes in fluorescence to catalysis. *J. Phys. Chem. B* **2014**, *118*, 6049−6061.

(21) Meadows, C. W.; Tsang, J. E.; Klinman, J. P. Picosecond-resolved fluorescence studies of substrate and cofactor- binding domain mutants in a thermophilic alcohol dehydrogenase uncovers an extended network of communcation. *J. Am. Chem. Soc.* **2014**, *136*, 14821−14833.

(22) Krishna, M. M. G.; Hoang, L.; Lin, Y.; Englander, S. W. Hydrogen exchange methods to study protein folding. *Methods* **2004**, *34*, 51−64.

(23) Hoofnagle, A. N.; Resing, K. A.; Ahn, N. G. Protein analysis by hydrogen exchange mass spectrometry. *Annu. Rev. Biophys. Biomol. Struct.* **2003**, *32*, 1−25.

(24) Liang, Z. X.; Tsigos, I.; Lee, T.; Bouriotis, V.; Resing, K. A.; Ahn, N. G.; Klinman, J. P. Evidence for increased local flexibility in psychrophilic alcohol dehydrogenase relative to its thermophilic homologue. *Biochemistry* **2004**, *43*, 14676−14683.

(25) Nagel, Z. D.; Cun, S. J.; Klinman, J. P. Identification of a long-range protein network that modulates active site dynamics in extremophilic alcohol dehydrogenases. *J. Biol. Chem.* **2013**, *288*, 14087−14097.

(26) Nagel, Z. D.; Dong, M.; Bahnson, B. J.; Klinman, J. P. Impaired protein conformational landscapes as revealed in anomalous Arrhenius prefactors. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 10520−10525.

(27) Nagel, Z. D.; Meadows, C. W.; Dong, M.; Bahnson, B. J.; Klinman, J. P. Active site hydrophobic residues impact hydrogen tunneling differently in a thermophilic alcohol dehydrogenase at optimal versus nonoptimal temperatures. *Biochemistry* **2012**, *51*, 4147−4156.

(28) Abbyad, P.; Shi, X. H.; Childs, W.; McAnaney, T. B.; Cohen, B. E.; Boxer, S. G. Measurement of solvation responses at multiple sites in a globular protein. *J. Phys. Chem. B* **2007**, *111*, 8269−8276.

(29) Fraser, J. S.; van den Bedem, H.; Samelson, A. J.; Lang, P. T.; Holton, J. M.; Echols, N.; Alber, T. Accessing protein conformational ensembles using room-temperature X-ray crystallography. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 16247−16252.

(30) Sawaya, M. R.; Cascio, D; Gingery, M.; Rodriguez, J.; Goldschmidt, L.; Colletier, J.-P.; Messerschmidt, M. M.; Boutet, S.; Koglin, J. E.; Williams, G. J.; Brewster, A. S.; Nass, K.; Hattne, J.; Botha, S.; Doak, R. B.; Shoeman, R. L.; DePonte, D. P.; Park, H.-W.; Federici, B. A.; Sauter, N. K.; Schlichting, I.; Eisenberg, D. S. Protein crystal structure obtained at 2.9 Å resolution from injecting bacterial cells into an X-ray free-electron laser beam. *Proc. Natl. Acad. Sci., U.S.A.* **2014**, *111*, 12769−12774.

(31) Anslyn, E. V.; Dougherty, D. A. *Modern Physical Organic Chemistry*; University Science Books: Sausalito, CA, 2005; p 188.

(32) Nagel, Z. D.; Klinman, J. P. A 21st century revisionist's view at a turning point in enzymology. *Nat. Chem. Biol.* **2009**, *5*, 543−550.