New Concepts

Free-Energy Landscape of Enzyme Catalysis[†]

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ABSTRACT: The concept is developed that enzyme mechanisms should be viewed as "catalytic networks" with multiple conformations that occur serially and in parallel in the mechanism. These coupled ensembles of conformations require a multi-dimensional standard free-energy surface that is very "rugged", containing multiple minima and transition states. Experimental and theoretical evidence is presented to support this concept.

Understanding the role of protein conformational changes in enzyme catalysis has been a long standing goal of enzymologists, and recent advances in experimental methodology and theory have provided new insight into this undertaking (*1–7*). Dynamic fluctuations in protein structure occur over a wide range of times, typically femtoseconds to seconds. However, the observed time scale for enzyme catalysis and the interconversion of reaction intermediates is in the millisecond−microsecond range, so that structural changes in this time regime are most likely to be of significance in catalysis. Unliganded enzymes often exist in multiple conformations, and the binding of substrates and/or coenzymes elicit changes in conformation. Moreover, as the reaction proceeds, some structural reorganization of the enzyme–substrate complexes must occur as the enzyme proceeds through the transition states to the product. Indeed, the structures of enzymes in different conformations (typically different substrate- or substrate analogue-bound states) have been solved by X-ray crystallography and nuclear magnetic resonance (NMR). However, the chronological order of the multiple conformational transitions along the reaction coordinate and their relationship to enzyme catalysis are just beginning to be understood.

At the present time, a large number of different tools exist for probing the conformations of enzymes during catalysis. Predominant among these efforts are steady-state and transient kinetics (*2, 3, 6, 8*), single-molecule kinetics (*9, 10*), and NMR (*11–15*). In the former cases, the ability to attach fluorescent probes at various positions in the enzyme has been of great importance in probing different regions of the enzyme, whereas in the case of NMR, dipolar coupling techniques have permitted kinetic measurements to be made at longer times than previously, i.e., microseconds (*11*). In concert with experimental developments, advances in theory have permitted atomic-level molecular dynamics simulations of enzyme reactions, providing information about the movement of individual amino acid residues as the reaction proceeds (*16–19*). The use of transition-state theory to discuss enzymatic reactions is long standing, and a familiar perspective is that enzymes lower the standard free energy of activation relative to the uncatalyzed reaction. Although this is not an explanation of enzyme catalysis in itself, the framework of transition-state theory is often convenient for describing the energetics of a reaction as it proceeds from reactants to products.

In this paper, we synthesize recent results to provide a unifying picture of enzyme catalysis. As an example, we use results obtained for dihydrofolate reductase, but the conclusions reached are applicable to enzyme catalysis in general. In brief, enzymes must be considered as existing in ensembles of coupled conformational states [this view has long been accepted in the field of protein folding (*20*)]. The compositions of the ensembles are altered as ligand binding and catalysis occur. In this work, we develop the concept that the free-energy description of enzyme catalysis cannot be described in two dimensions but requires a multidimensional free-energy landscape that is very rugged with multiple minima and transition states. Thus, enzyme reactions can be regarded as operating through "catalytic networks" to achieve their remarkable efficiency.

DIHYDROFOLATE REDUCTASE

Dihydrofolate reductase (DHFR)[1] catalyzes the reduction of dihydrofolate by NADPH to give tetrahydrofolate and $NADP^+$. It has been extensively studied by a variety of methods, including kinetics, X-ray crystallography, NMR, and theoretical calculations (*21–33*). Multiple conformations have been observed with all of these methods. On the basis of these studies,

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[1] Abbreviations: DHFR, dihydrofolate reductase; FRET, fluorescence resonance energy transfer.

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the mechanism of DHFR catalysis is believed to involve multiple conformational changes, including domain rotation and the motion of structural loops. It is a particularly attractive target for study because of the ease of site-specific mutagenesis and the ability to place fluorescent probes specifically on different parts of the molecule. In addition, whether or not a conformational change is directly coupled to the hydride transfer can be determined by direct observation through the spectral changes of a tryptophan residue and the accompanying isotope rate effect (*21*).

