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The Dynamical Nature of Enzymatic Catalysis

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CONCEPTUS: As is well-known, enzymes are proteins designed to accelerate specific life essential chemical reactions by many orders of magnitude. A folded protein is a highly dynamical entity, best described as a hierarchy or ensemble of interconverting conformations on all time scales from femtoseconds to minutes. We are just beginning to learn what role these dynamics play in the mechanism of chemical catalysis by enzymes due to extraordinary difficulties in characterizing the conformational space, that is, the energy landscape, of a folded protein. It seems clear now that their role is crucially important. Here we discuss approaches, based on vibrational spectroscopies of various sorts, that can reveal the energy landscape of an enzyme–substrate (Michaelis) complex and decipher which part of the typically very complicated landscape is relevant to catalysis. Vibrational spectroscopy is quite sensitive to small changes in bond order and bond length, with a resolution of 0.01 Å or less. It is this sensitivity that is crucial to its ability to discern bond reactivity.

Using isotope edited IR approaches, we have studied in detail the role of conformational heterogeneity and dynamics in the catalysis of hydride transfer by LDH (lactate dehydrogenase). Upon the binding of substrate, the LDH-substrate system undergoes a search through conformational space to find a range of reactive conformations over the microsecond to millisecond time scale. The ligand is shuttled to the active site via first forming a weakly bound enzyme-ligand complex, probably consisting of several heterogeneous structures. This complex undergoes numerous conformational changes spread throughout the protein that shuttle the enzyme-substrate complex to a range of conformations where the substrate is tightly bound. This ensemble of conformations all have a propensity toward chemistry, but some are much more facile for carrying out chemistry than others. The search for these tightly bound states is clearly directed by the forces that the protein can bring to bear, very much akin to the folding process to form native protein in the first place. In fact, the conformational subspace of reactive conformations of the Michaelis complex can be described as a “collapse” of reactive substates compared with that found in solution, toward a much smaller and much more reactive set. These studies reveal how dynamic disorder in the protein structure can modulate the on-enzyme reactivity. It is very difficult to account for how the dynamical nature of the ground state of the Michaelis complex modulates function by transition state concepts since dynamical disorder is not a starting feature of the theory. We find that dynamical disorder may well play a larger or similar sized role in the measured Gibbs free energy of a reaction compared with the actual energy barrier involved in the chemical event. Our findings are broadly compatible with qualitative concepts of evolutionary adaptation of function such as adaptation to varying thermal environments. Our work suggests a methodology to determine the important dynamics of the Michaelis complex.

INTRODUCTION

Enzymes accelerate the chemical reaction rates of cellular chemistry enormously over that of the same reaction in water. Roughly the goal of most enzymes is to bring reactions within cells to completion in about a millisecond because this is a typical time for diffusion of small molecules across cells. The uncatalyzed reactions in water can take days to millions of years depending on the specific reaction. The question of how enzymes bring about such enormous rate enhancements is a crucial one yet unresolved despite decades of intense research.

FORMULATIONS OF CHEMICAL RATE ENHANCEMENTS

It has long been recognized that chemical reactions are the result of rare events. The actual on-enzyme chemical transformation takes a very short time, on the femtosecond time scale, in comparison to the underlying dynamical process, typically on the nanosecond to microsecond time scales. Most formulations of enzymatic catalysis involve a separation of time variables: fast events are taken to be independent of slower processes. Theoretical formulations take on a form containing a variables: fast events are taken to be independent of slower processes. Theoretical formulations take on a form containing a probability Boltzmann factor-like distribution, dating back to the work of Van’t Hoff–Arrhenius of the late 1800s, since this recognizes explicitly the rarity of the event. Two formulations are common in understanding chemical events. One, developed by Enge, envisions the system as a hunt through phase space governed by Brownian motion dynamics driven by
thermal fluctuations. The crossing from one side of the reaction to the other is described as a diffusion process. Kramers considered separately the cases of weak and strong friction. This formulation has become widely used in condensed matter physics. Transition state theory is widely used to understand chemical and biochemical reactions, and recent advances in TST have provided new insights into enzymatic catalysis (e.g., ref 7). The transition state itself is defined as the dividing surface in phase space between reactants and products. It is generally assumed that all degrees of freedom are in thermodynamic equilibrium (a sometimes problematic assumption). The simplest form of the rate is given by

