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Flexibility, Diversity, and Cooperativity: Pillars of Enzyme Catalysis

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Abstract

This brief review discusses our current understanding of the molecular basis of enzyme catalysis. A historical development is presented, beginning with steady state kinetics and progressing through modern fast reaction methods, NMR, and single molecule fluorescence techniques. Experimental results are summarized for ribonuclease, aspartate aminotransferase, and especially dihydrofolate reductase (DHFR). Multiple intermediates, multiple conformations, and cooperative conformational changes are shown to be an essential part of virtually all enzyme mechanisms. In the case of DHFR, theoretical investigations have provided detailed information about the movement of atoms within the enzyme-substrate complex as the reaction proceeds along the collective reaction coordinate for hydride transfer. A general mechanism is presented for enzyme catalysis that includes multiple intermediates and a complex, multidimensional standard free energy surface. Protein flexibility, diverse protein conformations, and cooperative conformational changes are important features of this model.

Introduction

Understanding the incredible catalytic efficiency of enzyme catalysis in molecular terms has been a goal of biochemistry for over half a century. Although considerable progress has been made, we are still working toward this elusive goal, demanding a deeper and deeper understanding of the molecular interactions and dynamics underlying the catalytic process. This subject has been extensively reviewed (cf. references (1–4)), and this brief perspective makes no attempt at a complete review of the literature. Instead we focus on the evolution of our understanding of the catalytic process, primarily as exemplified by research done by the authors. Although we have started from different points, we have arrived at a common understanding of enzyme catalysis through our individual research programs and cooperative efforts among us.

In this perspective, a brief historical outline of the research aimed at a molecular understanding of enzyme mechanisms is presented. This is followed by results demonstrating the importance of multiple intermediates and conformations in the catalytic process. The development of new experimental methods, along with theoretical analyses, has led to a detailed understanding of the molecular events underlying enzyme catalysis. The picture of the catalytic process that emerges rests on three interconnected underpinnings: flexibility of protein structure, diversity of protein/enzyme conformations, and cooperative conformational changes within the enzyme.

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Historical Background

Initial attempts to understand enzyme mechanisms were based on steady state kinetic studies of enzyme catalysis. These studies require only small amounts of enzyme, not necessarily highly purified. However, steady state kinetic studies provide only limited information about reaction intermediates that cannot be directly observed at the low enzyme concentrations utilized. In general, the results furnish a measure of the catalytic efficiency, characterized by the turnover number, and the strength of substrate binding, characterized by the Michaelis constant. A careful analysis of the information that can be obtained from steady state kinetic studies was developed by Peller and Alberty (5).

Along with direct studies of enzymes, model reactions were investigated, primarily by organic chemists. This research typically attempted to develop models based on known aspects of enzyme structure and the amino acids at the active sites of enzymes. Much of this work is summarized in the books by Benkovic and Bruice (6). At this point in time, the 1950s and 1960s, most of the catalytic efficiency of enzymes was attributed to the enhanced concentration of catalytic groups near the substrate and the orientation/closeness of the catalytic groups to the substrate. However, the enhanced concentration cannot provide more than a factor of 100 to the catalytic efficiency, e.g., the concentration of water in pure water is 55 M. Furthermore, proximity of catalytic groups did not seem to assist catalysis greatly in model reactions. A series of experiments by Bruice and coworkers demonstrated that restriction of rotation of the substrate and catalytic groups contributes significantly to catalysis (7). This led to the concept of the enzyme as an “entropy trap” (cf. reference (8)). However, this work did not lend itself to a molecular description of precisely what happens during catalysis. Such a description requires knowledge of the intermediate states occurring during catalysis, both in dynamical and structural terms.

Multiple Intermediates in Enzyme Catalysis

Because of the speed of enzyme reactions, fast reaction techniques are needed to observe intermediates occurring during catalysis. This requires high concentrations of pure enzyme, a formidable requirement in the 1960s. Today with the routine cloning and production of enzymes, this is not a great problem. Stopped flow methods proved to be the method of choice in the initial transient kinetic studies. Chance pioneered the development of this technique and applied it to a variety of electron transfer reactions (e.g., catalase and peroxidase) for which prosthetic groups had high extinction coefficients and dramatic color changes (cf. reference (9)). However, the first success in identifying a chemical intermediate derived from the substrate occurred with chymotrypsin, where the presence of a relatively stable acyl enzyme was demonstrated (10, 11). Stopped flow kinetics, steady state kinetics and chemical analysis led to the identification of an acylated serine (cf. reference (12)). This early work proved that some enzyme mechanisms go through detectable intermediates. However, these results were only the tip of the iceberg. Today we know that virtually every enzyme mechanism involves multiple intermediates of comparable stability that often can be detected. In fact, many years after the initial studies, the chymotrypsin mechanism was shown to involve several detectable reaction intermediates by using a combination of stopped flow and temperature jump techniques (12).

A major breakthrough in methodology occurred with the advent of relaxation kinetics (13). The time range available was extended from milliseconds with the stopped flow to microseconds with the temperature jump method and even to nanoseconds and picoseconds with other methods. The application of these methods proved especially fruitful in the 1960s and 1970s.