As a starting point, the mechanism proposed for DHFR in 1987 is presented in Figure 1 (*21*). This mechanism is based on a variety of thermodynamic and kinetic studies and presents clear-cut evidence for a multiplicity of enzyme–substrate/product species and parallel paths in the catalytic process. A further important point to note is that site-specific mutations far from the catalytic site profoundly affect the rate of hydride transfer, e.g., G121V (*34, 35*). This strongly suggests that motions of the entire molecule are essential for the catalytic process. A fluorescence probe has been inserted at amino acid 18, which is on a loop that opens and closes over the active site, so that the fluorescence is sensitive to the environment (*25, 36*). DHFR with a fluorescence resonance energy transfer (FRET) pair at residues 17 and 37 also has been studied; the fluorescence in this case is sensitive to distance changes between the labels, i.e., the internal motion of the enzyme (*27*) (residue 37 is not near the catalytic site). The structure of the modified enzyme is shown schematically in Figure 2. Stopped-flow, steady-state, and single-molecule fluorescence microscopy have been carried out with both of these modified enzymes. In brief, conformational changes have been observed that are associated with NADPH and dihydrofolate binding. In addition, conformational changes are observed with single-molecule experiments in an equilibrium mixture of enzyme and all of the substrates. Direct coupling of the conformational changes to hydride transfer does not always occur, but rates of the conformational changes are altered in the G121V mutant, for which the hydride transfer rate decreases by a factor of more than 100 (*34, 35*).

These results suggest that the catalytic process involves a multitude of conformations and numerous parallel reaction paths. For a single substrate−single product reaction, the mechanism can be represented as (*27*):

$$\begin{array}{ccccccccccc} E_1 & \rightleftarrows & A_1 & \rightleftarrows & B_1 & \rightleftarrows & \cdots & \rightleftarrows & X_1 & \rightleftarrows & E_1 \\ \updownarrow & & \updownarrow & & \updownarrow & & & & \updownarrow & & \updownarrow \\ E_2 & \rightleftarrows & A_2 & \rightleftarrows & B_2 & \rightleftarrows & \cdots & \rightleftarrows & X_2 & \rightleftarrows & E_2 \\ \updownarrow & & \updownarrow & & \updownarrow & & & & \updownarrow & & \updownarrow \\ \cdots & & \cdots & & \cdots & & \cdots & & \cdots & & \cdots \\ \updownarrow & & \updownarrow & & \updownarrow & & & & \updownarrow & & \updownarrow \\ S + E_m & \rightleftarrows & A_m & \rightleftarrows & B_m & \rightleftarrows & \cdots & \rightleftarrows & X_m & \rightleftarrows & E_m + P \\ \updownarrow & & \updownarrow & & \updownarrow & & & & \updownarrow & & \updownarrow \\ \cdots & & \cdots & & \cdots & & \cdots & & \cdots & & \cdots \\ \updownarrow & & \updownarrow & & \updownarrow & & & & \updownarrow & & \updownarrow \\ E_n & \rightleftarrows & A_n & \rightleftarrows & B_n & \rightleftarrows & \cdots & \rightleftarrows & X_n & \rightleftarrows & E_n \end{array}$$

For simplicity, only a single substrate, S, and product, P, are shown. In this schematic equation, the E_i represent conformation ensembles of the enzyme, the A_i, B_i, . . . X_i represent conformation ensembles of enzyme–substrate complexes that are sequential in the catalytic pathway, and parallel catalytic pathways exist, e.g., $A_1 \rightarrow B_1 \rightarrow \ldots \rightarrow X_1$ and $A_2 \rightarrow B_2 \rightarrow \ldots \rightarrow X_2$. Unpublished results with several other fluorescent DHFRs with probes strategically placed around the molecule provide further data of a similar nature to support such a mechanism (*37*). An analogous mechanism has been proposed on the basis of a theoretical analysis of single-molecule measurements (*38*).