$$k_{\text{cat}} = \left[ \frac{kT}{\hbar} \right] e^{-\Delta G^\ddagger/(kT)} $$

where \(\Delta G^\ddagger\) is the transition state (TS) energy, the fundamental parameter describing the probability of the system to reach reactive states, and \(kT/\hbar (k, \text{the Boltzmann constant}; T, \text{absolute temperature}; \text{and } \hbar, \text{Planck's constant})\) is a frequency factor approximating the barrier crossing rate. Hence, determining and calculating how enzymes lower the TS Gibbs free energy, \(\Delta G^\ddagger\), from its corresponding value in water is of central importance from this set of organizing principles.

### ENZYMES ARE INHERENTLY DYNAMICAL ENTITIES

In this Account, we explore dynamics as a fundamental property of proteins and how it is related to enzymatic mechanism. It has long been recognized that a protein does not occupy a unique folded three-dimensional array of atoms, despite the impression that is common to almost all textbooks. Rather, a protein’s structure is best described as a hierarchy or ensemble of interconverting conformations on all time scales from femtoseconds to minutes, and spatial extent from small atom displacements to large scale domain motions. Most of the conformations occupy a metastable energy basin within the overall phase space describing the protein’s structure. This physical picture flows from the nature of the folded structure, whose stability and structural integrity is dictated by a large number of weak forces acting together. The role of a protein’s dynamical nature is described by its so-called “energy landscape” (e.g., ref 9), a mapping of the possible conformations of the system with the Gibbs free energy.

As such, enzymes are dynamic entities exhibiting distributions and fluctuations of catalytic rate constants, a notion termed dynamic disorder. The existence of dynamic disorder in enzymes has been long inferred from ensemble measurements and has been made abundantly clear by recent single-molecule experiments (see refs 10–13). It has been shown that an ensemble averaging of the single molecular results can yield Michaelis–Menten kinetics. Dynamic disorder is not taken into account by either Kramers’ theory or transition state theory. However, the rate of catalysis for most enzymes depends on the rates of protein conformational changes.

Given this, we need to ask whether the Michaelis complex, the enzyme–substrate construct that enters the Michaelis–Menten kinetic description of enzymatic catalysis, can be considered a single species. The most physically credible situation is that the Michaelis complex consists of an ensemble of conformations, each with its own effective \(k_{\text{cat}}\), the single molecule experiments mentioned above provide strong evidence for this notion. Single molecule experiments have also demonstrated that when interconversion among the Michaelis conformations is slow compared with the chemical step, the system still exhibits Michaelis–Menten kinetics, but in this case the observed \(k_{\text{cat}}\) is the weighted harmonic mean of \(k_{\text{cat}}\) for all of the conformers (depending on both the mean and distribution).10 Nevertheless, a single active Michaelis conformation is usually assumed in virtually all textbook treatments of the subject. A single conformation Michaelis complex is the easiest assumption given that so little is known, either experimentally or theoretically, about the nature of the chemical activities of a protein’s ensemble set of conformational substates. From an experimental point of view, isolating that atomic motion of atoms associated with the chemical event against the vast background of motions is very hard indeed. Moreover, if part of the ensemble of the Michaelis complex is not very active by itself (must convert to other substates that are active before chemistry can efficiently occur), this implies that the effective transition state energy of active conformations must be even correspondingly lower. It seems difficult enough to lower transition state energies to values permitting millisecond chemistry; why consider even lower values (cf.14) unless either theoretical or experimental findings require it.

### CHARACTERIZATION OF THE ENERGY LANDSCAPE OF ENZYMES

It is difficult, both theoretically and experimentally, to determine the ensemble of substates that a folded protein adopts. For one thing, the number of states within the ensemble is huge. The issue at hand, to discern the functional relevance of the various substates, is even more demanding. One successful approach to picking out relevant conformations from the enormous background is to discern specific coordinates that are clearly associated with reactivity. For this purpose, vibrational spectroscopy has proved promising and very useful. When ligands bind to proteins, there are small but clear structural changes that show up in bond orders and concomitant changes to vibrational frequencies. For an enzyme–substrate complex, key bonds associated with the reaction coordinate are generally affected, and these become spectroscopic markers for catalytic propensity.