Ribonuclease A was one of the first enzymes to be studied with a wide array of kinetic and structural methods. It was particularly attractive because it is relatively small (molecular weight 13,683), is very stable, and could be obtained commercially in large quantities. Ribonuclease catalyzes the breakdown of RNA in two steps: the diester bond is cleaved to form a pyrimidine 2′,3′ cyclic phosphate; and the cyclic phosphate is hydrolyzed to give a terminal pyrimidine 3′ phosphate. The ribonuclease structure is kidney-shaped with a long N-terminal helical segment and a central β-pleated sheet (14). The unusual stability of the structure is due to four disulfide bonds. A groove in the enzyme at the interface of the two lobes of the kidney contains the binding pocket for substrates. High resolution structures have identified the specific interactions between the enzyme and substrates/substrate analogues (14).

Many steady state kinetic studies of this enzyme have been carried out with dinucleosides and pyrimidine 2′,3′ cyclic phosphates (15). The turnover numbers are in the range of 1 – 10^3 s^{-1} and the Michaelis constants are 1 – 5 mM. Extensive transient kinetic studies of the enzyme were carried out using stopped-flow, temperature jump, and, for the first time, a combined stopped flow-temperature jump (16). The results indicate that the enzyme exists in at least two conformations that are in dynamic equilibrium: this represents an opening and closing of the two flaps of the kidney around the active site with characteristic rate constants of about 100 – 3000 s^{-1}. When the substrate binds, the hinge closes, creating a hydrophobic environment. The catalytic reaction proceeds through a series of proton transfer reactions involving the imidazoles of His12 and His119, with a postulated pentacoordinated phosphorous intermediate. The hinge opens to release products to complete the catalytic cycle. The transient kinetic experiments determined the rate constants for the initial binding of substrates to enzymes and demonstrated that a conformational change followed the binding process. The second order rate constants for binding of the substrate are close to the diffusion controlled limit, and the rate constants associated with the conformational changes are 10^3 – 10^4 s^{-1}. Altogether six distinct conformational intermediates were identified for the overall reaction, along with two different conformations of the enzyme. These results emphasize the multiplicity of enzyme conformations and intermediates that are an essential part of the enzymatic mechanism. Structural studies of the enzyme and its complexes at low temperatures have been used to postulate a structural model for the reaction (17).

Two additional aspects of the ribonuclease reaction are worth mentioning. Even though His48 is not at the active site of the enzyme, its pK_a is altered as the hinge opens and closes (16). Also, if tyrosines distal to the active site are iodinated, the catalytic properties of the enzyme are altered (18). These results indicate that communication within the macromolecule occurs during catalysis, even relatively distant from the active site, and that the integrity of the macromolecule is important.

A second illustration of the evidence for the occurrence of multiple conformations and intermediates in enzyme catalysis is aspartate aminotransferase. This enzyme uses enzyme-bound pyridoxal phosphate as an integral part of the catalysis: the overall reaction is the transfer of an amino group from aspartate to ketoglutarate to give oxalacetate and glutamate. The mechanism involves the transfer of the amino group to pyridoxal phosphate to give a pyridoxamine phosphate intermediate. This intermediate then transfers the amino group to the keto acid to complete the reaction. The enzyme bound pyridoxal phosphate provides a convenient spectral probe to monitor the reaction progress. This enzyme from many different sources has been intensively studied with a variety of kinetic, spectroscopic, and structural techniques (cf. references (19–21)).

Transient kinetics and spectroscopic measurement have shown that the reaction proceeds by formation of an aldimine between the amino acid and pyridoxal phosphate. The aldimine

forms a quinoid structure that breaks down into a ketimine, which in turn forms pyridoxamine and the keto acid. The second half of the reaction reverses this process: the pyridoxamine reacts with the keto acid to form a ketimine, which ultimately breaks down into pyridoxal phosphate and the amino acid. A temperature jump study of the reaction of the mitochondrial enzyme with the substrate analogue *β-erythro*-aspartic acid has permitted the development of a detailed mechanism, including the associated rate constants and spectral properties of the intermediates (22). Briefly, the initial formation of the enzyme-substrate complex is followed by a conformational change. Next, the aldimine is formed, followed by the quinoid and ketimine in sequence. A conformational change then occurs that allows the product to dissociate. In all, seven intermediates have been observed and characterized for half of the transamination reaction. Fifteen reaction intermediates have been identified for the overall reaction mechanism!

Many years after the kinetic studies, structures of the enzyme and the enzyme complexed with various ligands provided structural evidence for all of the proposed intermediates (23). The conformational change following binding of the substrate was shown to be a closing of domains that sequesters the enzyme-substrate complexes from the solvent. Thus, the mechanism of action of aspartate aminotransferase, similar to that for ribonuclease, involves multiple intermediates and conformational changes. Evidence has also been obtained that residues outside of the active site can modulate the enzyme activity (24). Indeed, these are common themes for virtually all enzyme mechanisms that have been studied in detail.