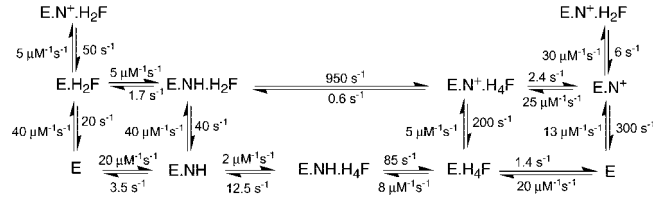

FIGURE 1: The pH-independent kinetic scheme for DHFR catalysis at 25 °C. This scheme pertains to the reaction at pH less than 7. E is DHFR; NH is NADPH; N^+ is $NADP^+$; H_2F is dihydrofolate; and H_4F is tetrahydrofolate. The figure was reproduced with permission from ref *21*.

FIGURE 2: Structure of the double-labeled *Escherichia coli* DHFR with bound $NADP^+$ (green) and folic acid (purple; PDB code 1rx2). The amino acids labeled with a fluorescent probe, amino acid 37, and a fluorescence energy acceptor, amino acid 17, are indicated. Also shown is amino acid 121; the mutation of this residue is discussed in the text. The figure was reproduced with permission from ref *27*.

Recent NMR experiments with DHFR using dipolar coupling have been interpreted in terms of the mechanism in Figure 1 (*12*). These results not only demonstrate the existence of multiple conformations but also suggest that these conformations are pre-existing in the molecule and become differentially populated as the reaction proceeds. Furthermore, the conformations interconvert at rates consistent with their involvement in catalysis. Similar results have been obtained in NMR studies of other enzymes (*13–15*). In earlier NMR experiments with DHFR, very fast motions on the nanosecond time scale were observed and were found to be altered in the G121V mutant (*39*). These fast motions should be differentiated from those occurring on the catalytic millisecond time scale. Typically, these fast motions are associated with

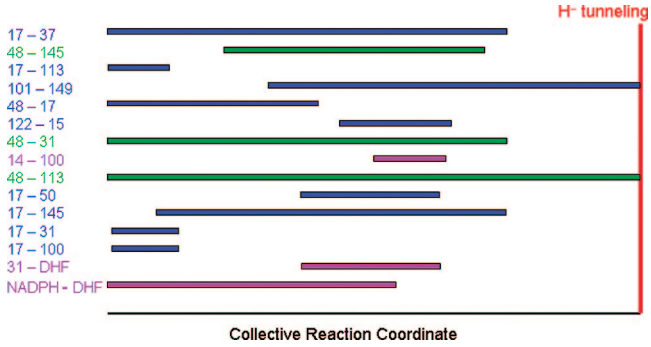

FIGURE 3: Analysis of thermally averaged distances in DHFR from hybrid quantum/classical molecular dynamics simulations, illustrating that the motions form a complex coupled network of distinct yet overlapping segments along the collective reaction coordinate. The bars indicate the region of the collective reaction coordinate over which the distance between the specified residues/ligands changes. Only the region of the collective reaction coordinate from the reactant to the hydride tunneling is shown, and the hydride tunneling is indicated by a red vertical line. Here, blue indicates an increasing distance; purple indicates a decreasing distance; and green indicates a distance that both increases and decreases over this region.

thermal fluctuations of single or several amino acid residues and are altered during catalysis because of changes in their local environments. However, these motions are not directly coupled to catalysis in a dynamic sense: because of the much slower time scale for catalysis, an average conformation of these residues is seen by the ensemble of molecules during catalysis (i.e., the fast motions can be considered to be at equilibrium for the slower ensemble reaction). On the other hand, the equilibrium (i.e., thermally averaged) positions associated with these residues may be altered during the catalytic cycle. Consequently, these types of conformational changes can be of importance in shaping the free-energy landscape of the catalyzed reaction and are thought to be of mechanistic significance (*5, 29*).