For example, we determined a broad linear correlation of the bridging P–O(R) bond length in monosubstituted dianionic phosphates and the pKₐ of the substituent alcohol. We addressed the relationship between ground-state bonding properties and reactivity and found that a change of just 0.02 Å of the bridging P–O(R) bond length is quantitatively correlated to orders of magnitude in change to reactivity. Importantly, vibrational spectroscopy can discern these quite small changes in bond length. Hence, a vibrational analysis of this bond as the phosphate substrate binds to an enzyme and forms the Michaelis complex and its ensemble of structures is a monitor of functionally important substates. The bond itself can be specifically picked out from all other bonds within the Michaelis complex by employing isotope editing procedures. For example, the IR spectra of E-P–O(R) and E-P–¹⁸O(R) are obtained and differenced. Since all vibrational modes not involving the isotope label subtract out, this procedure yields the spectrum of the enzyme bound phosphate modes.

Our work has concentrated on the hydride transfer proteins, particularly lactate dehydrogenase (LDH). The rate of catalysis, \(k_{\text{cat}}\) by LDH depends on the rates of protein conformational changes. For this reason and because
much is known about its biochemical and structural nature (see refs 23 and 25–27), this enzyme offers an excellent system for the study of how dynamics affects function. Using isotope edited IR approaches,²⁶,²⁷ we have studied in detail the role of conformational heterogeneity and dynamics in the catalysis of hydride transfer by LDH. This enzyme catalyzes the reduction of pyruvate to lactate mediated by the transfer of a hydride from NADH to C-2 of pyruvate as shown in the cartoon of the active site (Figure 1). There are several vibrational localized modes that report on catalysis, particularly, the C₂=O group of the bound pyruvate, which is polarized by active site interactions. Another important structural feature of the active site is the electronic orbital overlap between pyruvate’s C₂=O bond and the nicotinamide ring of NADH, as shown from the observation of a delocalized vibrational mode involving motions from both moieties.²² Finally, the nicotinamide C4-H stretch is sensitive to changes in nicotinamide ring geometry related to reactivity.²⁰,²¹

Clearly the C₂=O group of the bound pyruvate substrate monitors the strength of the hydrogen bonding and electrostatic interactions (Figure 1, shown in red), which is an important factor in the mechanism of the hydride transfer.²⁷ Indeed, it has been shown that the frequency of the C₂=O stretch is a quantitative monitor of propensity toward catalysis.¹⁶ The C₂=O stretch frequency of a specific conformation of bound pyruvate within the LDH-NADH-pyruvate Michaelis complex is an excellent monitor of the protein's ensemble nature since the stretch value is highly correlated with the propensity toward the on-enzyme chemical reaction (production of lactate) of that conformation.¹⁶ The C₂=O stretch is quite localized to just motions of the C2 carbon and the O2 oxygen. Thus, we can use the C₂=O stretch frequency as a quantitative measure of the energy landscape of the enzyme-substrate complex compared with that found in solution.

Figure 2 shows the IR absorbance band of pyruvate’s ¹²C₂=O stretch mode for three cases: (a) pyruvate in water; (b) pyruvate bound in the Michaelis complex (i.e., LDH-NADH-pyruvate complex) of a catalytically impaired LDH; (c) pyruvate bound to the Michaelis complex of native LDH. Some key features to keep in mind to interpret these data are as follows. First, the homogeneous bandwidth of the C₂=O stretch is around 1 cm⁻¹. All the band profiles of Figure 2 are much broader so that the spectra represent a heterogeneous mixture of substrates. The pyruvate molecule finds itself in