Conformational Changes in Enzyme Catalysis

Thus far we have demonstrated that conformational changes are frequently observed as part of enzyme mechanisms. Almost all, if not all, substrate/specific ligand binding is associated with a conformational change (25). Moreover, additional conformational changes are frequently involved in the mechanism. For the binding of ligands, the mechanistic distinction is often made between a conformational change following binding ("induced fit", (1)) and the binding of a ligand to a conformation of the enzyme present in low concentrations ("conformation selection"). In fact, these two mechanisms are all part of a more general scheme, and the specific mechanism occurring depends on the concentrations of the reactants (2, 26). More important than this subtle mechanistic fine point is understanding the molecular nature of the conformational changes.

The conformational transitions observed are generally in the time range of ms or μs. Yet the interactions within the protein that are changing are noncovalent interactions, such as hydrogen bonding, electrostatics, and alteration of water structure. Studies with model systems have shown that the time scale of making/breaking such interactions are in the ns and ps time domains (cf. references (3, 4)). The reason that the time scale for the conformational changes associated with enzyme catalysis is so slow is that the conformational changes involve a large number of noncovalent interactions, i.e., they are cooperative in nature. In simple terms, this means that the initiation of the conformational change is rate limiting rather than the rates of the ensuing individual steps. From consideration of the time scale alone, the conformational changes associated with enzyme catalysis must be cooperative.

This cooperative, or coordinated, motion within the protein involves a large portion of the macromolecule. This can be deduced by the fact that enzyme structural alterations through chemical modification or site specific mutagenesis can dramatically alter the rates of enzyme reactions, even when these alterations are far from the active site. In addition, all enzymes are macromolecules. Many attempts have been made to reduce the size of enzymes, but thus far the minimum size of an efficient enzyme is about 10,000 daltons.

Moreover, attempts to design or synthesize molecules approaching the catalytic efficiency of enzymes have been unsuccessful thus far. Finally, as discussed later, theoretical calculations also indicate that the entire macromolecule is involved in the catalytic process.

Of course, noncooperative conformational transitions also occur within the enzyme, e.g., restricted rotations and vibrations. These transitions, similar to the elementary steps for hydrogen bonding, the making and breaking of water structure, and molecular vibrations, are very rapid, typically in the ns to ps time region. These motions are undoubtedly altered by mutations and cooperative conformational changes, but they are unlikely to be directly involved in the dynamics of enzyme catalysis: they are orders of magnitude faster than the time scale of enzyme catalysis so that noncooperative conformational changes are essentially at equilibrium during catalysis. Note that these noncooperative motions are distinct from the very rapid motions corresponding to passage over the top of the free energy barrier (i.e., through the dividing surface separating reactants and products), which are required for the chemical reaction but also are unlikely to be rate determining for enzyme catalysis.

Why are cooperative conformational changes and multiple reaction intermediates prevalent in the mechanism of action of enzymes? In many cases, the conformation following the initial binding of substrate "closes" over the active site and drastically alters the environment, e.g., solvent water is excluded, the dielectric constant is reduced, and pK_a's are altered. These factors all may be important for the subsequent catalysis. The presence of multiple intermediates in enzyme mechanisms suggests that the enzyme functions by creating multiple steps with low standard free energies of activation, as contrasted to one-step, or few-step, reactions with relatively high standard free energies of activation. An enzyme is able to do this because of its *structural flexibility* or *conformational adaptability*. The enzyme can optimize its structure for each step in the reaction sequence.

Finally, the simultaneous making and breaking of noncovalent interactions within the protein in conjunction with the making and breaking of substrate chemical bonds may be an effective means of reducing the standard free energy of activation and also provides thermodynamic compensation during the course of the reaction (27). For example, the standard free energy barrier for bond breaking within a substrate can be lowered by occurring simultaneously with the formation of free energy favorable interactions within the protein. Similar compensation can occur within the protein in conjunction with conformational changes. This type of compensation also can occur at the level of thermodynamics. These multiple cooperative changes serve to provide an optimal structure for the next step in catalysis, but to our knowledge, there is no direct evidence that such changes are directly coupled to the actual catalysis. However, *compensation* is an important concept to keep in mind.

A more detailed picture of enzyme catalysis for a specific enzyme, dihydrofolate reductase (DHFR), is now developed, along with the concept of conformational ensembles to provide a comprehensive description of the catalytic process.

