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A Biophysical Perspective on Enzyme Catalysis

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Abstract

Even after a century of investigations, our knowledge of how enzymes work remains far from complete. In particular, several factors that enable enzymes to achieve the high catalytic efficiency still remain only poorly understood. A number of theories have been developed, which propose or reaffirm that enzymes work as structural scaffolds, serving to bring together and properly orienting the participants so that the reaction can proceed; therefore, only viewing enzymes as passive participants in the catalyzed reaction. Increasing evidence shows that enzymes are not rigid structures but they are constantly undergoing a wide range of internal motions and conformational fluctuations. In this perspective, based on studies from our group, we discuss the emerging biophysical model for enzyme catalysis that provides detailed understanding of the interconnection between internal protein motions, conformational sub-states, enzyme mechanisms and catalytic efficiency of enzymes. For a number of enzymes, networks of conserved residues have been discovered that span from the surface of the enzyme all the way to the active-site. These networks are hypothesized to serve as pathways of energy transfer that enables thermodynamical coupling of surrounding solvent with enzyme catalysis, and play a role in promoting the enzyme function. Additionally, the role of enzyme structure and electrostatic effects is already well known for quite some time. Collectively, the recent knowledge gained about enzyme mechanisms suggest that the conventional paradigm of enzyme structure encodes function is incomplete and needs to be extended to structure encodes dynamics and the catalytic rate-acceleration, and together these enzyme features encode function.

Graphical abstract

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Keywords

Enzyme dynamics; conformational sub-states; energy flow; solvent conditions; enzyme engineering

Introduction

Enzymes are known to accelerate reactions by more than 10^{17} folds.¹ Understanding of the detailed mechanism of enzyme catalysis and the factors that enable enzymes to achieve the remarkable catalytic efficiency has now been sought for more than a century.² A wealth of information about enzymes has been obtained through experimental and computational investigations, particularly revealing the important role of enzyme structure in catalysis. In the original *lock-and-key* hypothesis and in the revised forms including the *induced-fit* and the *transition-state stabilization* hypotheses, enzymes are proposed to serve as structural scaffolds that selectively recognize and bind their substrates.²⁻⁴ After binding and proper positioning of the participants, enzymes are proposed to create a reaction environment with properties that differ significantly from the properties in the bulk solvent, and favor the reaction to proceed faster than possible in solution.⁵ Enzyme residues that make direct contacts with the substrate (and cofactor) have been shown to have critical roles in holding the reaction participants in fixed positions;^{6, 7} and mutations of these residues are known to alter the speed and/or outcome of the catalyzed reaction.⁷ It is widely discussed that the shapes of active-sites are complementary to the transition-state of the catalyzed reaction, and the electrostatic environment of the active-sites have been shown to be different from that in the bulk solvent, such that they favor the progress of reactions.⁸ Therefore, enzyme function has long been understood in terms of structural scaffolds where enzyme active-sites serve to provide a complementary structural and electrostatic environment to preferentially bind and/or stabilize the transition-state.

Even after many decades of investigations, a number of important aspects of enzyme catalysis still remain unexplained. Enzymes have large complex structures, what roles do the enzyme regions beyond the active-site play in catalysis? It is well-known that residues located far away from the active-site when altered through mutations can have drastic consequences for the enzyme catalyzed reaction;⁹ the role of these residues in the catalytic mechanism is poorly understood.¹⁰ Evidence suggest that the bulk and hydration-shell solvent can have drastic impact on enzyme dynamics and activity.^{11, 12} However, the understanding of the linkage between solvent, and enzyme structure and function remains rather limited. Moreover, bio-organic and even protein molecules that are specially designed to mimic the active-site, with complementary environment to the transition-state of catalyzed reaction, have much lower catalytic efficiencies than their naturally occurring counterparts.^{13, 14}

The search for a fundamental understanding of factors that control enzyme catalytic efficiency has led investigators to look beyond viewing enzymes only as rigid structural scaffolds. In pioneering work Arie Warshel has described the critical role of electrostatic effects in mechanisms of many enzymes.⁸ More recently, other factors including the internal

motions of proteins have attracted considerable interest for their role in enzyme function.^{15–36} Like all objects, enzyme molecules also undergo a range of internal motions driven by the surrounding environment and temperature. Emerging evidence indicates that enzymes, as suggested by the conventional paradigm, are not rigid structure but are intrinsically flexible molecules and the internal motions play an active role during the enzyme catalyzed reactions. Recent investigations have shown that a number of enzymes contain network of conserved residues that connect distal region of the enzyme to the active-site.^{37–40} These networks show the presence of protein motions coupled to the enzyme reaction that play a promoting role in catalysis.