Figure 1. LDH catalyzes the interconversion of NADH + pyruvate + H⁺ with NAD⁺ + lactate (see refs 8 and 27). Binding is strictly ordered with cofactor binding preceding substrate. It is widely believed that hydride and proton transfer are concerted. Calculations show multiple routes for proton and hydride transfer occurring in traversal of the transition state within the time frame of a single bond vibration (ca. 5 fs).²³ Shown is a schematic of the LDH active site showing the residues stabilizing the substrate pyruvate and the proximity of the cofactor, NADH. The catalytically key surface loop (residues 98–110) closes over the active site, bringing residue Arg109 in hydrogen bond contact with the ligand; water leaves the pocket. Creation of the pocket is accompanied by the motions of mobile areas within the protein, rearranging the pocket geometry to allow for favorable interactions between the cofactor and the ligand that facilitate on-enzyme catalysis.²¹ Of particular interest to this work are the hydrogen bonds formed between Arg109 and His195 to the C2 carbonyl of pyruvate (emphasized in red). These bonds dictate the polarity of the carbonyl when pyruvate is bound. Figure taken from ref 35.

Figure 2. (a) IR spectrum of pyruvate in water. (b) FTIR isotope edited difference spectrum (D168N)LDH-NADH-[¹³C₂]pyruvate subtracted from that of (D168N)LDH-NADH-[¹²C₂]pyruvate. The D168NLDH mutants shows a kcat about a factor of 800 lower than native LDH. (c) FTIR isotope edited difference spectrum with the spectrum of LDH-NADH-[¹³C₂]pyruvate subtracted from that of LDH-NADH-[¹²C₂]pyruvate. The spectral region of the ¹³C=O stretch is considerably downshifted from ¹²C=O stretch and is not shown in the figure. The difference spectra are measured using [¹³C/¹⁵N]LDH labeled protein to move the intense amide-I protein IR band out of the way of pyruvate’s ¹²C=O stretch.²² Figure adapted from ref 22.
The distribution is intermediate for pyruvate bound to the enzyme either in solution or when bound. Also note that the dipole moment of the C=O moiety changes little. The underlying time scale of vibrational spectroscopy is very fast (on the femtosecond time scale) so that, using NMR language, the spectra are in “slow” exchange.

The important conclusion from all this is that the concentration of a specific conformation is proportional to the intensity of its IR band to a good approximation. Hence, the IR band profile acts as a measure of the density of states per unit C=O stretch frequency. A lower value of the C=O stretch frequency of a specific conformation of bound pyruvate within the LDH-NADH-pyruvate Michaelis complex is exponentially correlated to a faster hydride transfer rate; a 35 cm⁻¹ downward shift in frequency corresponds to a rate enhancement of about one million. This is an empirical relationship inferred by parallel kinetic studies of a series of LDH mutants where hydride transfer is largely rate limiting (primary H/D isotope effect of 2−4) with static vibrational studies of Michaelis complex mimics of the same mutants. Thus, the C=O stretch frequency spectrum is a quantitative measure of the energy landscape of the enzyme-substrate complex projecting out key substrate conformations and the propensity of a particular substrate toward on-enzyme chemistry.

The hydride transfer reaction between NADH and pyruvate in water proceeds very slowly. The two molecules have to come to a correct geometry, with the C4=H bond of NADH pointed toward the C=O moiety of pyruvate in the correct geometry and also in very close approach. All this happens via a statistical search. Moreover, the arrangement of water molecules around the two molecules must be such to form strong hydrogen bonds that polarize the C=O bond so as to lower the effective transition state energy sufficiently for the hydride ion to transfer. There is a low probability of finding highly polarized C=O bonds for pyruvate in water according to the distribution of molecules indicated by the solution IR spectrum of Figure 2a. When bound to LDH, the distribution of C=O bonds is very strongly skewed toward substantially more polarized (Figure 2c). Note, there is not a single conformation of molecules either in solution or when bound. Also note that the distribution is intermediate for pyruvate bound to the impaired LDH isozyme (Figure 2b). LDH enhances the rate of hydride transfer between NADH and pyruvate by about 10¹⁴ M⁻¹. A substantial portion of this rate enhancement is clearly a consequence of constraints imposed by the protein structure that bring about a restricted ensemble of more reactive conformational substates in the enzyme system.