Dihydrofolate Reductase: Experiments

DHFR is a key enzyme essential for the synthesis of purines, some amino acids, and thymidine. This enzyme catalyzes the stereospecific transfer of a hydride from C4 of NAPDH (NH) to the C6 position of 7,8-dihydrofolate (H_2F) to form 5,6,7,8-tetrahydrofolate (H_4F) (Figure 1A) and $NADP^+$ (N^+). A detailed kinetic scheme for the *E. coli* DHFR has been determined, as shown in Figure 1B (28). Under cellular conditions of substrate and cofactor, the enzyme cycles through five intermediates: E.NH.H_2F, E.N^+.H_4F, E.H_4F, E.NH.H_4F, and E.NH with the rate-limiting step being the release of H_4F from the

E.H_4F.NH complex. Large conformational changes are observed for the Met 20 loop (residues 9 to 24) that is found in a closed conformation for the E.NH and surrogate Michaelis complex (E.N^+.F) for E.NH. H_2F and in an occluded conformation for the E.N^+H_4F, E.H_4F, and E.NH.H_4F product complex. In the closed conformation, the loop packs tightly against the nicotinamide ring of the cofactor; in the occluded conformation, the loop protrudes into the active site pocket, extruding the nicotinamide. These conformational changes are depicted in Figure 2 (29).

Kinetic analysis of single mutations distal to the active site revealed significant effects on several steps in the cycle. Restricting our analysis to the step associated with hydride transfer, for example, the mutant protein G121V exhibited a 200-fold decrease in the step associated with the hydride transfer (30). When paired with S148A, the effect on this step was non-additive, suggesting a coupled interaction. Collectively, such data support the hypothesis of coupled conformational changes occurring throughout the protein that accompany progress along the reaction coordinate.

Nuclear magnetic resonance relaxation dispersion was used to characterize the five reaction cycle complexes described above (31). Each intermediate in the cycle samples low-lying excited states whose conformations resemble the ground-state structures of the preceding and following intermediates (Figure 3). The rate constants obtained by pre-steady state and competitive trapping experiments are in remarkable agreement with those measured for the transition between the low-lying excited states and the present ground-state structures. These experiments are consistent with the hypothesis that ligand release, as well as the chemical step, is governed by the rates of conformational changes in the reaction cycle. This hypothesis does *not* imply, however, that the conformational changes influencing the ligand release are related to the conformational changes influencing the chemical step.

Furthermore, stopped flow and single molecule experiments with fluorescent labeled DHFR suggest that the mechanism depicted in Figure 1B needs to be expanded to accommodate more enzyme conformations (32, 33). In these experiments, two constructs were prepared. In construct A, a fluorescence probe was placed on a residue for which the environment was expected to change during the catalytic cycle (residue 18 of the *E. coli* enzyme). In construct B, a fluorescence resonance energy transfer pair was placed on residues 17 and 37 of the *E. coli* enzyme. Measurements were made of the turnover number, the hydride transfer rate when NADPH and H_2F were added to the enzyme (stopped-flow), the rate of fluorescence change when the enzyme was mixed with NADPH and DHF (stopped-flow), and single molecule fluorescence changes in the presence of saturating concentrations of all substrates. In addition, measurements were made with constructs in which glycine121 was changed to valine. As previously mentioned, this mutation, although not at the active site, significantly lowers the activity of the enzyme. In some cases, the hydrogen/deuterium isotope rate effect was also measured by replacing the transferring hydrogen in NADPH to deuterium.

Selected results are shown in Table 1, where the first order rate constants are summarized for a variety of enzyme derivatives, as obtained for the reactions described above. In some cases, the fluorescence changes measure the rate of hydride transfer, whereas in other cases they do not. For the mutant enzymes, this is also true, but the rate changes do not always parallel what is seen with the “native” fluorescent enzymes. The same situation prevails for the single molecule results. The simplest interpretation of these results is that multiple conformational changes are being observed. In some cases, the three methods of detection measure the same rate constant, whereas in other cases they measure the rate constants for different conformational changes. Moreover, mutations may cause different conformations to be populated, and different conformational changes may be detected than with “native” fluorescent constructs.

These results can be understood in terms of the concept that proteins exist in a scaffold of conformations with different population distributions existing under different conditions. Furthermore, the population distributions change as the catalytic cycle proceeds. This is consistent with the NMR results previously discussed. To accommodate these findings, the traditional Michaelis-Menten mechanism must be expanded to accommodate multiple conformations and parallel paths. For a single substrate(S)-single product(P) reaction, this expanded mechanism can be written as in Scheme 1, where E_i is the free enzyme, and A_i, B_i, … are reaction intermediates. A discussion of the implication of this mechanism for the standard free energy profile of the catalytic reaction, along with theoretical aspects of enzyme catalysis, is presented in the next section.

Dihydrofolate Reductase: Theory and Concepts

The hydride transfer reaction catalyzed by DHFR has been studied with a wide range of theoretical methods (34–37). Here we focus on hybrid quantum/classical molecular dynamics simulations of this reaction (34, 35). In these simulations, an empirical valence bond potential and umbrella sampling methods were used to generate the free energy profile along a collective reaction coordinate that includes motions of the enzyme, substrate, and cofactor (Figure 4). In general, the free energy barrier corresponds to the relative probabilities of sampling configurations at the top of the barrier compared to sampling those at the reactant minimum. The impact of site-specific mutations on the free energy barrier has also been investigated with this hybrid quantum/classical molecular dynamics approach (38, 39).