Based on investigations of enzymes performed in our group, we present a biophysical model of how enzymes function (see Figure 1), with the following hypothesized aspects:

1. Internal motions, particularly in the conserved network of residues, play important roles in enzyme function;
2. A hierarchy of conformational fluctuations enable energetic coupling between the external solvent and the enzyme's active-site;
3. The thermodynamical energy selectively directed into the active-site from solvent allows access to functionally relevant conformational sub-states and allows overcoming the activation energy barrier;
4. The functionally important dynamical parts of the enzyme (particularly the ones that exhibit slow conformational fluctuations) are conserved over evolution; and
5. Using this new understanding of enzymes, it is possible to identify and modulate reaction promoting conformational fluctuations to develop hyper-catalytic enzymes.⁴¹

These aspects of this hypothesized model are discussed in this perspective. This review describes a perspective about how enzymes work based on studies performed in our group. Note, this perspective is not meant to claim that we completely understand all aspects of enzyme catalysis; and it is also noted that investigations from many other research groups also continue to provide new and vital insights into enzyme function as well.^{15–25} Moreover, various enzyme structural elements and electro-static environment, enabled by the optimal positioning of the active-site and distal residues, play critical roles during enzyme catalyzed reactions (Figure 1), and no biophysical models would be complete without inclusion of their significant contributions to enzyme mechanisms.^{5, 8}

I. Protein motions and conserved networks in enzyme catalysis

Internal motions of proteins span at least 12 orders of magnitude, ranging from femtoseconds (10^{-15} s) to microseconds and higher ($>10^{-6}$ s).^{16, 39} The process of enzyme function involves internal movements of participating molecules during the binding and positioning of substrate (and cofactor) and the removal of product(s). Inherently these steps involve motions of enzyme residues, opening and closing of large regions due to movements at hinge regions or even entire domains in facilitating these events to occur. These types of concerted motions of protein parts and their role in enzyme function are well documented.

^{42, 43} The more interesting question is regarding the direct role of enzyme motions in accelerating the rate limiting step, which determines the overall efficiency of enzyme catalysis. The rate limiting step could be the binding step, the chemical (substrate turnover) step or removal step. The identification of enzyme motions or conformational fluctuations that actively participate and promote the rate limiting step will have important implications in understanding the catalytic efficiency.¹²

The role of internal motions in enzyme function a highly debated topic with conflicting views being reported.^{6, 26, 40, 44–46} The confusion, in part, comes from different definitions of protein motions.²⁶ The term *protein dynamics* has been used broadly to describe a wide variety of motions often leading to confusing and conflicting results. We distinguish between the following types of protein motions (Figure 2):

1. The motions at femtoseconds to sub picoseconds time-scales involving a small number of atoms or a few residues, which repeatedly sample the same area of the conformational landscape and over time average around the same mean structure, and more importantly can be described by harmonic potential wells are considered as *fast motions* or dynamical motions (see Figure 2A). These types of motions are random in nature and are driven by thermodynamical fluctuations associated with temperature.
2. *Conformational fluctuations* are movements that occur at intermediate (nanoseconds) to slow time-scales (microseconds and higher) and enable sampling of distant areas of protein landscape. In particular, these types of motions involve movements of large protein domains (loops, secondary structure elements) and allow overcoming barriers between various conformational wells. These types of motions require more energy than what is available from temperature related thermodynamical fluctuations. Most likely these conformational fluctuations are driven by a group of faster motions, through an enslaving type process.⁴⁷ As shown in Figure 2B, a single conformational fluctuation can allow sampling of conformational wells located along an anharmonic landscape.
3. A movement that results in temporary (or even permanent) change in protein structure is considered as a *structural change*. This type of movement differs from the previous two types of motions, as it entails a shift in the thermodynamic equilibrium. For example, as shown in Figure 2C binding of a small molecule changes the average structure that is sampled by enzyme (such as the binding of adenosine triphosphate to enzyme adenylate kinase).⁴⁸ Such changes mostly commonly occur on binding and/or release of reactants and products, leading to a change in average structure. Most likely they are a result of changes in interaction between the protein and binding molecule(s). Structural changes also results in change of types of motions that are sampled, but structural change is fundamentally different from sampling of equilibrium motions. Binding or removal of substrate/cofactor or ligands causes changes in the energetic landscape and, therefore, corresponds to major shifts in conformational sub-states and the related sampling of motions (see section III below).