**THE DYNAMICS OF THE ON-ENZYMES STATISTICAL SEARCH FOR REACTIVITY**

Protein structural fluctuations may be important both in the search for catalytically competent conformations and within such conformations, in the transition over the barrier that defines the chemical step. These structural dynamics span a wide range of times, from the femtosecond time scale of crossing the transition state to the millisecond time scale of overall enzyme turnover, or slower. Here we focus on the protein dynamics of the search for reactive conformations, expected to occur on the microsecond to millisecond time scale. Heterogeneity in the Michaelis state as described above for LDH may manifest in different ways in the observed enzyme turnover kinetics, depending on the relative time scale of the conformational dynamics. Heterogeneity in T̄2,13,34 is usually hidden in ensemble kinetics experiments because they only observe an average of the rate. In contrast, single molecule enzyme studies have demonstrated the existence of dynamic disorder in enzyme structure and its influence on T̄2.12,13,34 These studies observe heterogeneity in T̄2 and attribute it to protein conformational fluctuations that are slow compared with the chemical step, but they do not reveal the physical origin of this behavior. Thus, an important question that remains is how protein conformational fluctuations change the rate of the chemical reaction.

Recently it was shown that with LDH it is possible to resolve the heterogeneity in T̄2 due to conformational fluctuations of the Michaelis complex in ensemble experiments. These studies reveal how the dynamic disorder in the protein structure modulates the on-enzyme reactivity. Infrared spectroscopy was used to probe independently the differing reactivity of each Michaelis substate shown in Figure 2. Using a laser-induced temperature jump to perturb the enzyme equilibrium, the relaxation times for establishing new equilibria among the IR detected substates and the product states were determined by IR probes at infrared frequencies corresponding to each of the substates noted above (Figure 2c). Figure 3 shows the relaxation transient at each probe wavelength from 10 μs to 1 ms. The lower limit of this range is set by the response time of the instrument, and the upper limit is determined by the cooling time of the sample after the temperature-jump occurs (typically several milliseconds for this sample configuration). The simplest model that fits the IR data is presented in Scheme 1, which has multiple enzyme conformations at both the encounter and tightly bound complex stages of the reaction pathway. This model is also substantiated by significant previous work, which supports formation of a weakly binding encounter complex as the initial step,19,29,36 protein structural fluctuations associated with forming the Michaelis complex,21

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**Figure 3.** Isotope-labeled IR difference temperature-jump relaxation transients of LDH-NADH-[13C₂]pyruvate minus LDH-NADH-[13C₂]-pyruvate at various probe frequencies. Each probe frequency is plotted as a different color as specified in the legend, and the exponential fits using kinetic Scheme 1 are plotted as black lines. The Michaelis state transients each show a negative amplitude signal with a submillisecond relaxation lifetime that depends on the probe frequency: 254 μs (1685 cm⁻¹), 128 μs (1679 cm⁻¹), and 44 μs (1670 cm⁻¹). The difference transients are measured using [13C₂]/[15N]LDH uniformly labeled protein to move the intense amide-I protein IR band out of the way of pyruvate’s 12C=O stretch. Graph adapted from ref 35.
and multiple conformations within the Michaelis state that do not directly interconvert, with one of these populations being incompetent toward conversion to lactate.\textsuperscript{36,50,37} Three critical features of the enzyme mechanism emerge from this model. Early on the reaction pathway, LDH forms an encounter complex with the pyruvate substrate, which then rearranges to the tightly bound states. This intermediate is obligatory, because there is no direct pathway between free substrate and the reactive conformations. The time scale of various protein structural rearrangements, including those that are somewhat slower than the chemistry step (such as closure of the surface loop, residues 98–110, that brings the key residue, Arg109, into the active site; see Figure 1), is similar to the time scale of the chemical step, such that they are strongly coupled kinetically. The different Michaelis states do not directly interconvert, and most importantly, they exhibit different rates of conversion of pyruvate to lactate.