Based on these simulations, as well as experimental evidence, we have proposed that stochastic thermal motions throughout the enzyme, substrate, and cofactor lead to conformational sampling of configurations that facilitate hydride transfer by bringing the donor and acceptor closer together, orienting the substrate and cofactor properly, and providing a favorable electrostatic environment (34, 40, 41). These thermal motions typically occur on the pico- to nanosecond timescales and are Brownian in nature, although they occur within the confines of the protein structure. To our knowledge, there is no evidence that these fast motions are directly coupled to the chemical reaction (i.e., the bond breaking and forming) or to the barrier crossing. However, these fast thermal motions lead to overall conformational changes that occur on a slower timescale along the collective reaction coordinate, i.e., cooperative conformational changes. For example, Figure 4 depicts thermally averaged structures along the collective reaction coordinate and indicates that the average hydride donor-acceptor distance decreases as the reaction progresses from the reactant minimum to the top of the free energy barrier, often denoted the “transition state” in these types of calculations. Note that very fast motions are associated with passage through the dividing surface separating reactants and products, typically at the top of the free energy barrier. Rapid motions through this surface are required for all chemical reactions but are unlikely to be rate determining for enzyme catalysis.

According to this perspective, the experimentally measured millisecond timescale for hydride transfer in DHFR is related to the average time required to sample the configurations that are conducive to the hydride transfer reaction (40, 41). This perspective is consistent with the standard definition of the free energy barrier as the probability of sampling configurations at the top of the barrier relative to those in the reactant minimum. The actual chemical bond breaking and forming, or barrier crossing, occurs virtually instantaneously relative to the millisecond timescale required for the conformational sampling. In other words, the chemical reaction is a rare, yet rapid, event that occurs only after sufficient conformational sampling to generate a configuration conducive to the chemical reaction. We emphasize that the conformational changes along the collective

reaction coordinate may occur on the millisecond time scale measured experimentally, but they should *not* be viewed as millisecond vibrational motions that are *dynamically* coupled to the hydride transfer reactions. In fact, there is no evidence of protein vibrational motions, on any timescale, that are directly coupled to the chemical reaction in DHFR to the best of our knowledge.

According to transition state theory, the rate constant of a chemical reaction depends exponentially on the associated free energy barrier. The transmission coefficient can be calculated to account for the dynamical barrier recrossings. Hybrid quantum/classical molecular dynamics simulations of the hydride transfer reaction catalyzed by DHFR indicate that the transmission coefficient is nearly unity, so these dynamical barrier recrossings do not play an important role in this system (35, 36). Thus, the free energy barrier along the collective reaction coordinate for hydride transfer primarily determines the hydride transfer rate constant. In general, enzyme reactions should be viewed in a multidimensional free energy landscape, as in Figure 5, which depicts axes corresponding to the reaction coordinate and to ensembles of conformations (42). Note that conformational changes occur along both axes. The conformational changes occurring along the reaction coordinate axis correspond to those changes that facilitate the hydride transfer reaction. In contrast, the conformational changes occurring along the ensemble conformations axis do not directly impact the free energy barrier for hydride transfer.

Within this framework, the impact of mutations on the hydride transfer rate constant can be understood in terms of the free energy barrier along the collective reaction coordinate for hydride transfer (38, 39). The mutation alters the potential energy surface on which the protein, ligand, and substrate move, thereby altering the relative probabilities of conformational sampling in the various regions of the multidimensional surface. Thus, the mutation changes the relative probability of sampling configurations at the top of the barrier (i.e., those conducive to hydride transfer) relative to those at the reactant minimum, leading to a change in the free energy barrier. Because the rate constant is related exponentially to the free energy barrier along the reaction coordinate, increasing this free energy barrier will decrease the rate constant.

The proposal that mutations alter the “reorganization energy” (43) is consistent with this view. By definition, reorganization implies motion; if the environment were completely frozen, the reorganization energy would be zero. In Marcus theory for electron transfer (44), the reorganization energy is related to the change in the solvent polarization, which involves reorientation of the solvent molecules. For electron transfer, these motions occur along the energy gap reaction coordinate, defined as the difference between the energies of the reactant and product diabatic electron transfer states. Analogously, in a protein, the reorganization energy is related to motions of the protein and ligands. For a hydride transfer reaction, these motions can be viewed to occur along the collective reaction coordinate defined as the difference between the energies of the reactant and product diabatic hydride transfer states (i.e., the valence bond states), as shown in Figure 4. Thus, reorganization is simply another term for the conformational changes along the collective reaction coordinate that facilitate hydride transfer, particularly those that lead to a conducive electrostatic environment.

Preorganization has also been proposed as an important aspect of enzyme catalysis that may be altered by mutation (43). Preorganization of the active site allows the enzyme to effectively bind the substrate and cofactor and orient them properly. Subsequently, however, typically further *reorganization* of the active site is required for the reasons discussed above. For example, in DHFR, the hydride donor-acceptor distance is too large for effective hydrogen transfer in the equilibrium reactant state of the ternary complex. Further

reorganization of the environment leads to configurations that are conducive to hydride transfer, e.g., the hydride donor-acceptor distance becomes smaller (Figure 4). In principle, a mutation could affect both the preorganization and the reorganization of the enzyme active site, or possibly mainly one or the other.