A number of recent studies and reviews have described theoretical approaches for quantifying the contributions of various types of dynamical motions in enzyme kinetics.^{6, 49–52} Experimental investigations including nuclear magnetic resonance (NMR) experiments,^{33, 44, 53} single molecule techniques,⁵⁴ hydrogen/deuterium exchange,⁵⁵ neutron scattering⁵⁶ and computational techniques^{15, 33} have been used to identify motions that are closely coupled to the reaction mechanism of enzyme reactions.^{31, 44} These techniques have also led to the discovery that enzyme residues that show functionally relevant motions are arranged in networks that connect surface loop regions of the enzymes to the active-sites,^{37, 38, 40, 41, 57} such as shown in Figure 3. As an illustration we briefly discuss enzyme cyclophilin A (CypA), which has been investigated extensively for the link between protein motions in enzyme catalysis.

Cyclophilin A:

The enzyme human CypA catalyzes the *cis/trans* isomerization of peptidyl-prolyl amide bonds in peptides and proteins substrates.^{33, 58} NMR spin relaxation studies by Kern and coworkers provided important insights into the movement of the protein residues in presence and absence of the substrate which coincides with the catalytic cycle.^{59, 60} The data indicated that the intrinsic dynamics of the protein in this enzyme coincides with the time-scale (about 9000/second) of the substrate turnover step (the rate limiting step) and promotes the reaction mechanism.

Computational studies by Agarwal and coworkers identified a hierarchy of motions coupled to the reaction.³⁸ Analysis of fast motions, slow conformational fluctuations and cross-correlation of the motions over the course of isomerization reaction led to the discovery of a *network* of protein vibrations in CypA coupled to its reaction mechanism (Figure 3). This network extends from surface loop residues of the enzyme all the way to the active-site, through a series of hydrogen bonds and hydrophobic interactions.^{38, 57} The protein motions in this network occur on the time-scales ranging from picosecond to microsecond-millisecond (time scale of the reaction). Genomic and structural analyses indicated that the residues and hydrogen-bonds forming crucial points in the network are conserved in several cyclophilin structures of species ranging from bacteria to human. NMR relaxation studies have observed motions in the network residues coupled to the substrate turnover step.⁵⁹

The biophysical impact of discovered network on the CypA reaction mechanism was revealed by monitoring changes in the active-site environment that are introduced by the conformational fluctuations of the network residues.³⁸ Computational analysis of these changes showed that the progress of reaction is correlated with the fluctuations in the substrate-enzyme interactions, which in turn are controlled by the motions and conformational fluctuations in the CypA network.¹² The relative orientations and the motions of these network residues have shown to be conserved aspect of isomerase fold.⁴⁰ These observations are consistent with the reaction mechanism from previous X-ray crystallographic studies that indicate that the interaction between target proline residue of the substrate and active-site hydrophilic and hydrophobic residues of enzyme play a crucial role during the reaction.⁶⁸ An interesting observation is that the maximum enzyme stabilization of the substrate occurs close to transition-state (consistent with the transition-

state stabilization theory). The role of these conformational fluctuations could, therefore, be also interpreted as concerted internal protein movements, which facilitate in stabilization of the transition-state. Hence, these are conformational fluctuations are termed as *reaction promoting* as they: (1) bring together the enzyme active-site in an organized state such that the all the structural elements are correctly positioned and (2) allow more reaction trajectories to become productive. Note, other investigators have discovered that active-site electrostatic environment also plays an important role in CypA mechanism.⁶⁹