The key characteristic of the kinetics model in Scheme 1 is the emergence of branched pathways from the initial encounter complex, having Michaelis states of differing reactivity. The reactive states do not interconvert; therefore they are only populated through the initial encounter complex. Furthermore, the rate of chemical conversion of each Michaelis state is inversely proportional to the frequency of the pyruvate C=O carbonyl stretch frequency. This rate dependence is a consequence of the differing degree of polarization of the carbonyl bond for the different substrates, as indicated by the stretch frequency. From empirical correlation of force constants with bond distances, a shift of the pyruvate carbonyl mode from its solution value of 1710 cm\(^{-1}\) to the major reactive population at 1679 cm\(^{-1}\) represents a lengthening of the C=O bond by 0.01 Å.\textsuperscript{38} The polarization of this bond makes it more susceptible to nucleophilic attack by the hydride; thus the lower the frequency the faster the chemistry step, which is evident from Figure 3. Therefore, these results directly correlate the heterogeneity in \(k_{\text{cat}}\) with a specific structural feature of the Michaelis complex. Since these substrates do not interconvert directly, the net flux through each depends on the branching from the initial encounter complex, and the overall turnover rate is a population weighted average of the multiple parallel pathways. Another important conclusion from this work is that the most reactive substrate is not the most populated one.\textsuperscript{55,39} LDH does not appear to be optimized to use mainly the fastest pathway, implying that the conformational search is not necessarily for one optimal pathway, but simply for an average pathway that is fast enough to satisfy the functional demands in the context of the cell. It is interesting to consider whether this incomplete optimization of the conformational search is a consequence of evolutionary fine-tuning driven by the requirements of homeostasis.\textsuperscript{40} If so, it seems likely that such catalytic heterogeneity will be an important conserved feature of many enzymes.

\section*{The Nature of Dynamical Disorder Revealed for LDH}

A kinetic picture of the flux through the enzyme emerges from this work, from the binding of substrate to the hunt though phase space to conformations that can undergo efficient chemistry, to the actual on-enzyme chemical event. The ligand is shuttled to the active site via first forming a weakly bound enzyme-ligand complex, probably consisting of several heterogeneous structures. This encounter complex (ensemble) undergoes numerous conformational changes spread throughout the protein that shuttles the enzyme-substrate complex to a range of conformations where the substrate is tightly bound.\textsuperscript{21} The conformations in this ensemble all have a propensity toward chemistry, but some are much more facile for carrying out chemistry than others. The search for these tightly bound states is clearly directed by the forces that the protein can bring to bear, very much akin to the folding process to form native protein in the first place. For our system, the tightly bound conformations interconvert on the microsecond time scale, although the interconversion is not direct but rather through more loosely bound conformations. The search through conformation space on the nanosecond and slower times is very probably Markovian since the thermal fluctuations, occurring on the picosecond time scale, almost certainly disrupt any coherence in the system. That is, the system retains no or little memory of where it came in the interconversion from one substrate to another. The time scale of the conformational search is dictated by the energy barrier of a particular substrate toward conformational rearrangement toward other states on the protein’s energy landscape. The dynamical fluctuating nature of the complex is quite directly revealed by the studies. The enzyme does not appear to be optimized to use the fastest pathway for on-enzyme chemistry preferentially but rather accesses multiple pathways in a search process that often selects slower ones. Work of our collaborators has convincingly shown that for this system, but perhaps not universally, coherent femtosecond “promoting vibrations” carry the system over the effective transition state barrier separating substrate from product.\textsuperscript{41–43}

Consistent with these dynamics, the kinetic pathway can be separated into two parts: (1) the time it takes to form active conformations and the search time from less active to more active conformations and (2) the actual traversal from substrate to product. The first occurs on the nanosecond–microsecond time scale, while the latter occurs on the femtosecond time scale.
scale. Both the search process in the ground state through various reactive conformations and the chemical event are complicated, adopting multiple paths. Hence, it is useful to partition the effective Gibbs free energy, $\Delta G^\ddagger$, that appears in formulation of chemical rates using TST (eq 1 above) by decomposing it into two separate thermodynamic parameters, one representing the ground state search and the other the actual chemical event, $\Delta G_{\text{ground}}$ and $\Delta G^\ddagger$, respectively:

$$k_{\text{catalysis}} = e^{-\Delta G_{\text{ground}}/(kT)} \frac{kT}{h} e^{-\Delta G^\ddagger/(kT)}$$