Some controversy has arisen with respect to the interpretation of the experiments in Ref. (29). In this case, the N23PP/S148A mutations altered the conversion from the closed to occluded states of the Met20 loop (Figure 2), as well as the hydride transfer rate constant. Clearly the decrease in the hydride transfer rate constant is due to an increase in the free energy barrier along the collective reaction coordinate for hydride transfer. The increase in this free energy barrier arises from the alteration of the potential energy surface, which influences the relative probabilities of conformational sampling along this collective reaction coordinate. The fact that it also influences the relative probabilities of sampling along another reaction coordinate, namely the coordinate corresponding to the transition between the closed and occluded states of the Met20 loop, is not directly relevant to the change in the hydride transfer rate constant. This observation simply confirms that the potential energy surface, and therefore the free energy landscape, has been significantly altered by the mutation. This view appears to be consistent with the perspective of Ref. (43), despite the use of different terminology.

Finally, mention should be made that the theoretical calculations of hydride transfer in DHFR begin with a defined crystallographic structure. They presently do not take into account that the enzyme may in fact exist as a distribution of conformations at the beginning of and during the catalytic cycle (Scheme 1).

Concluding Remarks

In assessing our knowledge of enzyme catalysis, it is critical to ask what level of understanding is desired. In terms of standard free energy surfaces, the evidence is convincing that a multidimensional surface is required with many minima corresponding to reaction intermediates and/or conformations of the protein. The very shallow minima correspond to various side chain conformations, whereas the deeper minima correspond to conformations with significant changes in the relative positions of atoms within the enzyme and/or ligands. Unfortunately Figure 5 is woefully inadequate: to show a “real” free energy surface for enzyme catalysis, many more minima and maxima are needed.

In a strict sense, every possible conformation of a protein is always present, although most conformations are present in vanishingly small amounts. During catalysis, the population distribution of conformations changes as catalysis proceeds. This is, in fact, a good description of the catalytic process, i.e., sequential shifts in the population distribution of enzyme conformations. The multiplicity/diversity of conformations and reaction intermediates is now an accepted concept, amply demonstrated by the few examples discussed here. The ability of a protein to have many different conformations is due to its great flexibility. For catalysis, flexible but not too flexible, as well as rigid but not too rigid, is essential. Specifically, the protein must be rigid enough to maintain the required structure, but flexible enough to permit atomic movements as the reaction proceeds.

For a chemist, however, viewing catalysis in terms of its standard free energy surface does not suffice. We want to know how movement occurs between minima on the free energy surface and how the structure of the enzyme changes as catalysis proceeds. Moving between shallow minima is generally uninteresting, but moving between deeper minima requires cooperative conformational changes and significant readjustment of atomic coordinates within the enzyme and ligands. In this regard, tenths of Angstroms can be considered significant. Obtaining knowledge of these aspects of enzyme catalysis requires structural

and kinetic experiments, along with theoretical calculations. The results obtained with DHFR are illustrative examples of what can be achieved with modern techniques.

Our understanding of enzyme catalysis continues to evolve. We now know that a diverse population of protein conformations is required, which in turn comes about because of the incredible flexibility of protein structures. The prevalence of cooperative conformational changes makes use of the entire protein and implies stable (but not too stable) conformations/reaction intermediates. Thus, flexibility, diversity, and cooperativity are the pillars of enzyme catalysis. Yet much remains to be done – ultimately we want to know what every atom in the molecule is doing as catalysis proceeds. This is a formidable goal for the future.

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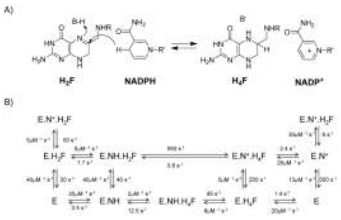


Figure 1.

The chemical reaction catalyzed by *E.coli* DHFR (A), and the pH independent kinetic scheme for DHFR catalysis (B). Notation is as follows: DHFR (E), NADPH (NH), $NADP^+$ (N^+), 7,8-dihydrofolate (H_2F), 5,6,7,8-tetrahydrofolate (H_4F). Figure modified from Ref. (28).