A number of other enzymes have also shown the presence of networks and the role of internal motions on enzyme catalysis including dihydrofolate reductase (DHFR), ribonuclease A, liver alcohol dehydrogenase, lipase B and others.^{7, 37, 40, 41, 66, 70–75} In some enzyme systems, the thermodynamical motions or fast motions have been shown to aid in enzyme catalysis.^{22, 76} However, CypA and DHFR specifically show the presence of motions and conformational fluctuations that are intrinsic motions associated with the topology of the enzyme. Therefore, these motions are not random in nature but are *designed* part of the enzyme structure as they have been observed to be present even in apo enzyme and enzyme complexes, and the rate of these repeated motions can be measured reproducibly using different techniques.^{44, 59, 77}

It should be noted that distal regions in all proteins can be connected through a series of interactions, however, experimental data (with controls) is required to before a series of interactions can be considered as a network impacting enzyme catalysis. In particular the effect of elimination or substitution of the network interactions (through mutations) on enzyme activity should be clearly demonstrated. For non-enzyme systems the existence of such networks is difficult to characterize and validate. The conversion of substrate(s) to product(s) by enzyme systems offer development of ways to monitor change in protein function associated with changes introduced in protein structure by techniques such as mutations. PDZ domain is an example of non-enzyme systems where the existence of such networks has been demonstrated using statistical coupling approach and tested with the use of mutational studies.^{78, 79}

II. Long-distance effects and energetic coupling

A puzzling aspect of enzymes is the presence of long-range effects and distal control of activity.^{27, 63, 78, 80} It is widely known that regions of enzymes located far away from the active-site, with no obvious connection between the distal regions and the functional site, can have drastic impact on protein and enzyme function. The allosteric and cooperative effects refer to the control of activity by binding of ligands or biological molecules at a site other than the primary site of enzyme function (the active-site).^{81, 82} Allosteric modulation is commonly used mechanism for feed-positive and feed-negative regulation of biochemical processes.⁸³ The understanding of biophysical mechanism of allosteric/cooperative effects in enzymes still remains another unsolved mystery. Investigations initially suggested that electrostatics and correlated structural interactions enabled allosteric modulation.⁸⁴ More recently, the long-range effects in enzyme function have been understood in terms of energy connectivity in the enzyme structure.^{10, 63, 80, 85}

It may initially appear un-related to long range effects but there is another important question about enzymes that still remains unanswered. Most enzyme catalyzed reactions have an activation energy barrier that needs to be surmounted for reaction to be productive. Note that even though enzymes lower the effective transition-state barrier (by raising the ground state and/or lowering the transition-state through stabilization), but still there are barriers typically of 5–10 kcal/mol that need to be overcome by the enzyme assisted reaction.⁸⁶ How do enzymes assist in overcoming these barriers? The random thermal fluctuations associated with enzyme motions (about 1 kcal/mol at ambient temperature) are not enough to provide enough energy. It has been hypothesized that the thermodynamical energy of the solvent on the surface of the protein provides the required energy to overcome these barriers.^{12, 39, 40} As described above, conserved networks which span from the surface regions of the enzyme to the active-site have been widely reported for a number of enzymes. It has also been hypothesized that these network pathways serve as channels of long-range connectivity between the solvent and the active-site. The role of these energetic pathways is to couple the surrounding solvent to the enzyme reaction.¹¹ Put another way, a hierarchy of protein motions allows the collection of energy from fast motions on the surface of the protein to intermediate motions associated with loop regions and ultimately this energy is transferred into the large-scale global conformations of the enzyme which are conserved part of the protein fold.^{12, 40, 41} Once the correct reaction promoting conformational fluctuations are activated they provide the energy for overcoming the activation energy barrier (Figure 4).³⁸ Such a model also provides a biophysical mechanism for long range effects including allostery. Supporting evidence for such a model of enzyme networks acting as energy channels is already available. The investigations of PDZ domain by Ranganathan and coworkers discovered that enzyme residues have co-evolved for long range connectivity.⁷⁹ Informatic theoretic approaches have also confirmed the presence of pathways of long-distance communication in protein structure.^{87, 88} The question arises if these pathways have a role in the designated function of the enzymes.