Recent theoretical studies of the DHFR reaction using hybrid quantum/classical molecular dynamics have elucidated the movement of specific amino acids during the hydride transfer reaction (*29–31*). These motions occur along a collective reaction coordinate representing the conversion of E−NADPH−H_3F^+ to E−$NADP^+$−H_4F (H_3F^+ and H_4F are protonated dihydrofolate and tetrahydrofolate, respectively). Some of the data obtained from these simulations that are particularly relevant to the FRET experiments are shown in Figure 3. The thermally averaged distances between pairs of atoms in the amino acids, the substrate, and the cofactor are altered significantly during the reaction. As expected, some distances increase, some decrease, some are unaltered, and some both increase and decrease. For example, the simulations indicate that the distance between the substrate and cofactor measured at the donor and acceptor carbons decreases to ~2.7 Å and levels off before hydride tunneling occurs. Other pairs of positions change at different regions along the collective reaction coordinate. Clearly some

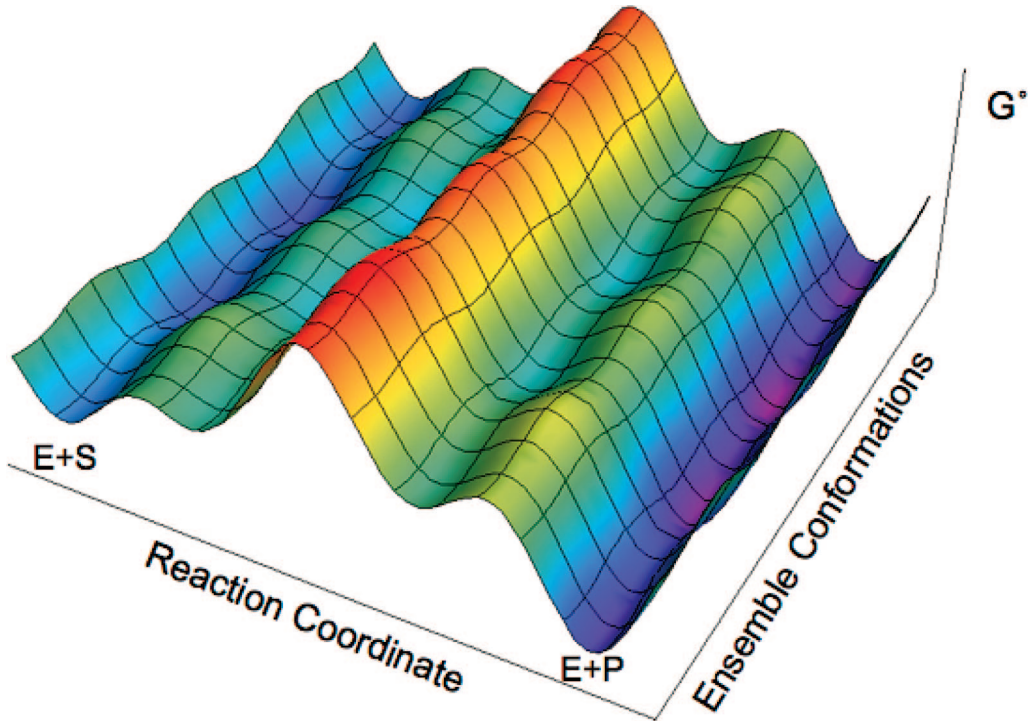

FIGURE 4: Schematic representation of the standard free-energy landscape for the catalytic network of an enzyme reaction. Conformational changes occur along both axes. The conformational changes occurring along the reaction coordinate axis correspond to the environmental reorganization that facilitates 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. If a plane parallel to the axis labeled "ensemble conformations" bisects this catalytic mountain range by cutting along the red mountain top, the substrates (E + S) are on one side of the plane and the products (E + P) are on the other. This figure illustrates the multiple populations of conformations, intermediates, and transition states. Strong coupling can occur between the reaction coordinates and the conformation ensembles; i.e., 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 dominant catalytic pathways will be altered by external conditions and protein mutations. The figure was created by Sarah Jane Edwards.