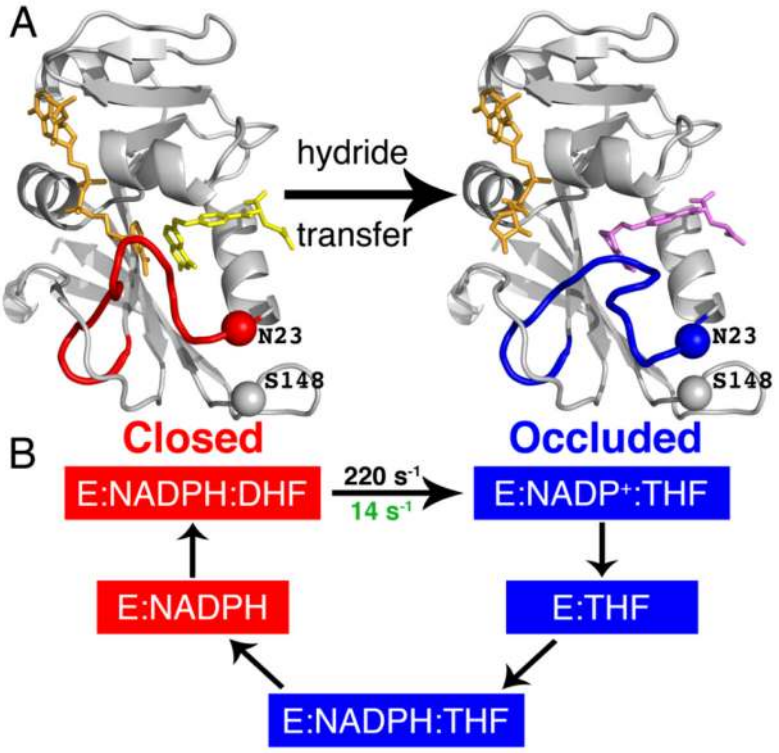


Figure 2.

Conformational changes that occur during the *E. coli* DHFR catalytic cycle. (**A**) Left, cartoon representation of E:NADP$^+$:FOL crystal structure (1RX2, model for the Michaelis complex, E:NADPH:DHF) in the closed conformation. Right, crystal structure of E:NADP$^+$:ddTHF (1RX4, model for the product complex) in the occluded conformation. NADP$^+$ is shown in orange, FOL in yellow, ddTHF in magenta, Met20 loop in the closed conformation in red, and Met20 loop in the occluded conformation in blue. The sites of mutation, N23 and S148, are shown as spheres. (**B**) Intermediates in the wild type *E. coli* DHFR catalytic cycle. Intermediates shown in red are in the closed conformation, and those in blue are in the occluded conformation. Prior to hydride transfer the Met20 loop is in the closed conformation, where it packs tightly against the nicotinamide ring of NADP$^+$. Following hydride transfer, the Met20 loop adopts the occluded conformation, in which the nicotinamide ring of NADP$^+$ is sterically hindered from binding in the active site. NADP$^+$ undergoes a concurrent conformational change in which the nicotinamide ring is expelled from the binding pocket, initiating NADP$^+$ release from the ternary product complex. The rates of hydride transfer in the wild type and N23PP/S148A mutant enzyme are indicated in black and green, respectively. Note that the mutation alters the pathway utilized for product and NADP$^+$ release. Figure and portions of caption reproduced from Ref. (29).

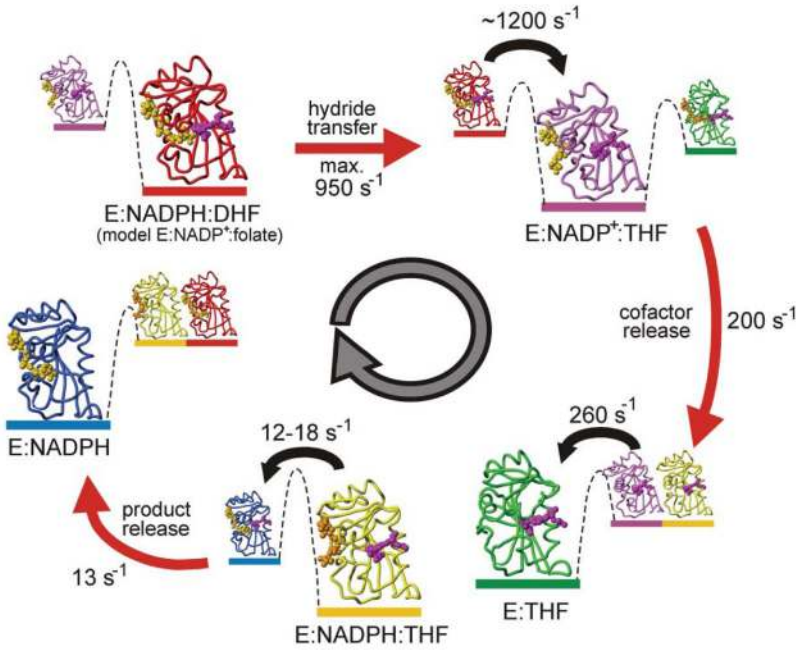


Figure 3.

The catalytic cycle for DHFR catalysis. Ground state (larger) and higher energy (smaller) structures of each intermediate in the cycle, modeled on the published X-ray structures, are shown with NADPH and $NADP^+$ shown in gold and substrate, product, and analogs shown in magenta. For each intermediate in the catalytic cycle, the higher energy conformations detected in the relaxation dispersion experiments resemble the ground-state conformations of adjacent intermediates; their interconversion rates, also obtained from the relaxation dispersion experiments, are shown with black arrows. Rate constants for the interconversion between the complexes, measured by pre-steady-state enzyme kinetics at 298 K, pH 6, are indicated with red arrows. R_2 relaxation dispersion measurements were made at pH 6.8 ($E:NADP^+$:folate) or pH 7.6 (E:NADPH:THF, $E:NADP^+$:THF, E:NADPH, and E:THF) at 281 K (E:NADPH), 300 K (E:NADPH:THF, $E:NADP^+$:THF, and E:THF), or 303 K ($E:NADP^+$:folate). Figure and portions of caption reproduced from Ref. (31).