Conformational fluctuations as pathways for energy flow within proteins:

In the hypothesized model of enzymes (Figure 1 and Figure 4) the energy is collected by fast motions of the surface protein residues through thermo-dynamical coupling with the solvent.^{31, 89–91} This energy is transferred to the intermediate motions and eventually fed to the slow protein motions, which are coupled to the reaction.¹² Note that this process is not dissipative (that is energy is not lost by random collisions) but rather leads to directed flow of energy through discovered networks of coupled motions.^{85, 92} Previous studies have already shown that vibrational energy from a specific normal mode is not lost by dissipation but instead transferred to a few other resonant modes, with a mode coupling term that is correlated with the geometric overlap between the modes.⁹² Similar studies have also been carried out for studying vibrational energy transfer between α -helices.^{85, 93} Additional evidence in support of the proposed model comes from another study showing that the slower global motions in proteins are enslaved by faster but local motions.⁴⁷

Thermo-dynamical energy from solvent enables overcoming the activation energy barrier:

Computational work performed by our group has shown that the discovered energy networks or pathways are closely connected to the overall functioning of the enzyme mechanism

particularly the rate limiting step where an energy barrier needs to be overcome.¹² The discovered pathways in enzyme structure function by channeling energy from different areas of the protein into the active-site. Terahertz spectroscopy has revealed protein-hydration shell coupling in human lysozyme at fast time-scales (picoseconds).⁹⁴ Neutron scattering and computational studies have already shown that the motions of solvent slave the motions of the protein motions therefore indicating thermo-dynamical coupling between the solvent and the protein motions.¹¹ The collected energy is channeled into the active-site through a detailed mechanism involving motions of the network residues.

The motions and energy in the networks alters the active-site environment by subtle changes in enzyme-substrate interactions so that more reactive trajectories become productive. For CypA, it has been shown that presence of extra kinetic energy in the reaction promoting conformational fluctuations improves the success rate of reactive trajectories, which corresponds to improving rate kinetics for the enzyme. It is only the presence of kinetic energy in the correct protein motions that shows improvement in reaction rate; which consists of only a small fraction of the total motions of a protein.¹² The majority of motions do not have any effect on the reaction. Supporting evidence for this energy flow model also comes from studies where microwave activation⁹⁵ has been used to improve the rate catalysis as well as photo-activation for improved catalysis through channeling energy into the active-site.⁴¹

Significance of these pathways and energy flow in enzyme catalysis could possibly be very wide. These networks provide a biophysical explanation of the presence of allosteric and long-range effects in protein structure including enzymes. It has been widely described that allosteric effects are possibly present in the enzyme molecules to provide nature a mechanism for regulation. However, the model presented here provides an explanation for the wide presence of these pathways and long-range effects in a variety of protein molecules, as they serve a mechanism for to enable the energy transfer for designated function of the protein, and the control through regulation is use of these pathways to modulate the activity of enzyme molecules.

III. Conformational sub-states in enzyme catalysis

An enzyme goes through a catalytic cycle involving a series of intermediate states that includes enzyme in unbound form, enzyme with substrate and cofactor bound (*ground state*), and enzyme in activated complex form with substrate and cofactor (*activated state*). In these states the enzyme exhibits different conformations, with functionally important residues and secondary structural elements arranged in different orientations. The probability of sampling these different conformations along the catalytic cycle is not equally distributed; instead depends on enzyme's energetic landscape. As illustrated in Figure 5, the fast time-scale motions of the protein allow sampling of the conformations in the near vicinity (within the wells) while the conformational fluctuations at long time-scales allow inter-conversion between different groups of populations or conformational sub-states (between conformational wells). These fluctuations that allow inter- conversion between different sub-states are known as *conformational transitions* and have been investigated in proteins. Austin and coworkers have reported a connection between slow anharmonic energy relaxation rate

at nanosecond time-scale and the rate of conformational transitions in proteins, which require multilevel vibrational excitations.⁹⁶

Recent studies indicate that certain conformational sub-states with small populations contain the structural and dynamical features that aid in promoting catalysis;⁹⁷ and these sub-states are known as *functionally relevant conformational sub-states*. A number of recent studies have shown that the intrinsic dynamics of the enzyme enables it to sample higher energy conformational sub-states (the activated complex), which contain geometric and dynamical features of residues that favor the reaction to proceed to the product state,³⁵ therefore, promoting the reaction. In other words, these conformational sub-states have the active-site residues correctly oriented for stabilizing the transition-state, providing the appropriate electrostatic environment, and allowing enzyme-substrate interactions to sample the right motions at the fast time-scales that enable successful completion of reactive trajectories.^{36, 98}