changes could be viewed as stepwise and perhaps causal (i.e., where an initial sequential movement could be required for a later one). Other changes happen in parallel, leading to a complex coupled network of motions. These coordinated (or cooperative) conformational changes are essential for catalysis. Note that these conformational changes are induced by the stochastic thermal motions (i.e., conformational sampling) of the enzyme. These results also predict that alteration of amino acids far from the catalytic site could greatly affect the reaction rate by changing the conformational sampling (*40, 41*), consistent with the experimental observations cited earlier.

Conformational changes are observed in both experiments and simulations. The conformational changes seen at equilibrium in the experiments must have standard free-energy differences on the order of RT, where R is the gas constant and T is the temperature, for both conformations to be significantly populated at thermal equilibrium. Conformational fluctuations also occur in the molecular dynamics simulations. In generating a standard free-energy curve along a reaction coordinate, however, averaging over an ensemble of configurations occurs and the configurations are "binned" along the reaction coordinate, so that a smooth curve is obtained. The entire free-energy curve can be generated by running a series of independent simulations with biasing potentials and umbrella-sampling techniques (*30*). Thus, the fast femtosecond−picosecond fluctuations are thermally averaged in generating a standard free-energy curve. The slower microsecond−millisecond conformational changes cannot be directly observed in the molecular dynamics simulations, but equilibrium conformational changes correlated to the chemical reaction can be deduced by analyzing the changes in the thermally averaged structures as the system evolves from the reactant to the product. Some of these conformational changes may be related to the experimentally observed conformational changes.

UNIFYING CONCEPT

When these results are taken together, they suggest a reassessment of how enzyme catalysis occurs and should be depicted. They suggest the existence of multiple sequential intermediates that occur as catalysis proceeds. In addition, each of these intermediates is associated with an ensemble of conformations, leading to parallel reaction paths. Some of these ensemble members/intermediates may be in rapid equilibrium (i.e., on the fast time scales observed with NMR), whereas others may equilibrate at rates comparable to catalysis and/or hydride transfer itself. Furthermore, the dominant pathways that are followed will undoubtedly change with reaction conditions. For example, in the case of DHFR, hydride transfer is rate-limiting at some pH values, whereas product dissociation is rate-limiting at other pH values (*21*).

In discussing enzyme catalysis, the standard free energy of activation is usually plotted versus the reaction coordinate in two dimensions, generating a one-dimensional free-energy curve. In some cases, intermediates may be explicitly shown in such diagrams. If the ensemble concept is to be embraced, however, the free-energy diagram should be presented in at least three dimensions, generating a two-dimensional free-energy surface, as shown schematically in Figure 4. This diagram represents a coupling of multiple reaction coordinates with multiple ensemble conformations. In terms of the reaction mechanism above, the reaction coordinate axis represents multiple horizontal paths and the ensemble conformations represents multiple vertical paths. This free-energy diagram can be viewed as a three-dimensional catalytic mountain range, with a plane bisecting the mountains. Substrates are on one side of the plane, and products are on the other: a "catalytic network". The paths up and down the mountains have multiple minima (reaction intermediates) that are connected by "hills" (standard free-energy maxima) along the mountain sides (parallel paths). The stochastic thermal motions of the enzyme and ligands enable the system to sample the conformational space represented by this free-energy landscape. We suggest that multi-dimensional free-energy diagrams characterize enzyme catalysis in general and enzyme catalysis should be considered in terms of such "catalytic networks".

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