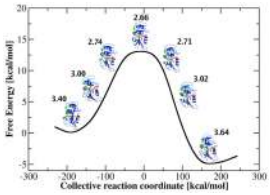


Figure 4.

Free energy profile of the DHFR-catalyzed hydride transfer reaction as a function of a collective reaction coordinate defined in terms of the energy difference between the reactant and product valence bond states. This reaction coordinate includes motions of the enzyme, substrate, and cofactor. The magnitude of the free energy barrier is determined by the relative probabilities of sampling the transition state and the reactant configurations. The thermally averaged equilibrium structures, as well as the average donor-acceptor distances in Angstroms, are provided for selected values of the reaction coordinate. Note that the donor-acceptor distance decreases as the reaction evolves from the reactant to the transition state. The conformational changes along the collective reaction coordinate are attained by equilibrium, stochastic, thermal motions occurring within the confines of the protein fold. These conformational changes facilitate the hydride transfer reaction by bringing the donor and acceptor closer together, orienting the substrate and cofactor properly, and providing a favorable electrostatic environment. Figure and portions of caption modified from Ref. (40).

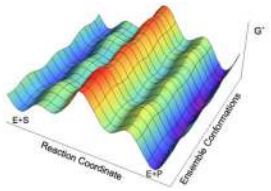
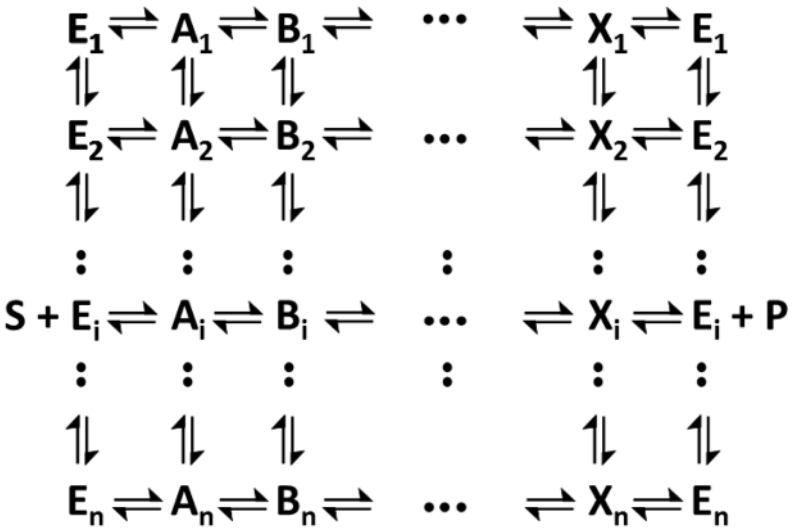


Figure 5.

Schematic representation of the standard free energy landscape for the catalytic network of an enzyme reaction. Conformational changes occur along both axes. The conformational changes occurring along the Reaction Coordinate axis correspond to the environmental reorganization that facilitates the chemical reaction. In contrast, the conformational changes occurring along the Ensemble Conformations axis represent the ensembles of configurations existing at all stages along the reaction coordinate, leading to a large number of parallel catalytic pathways. The reaction paths can slide along and between both coordinates. For real enzymes the number of maxima and minima along the coordinates is expected to be greater than shown. The free energy landscape and dominant catalytic pathways will be altered by external conditions and protein mutations. Figure and portions of caption reproduced from Ref. (42).



Scheme 1.
Schematic Mechanism for Enzyme Catalysis with Multiple Intermediates and Conformations

Table 1

Rate Constants for DHFR Catalysis

Construct	k_{ht} (s^{-1})	$k_{ensemble}$ (s^{-1})	KIE	$k_{singlemolecule}$ (s^{-1})	KIE
A[a]	146	130	2.8	180	2.3
A, G121V[b]	1.3	33	No	ND	
B[c]	210	280	No	210	No
B, G121V[c]	3.5	154	ND	145	ND

k_{ht} is the hydride transfer rate constant from NADPH to DHF, $k_{ensemble}$ is the rate constant for the change in fluorescence when enzyme is mixed with NADPH and DHF, $k_{singlemolecule}$ is the ensemble rate constant observed in single molecule assays when the enzyme is mixed with saturating concentrations of substrates. KIE indicates whether a kinetic isotope effect was observed. ND designates not determined.

[a] Reference (32), Alexa 488 at residue 18 of the *E. coli* enzyme.

[b] Unpublished results, N. M. Goodey, S. J. Benkovic, and G. G. Hammes.

[c] Reference (33), fluorescence resonance energy transfer pair at residues 17 and 37 of the *E coli* enzyme.

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