Characterizing the populations of enzyme conformations, particularly in relation to the designated function, has been extremely difficult. Experimental techniques such as X-ray crystallography and NMR provide some information about enzyme conformations associated with ground state, some intermediate states as well as the functioning ensemble of the activated complex.^{66, 99} However, obtaining quantitative information about the sub-states and conformational populations has been very difficult due to the transient nature of these short lived sub-states and the wide range of time-scales involved. Nonetheless, emerging evidence for a number of systems including ubiquitin, lysozyme, cyclophilin A have shown the connection between populations that contain the right structural and dynamical features facilitate in the reaction mechanism.⁹⁸

Most commonly projection of conformations on principal component vectors is used to obtain information about conformational sub-states. The underlying argument is that the vectors obtained from the principal component analysis identify the relevant conformational fluctuations, and projection of sampled protein conformations on lowest frequency modes would identify sub-states with meaningful populations. However, this approach suffers from a serious limitation that it provides only provides populations with mixed properties.⁹⁸ It is widely acknowledged that the conformations located in the same sub-state share similar properties including internal energy, geometrical and dynamical properties.¹⁰⁰ The principal component techniques have limited ability to identify conformational sub-states with homogeneous populations that correspond to the ones observed from experimental techniques. The break-through in conformational sub-state analysis came by realization that protein motions at long time-scales are anharmonic,^{89, 101, 102} and needs to be quantified using higher-order statistics.⁹⁸ Using this rationale a number of groups have developed techniques for identifying functionally important conformational sub-states, including a new computational technique known as quasi-anharmonic analysis (QAA) developed by Chennubhotla, Agarwal and coworkers.⁹⁸

This new technique, QAA, allowed the quantitative characterization of conformation sub-states associated with the reaction catalyzed by enzyme CypA, particularly identifying the ones associated with the transition-state.⁹⁸ Further, it also allowed identification of the long

time-scales conformational fluctuations (*conformational transitions*) that are required to access these transition-state enabling sub-states. These long time-scales conformational fluctuations identified by computational method QAA provide the same information corresponding to the NMR spin relaxation studies.^{59, 100} Results indicated that the enzyme regions which show large dynamical motions in these long-range and long time-scales fluctuations include the surface loop areas as well as loop in the proximity of active-site. Overall, QAA offers the unique opportunity for the identification and quantitative characterization of rare enzyme conformations that have the geometrical features that facilitate the attainment of transition-state. Note that this is related to the short-lived hidden conformational sub-states that have been discovered using X-ray and NMR techniques, and influence enzyme catalysis in CypA.⁷⁷

Enzymes in cell operate in different conditions than those of laboratory scale experiments. In laboratory, the enzyme studies are typically conducted in dilute aqueous conditions while in the cells the enzymes are surrounded by a wide variety of biomolecules. Recently, our group used miscible organic solvents to explore the linkage between protein dynamics, functionally relevant conformational sub-states and enzyme catalysis. Use of binary mixture of isopropanol and water indicated that the hydride transfer by *E. coli* DHFR under rate limiting conditions (measure by steady state kinetics) shows 2.2-fold decrease, at 20% isopropanol concentration. X-ray crystallography showed no change in enzyme structure, however, quasi-elastic neutron scattering shows that enzyme motions are suppressed with increasing concentration of isopropanol.¹⁰³ Computational modeling indicated that there is no change in active-site structural or electrostatic environment but the access to conformational sub-states altered is significantly. In particular, the rate of transitions into functionally relevant conformational sub-states was about 3-fold smaller, close to the experimentally observed decreased in hydride transfer rate. Collectively, this study using experimental techniques (including steady state enzyme kinetics, X-ray crystallography, neutron scattering and isothermal calorimetry) and computational methods indicates that solvent conditions altered the energetic landscape such that enzyme is unable to sample the natural motions required for accessing the functionally relevant conformational sub-states. These results have important implication for investigating enzyme mechanisms *in vivo* as the cellular conditions could impact dynamics and therefore catalysis.