(2)

where $e^{-\Delta G_{\text{ground}}/(kT)}$ represents the search process through the ground state ensemble of conformations while $(kT/h)e^{-\Delta G^\ddagger/(kT)}$ represents the ensemble of parallel pathways of on-enzyme chemistry from the ensemble of ground states, statistically weighted. The $e^{-\Delta G_{\text{ground}}/(kT)}$ term represents the “freezing out” effect of bringing reacting groups together in close proximity. It has a clear relationship to what is called intramolecular catalysis and effective concentration. For bimolecular reactions, such as that catalyzed by LDH, a rate enhancement of some 10$^9$ M can be realized from this effect.\cite{8,14,44} The same concepts can be applied to unimolecular reactions as well since key active site residues are almost universally brought into a close and orientated contact with the substrate; this is sometimes called “preorganization” of the active site structure.

For LDH, it has been estimated that out of the 10$^{14}$ M rate enhancement brought about by LDH, some 10$^6$ M (or more) is due to intramolecular catalysis.\cite{27} The size of the free energy at the transition state is unclear from particularly efficient “hot” ground state conformations. Calculations of our collaborators suggest that the TS energy may well be very small, even just a few kilocalories per mole. This then places much of the overall free energy for the on-enzyme catalyzed reaction within the ground state.

These findings are important for several reasons. One is that TST theory focuses on the energy barrier to the chemical event. It is very difficult to account for the dynamical nature of the ground state of the Michaelis complex by transition state concepts since dynamical disorder is not a starting feature of the theory. Taking dynamical disorder into account is typically not done or is rather ad hoc. Dynamical disorder may well play a larger or similar sized role in the measured Gibbs free energy of a reaction as the transition state energy associated with the chemical event. It is widely conjectured that evolutionary adaptation of function involves the adaptation of the enzyme’s dynamics, particularly the Michaelis complex ensemble population characteristics. Often hyperthermophilic and psychrophilic enzymes employ the same basic structural architecture as their mesophilic counterparts leading to the idea that the transition state of the chemical reaction is largely the same for the three classes, at least for many enzymes.\cite{24} Hence, modulation of the Michaelis complex ground state ensemble distribution and concomitant regulation of flux through the system along catalytic coordinates is indicated. It is also widely believed that allosteric regulation of enzymes has to do with a modulation of the Michaelis complex ensemble population characteristics. These notions generally and quite particularly for LDH are quite thoroughly discussed in ref 24. Our work suggests a methodology to determine the important dynamics of the Michaelis complex.

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**Author Contributions**

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**Notes**

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**Robert Callender** is Professor of Biochemistry at Albert Einstein College of Medicine since 1996. He received his Ph.D. from Harvard University in Applied Physics in 1969. After a year of postdoctoral studies in solid state physics in 1969–1970 at the University of Paris, he joined the physics faculty at The City College of New York in 1970, where he became Distinguished Professor of Biophysics in 1989. He joined the faculty at Albert Einstein in 1996. The focus of his studies has been spectroscopic studies of structure and dynamics of proteins using advanced approaches. His studies have included work on the photophysics of visual pigments and, more recently, enzymatic catalysis and protein folding. He developed sensitive difference spectroscopy to characterize the vibrational structures of enzymes and fast initiation methods to probe the dynamical nature of enzymes and the kinetics of protein folding.

**R. Brian Dyer** is Professor of Chemistry at Emory University since 2009. He received his Ph.D. degree in physical-inorganic chemistry from Duke University in 1985. Following a postdoctoral fellowship at Los Alamos National Laboratory in bioinorganic chemistry (with Woody Woodruff), he became a staff member in 1987. He was appointed a Laboratory Fellow in 2003. His science interests include bioinorganic chemistry, metalloenzymes, protein dynamics, and protein folding. In addition to these scientific interests, he has been active in developing new approaches to the study of dynamic processes, including time-resolved laser and spectroscopic techniques, fast reaction initiation methods, microfluidics, and molecular imaging. He pioneered the development of time-resolved infrared spectroscopy combined with new laser initiation methods and their applications to the study of protein dynamics.

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