IV. Conservation of functionally important dynamics

Evidence collected from an increasing number of enzyme systems where conformational fluctuations and internal motions play a promoting role in enzyme catalysis, leads to several questions – how common is this phenomenon and if protein dynamics makes significant contribution to the catalytic efficiency (rate enhancement) of enzymes? It is difficult to answer these questions conclusively for all enzymes as obtaining quantitative estimates is challenging. Evidence suggests that different enzymes may show different contributions of dynamics along with other factors.^{6, 46} However, a number of investigations have attempted to quantitatively assess the contribution of protein dynamics in enzyme catalysis.^{19, 104} In particular, enzyme super-families show preservation of functionally important dynamics.¹⁰⁴

A test for the proposed role of dynamics in catalysis has come from investigating the conservation of reaction promoting conformational fluctuations as a part of the enzyme fold.⁴⁰ The familiar paradigm for role of structure in enzyme catalysis suggests that if it is important for function then the structural element is conserved over evolution. The same question could be asked for reaction promoting motions, *if the motions and conformational fluctuations are important for function, then are they also conserved as a part of overall fold?* One of our studies identified the reaction promoting dynamics in three enzyme systems catalyzing diverse chemical reactions: *cis/trans* isomerization catalyzed by CypA, hydride transfer catalyzed by DHFR and single-stranded RNA hydrolysis by ribonuclease A (RNase A).⁴⁰ The study showed that even with low sequence similarity (as low as 30%) these enzymes that share the same overall fold show the conservation of reaction promoting dynamics as a part of the enzyme fold (Figure 3). The reaction promoting dynamics show movements in distal regions of the protein with large displacements and these regions are located in the same area of the fold, even though the sequences can be significantly different. These regions include surface loops showing high flexibility that are connected to the active-site through the conserved network of residues. The common features of these networks are that they include surface residues with long side-chains, possibly to increase thermodynamical coupling with the solvent. (Terahertz spectroscopy has shown coupling of protein-hydration shell solvent at picosecond time-scale.⁹⁴) It is interesting to note that the exact type of surface residue is not conserved as long as the replacement residue has the long side chain needed for the enzyme-solvent coupling. Additionally the networks show the presence of preserved hydrogen bonds and hydrophobic interactions all the way to the active-site (Figure 3). It is further interesting to note that these residues may themselves not be conserved as long as the interactions forming the networks are conserved as a part of the overall fold. It has been seen that mutation in these network residues, causing disruption of the network, can have drastic impact on enzyme catalysis.^{65, 105}

The enzymes with DHFR activity also present an interesting case for investigating the role of dynamics and catalysis. There are two structural forms of DHFR: the chromosomally encoded DHFR, present in bacteria to humans, with the Rossmann fold (including the *E. coli* DHFR). The other type of DHFR is encoded by a plasmid and is known as type II R67DHFR.¹⁰⁶ These two enzymes catalyze same reaction however share no structural or sequence homology. Detailed studies have shown that in addition for these enzymes in addition to bringing together the cofactor NADPH and substrate DHF through interactions in the active-site of the enzyme folds also contain designed features in the enzyme fold that promote the reaction through dynamical motions. As mentioned above, a network of coupled promoting motions in chromosomal encoded DHFR promotes the reaction.³⁷ Enzyme's assistance in the movement of donor carbon and the acceptor carbon towards each other are also part of the reaction mechanism in R67DHFR. However, in R67DHFR these movements are introduced by a residue position behind the nicotinamide ring and the flexibility of DHF tail which is present in a solvent exposed hole.¹⁰⁷ Therefore, function promoting dynamics is present in the two non-homologous enzymes that catalyze the same chemistry.

Pancreatic-type ribonucleases (RNases) are an interesting superfamily of enzymes which catalyze the cleavage of phosphodiester bonds in ribonucleic acid (RNA) substrates. The rate-limiting event in the catalytic cycle for the archetypical member of this family, namely

the bovine ribonuclease A (bRNaseA), is the product removal. The catalytic efficiency of phosphodiester bond cleavage among the 8 human RNases differs about 10^6 fold, which is intriguingly similar to range of the observed rates of conformational exchange which differs by 5 orders of magnitude. A joint computational-NMR study of 23 members of the RNases superfamily revealed some very interesting insights about linkage between conformational dynamics and function.¹⁰⁸ The superfamily members share the common chemical function of ribonucleolytic cleavage of RNA substrates, but their biological functions are diverse including antiviral, antibacterial and angiogenesis activities. Phylogenetic classification revealed the grouping of RNases into subfamilies with distinct biological functions, with members within a subfamily sharing similar biological functions. More interestingly, members within subfamilies that share similar biological functions also display conservation of dynamical properties, while significant variation in dynamics was observed between members from different subfamilies. The subfamily with the highest catalytic efficiency for the ribonucleolytic cleavage showed protein regions with the largest flexibility, on the other hand members of angiogenin subfamily that shows a million fold less catalytic activity shows most rigid structures in the entire RNase superfamily. Furthermore, swapping a loop region that was previously shown to be important for optimal catalysis, from bRNaseA with that from a different subfamily switched the dynamical profile of the chimeric protein, further highlighting the conservation of dynamical properties within a subfamily. The evidence from this study further indicates that functionally important dynamics is conserved over evolution as a part of the enzyme structural fold to optimize for function.

V. Designing hyper-catalytic enzymes through conformational modulation

In this article, we have described a biophysical model for enzyme catalysis. This model emphasizes enzymes not as rigid bodies that passively enhance catalysis but as internally flexible molecules that work closely in association with the surrounding environment to promote catalysis. In particular, examples from a number of soluble proteins show a hierarchy of motions with the surface motions enslaved by the surrounding solvent. The thermo-dynamical coupling between the solvent and the surface enzyme residues provide energy (through directed energy flow into the active-site) for the slow conformational fluctuations that have important role in promoting the enzyme catalyzed reaction. Moreover, it is also hypothesized that these set of reaction promoting conformational fluctuations are conserved part of the enzyme fold. Nevertheless, the role of protein motions or protein dynamics is a widely debated topic. Therefore, the biophysical model of enzyme catalysis described here needs to be tested thoroughly.

As an important first test of this model, it should be possible to identify and modulate the conformational fluctuations that are involved in promoting the reaction. In other words it should be possible to excite the functionally relevant conformations through addition of extra kinetic energy into the channels of the network pathways that are present in the enzyme (Figure 1), and if this model is correct then it should increase the efficiency of enzymes through conformational modulation of the enzyme. We used this concept to design a strategy for enzyme engineering for the development of hyper-catalytic enzymes.⁴¹ A challenging aspect of this design is to develop a strategy that allows selective modulation of the correct conformations that promote the reaction. The engineering strategy was made

possible by recent developments that show peptide/protein conformations can be modulated through attachment of a photo-activatable linker (containing azo-benzene group) and providing an external stimulus such as exposure to light. Under exposure of the appropriate wavelength of light azo-benzene molecule undergoes change from the low-energy *trans* form to the high-energy *cis* form, which induces conformational modulation of the protein through mechanical strain.¹⁰⁹ A number of investigators have attached linkers containing azo-benzene on peptides and proteins to change the conformation at fast time-scales enabled by exposure to light.^{109, 110}

Using the enzyme lipase B from *Candida antarctica* (CALB) as a model system, we were successfully able to design the hyper-catalytic enzyme.⁴¹ The reaction coupled (and reaction promoting) conformational fluctuations were identified using computations, and a photo-activatable linker containing the azo-benzene was conjugated to enable modulation of the enzyme conformations. Once the photo activatable bridge is correctly conjugated on the enzyme, external stimulus through exposure of two wavelength of light allows the conformational modulation of the function promoting conformations. To estimate the increase in catalytic efficiency of the engineered enzyme, it is important to quantify the amount of enzyme that has conjugated bridge in the correct position. Estimates show that in our preliminary and un-optimized chemical strategy, the yield is expected to be 0.48% –3.35%. Using this yield, we estimated that the engineered enzyme is able to achieve 8 to 52 fold (or ~3000% average) improvement in enzyme activity over the naturally occurring form of the enzyme. It should be noted that a number of control experiments, including the exposure of wild type enzyme to light and attaching photo-activatable group to non-specific locations and exposing them to light, did not show any increase in catalytic activity.

The successful design of this hyper-catalytic enzyme provides support for the various aspects of the biophysical model of enzyme catalysis. As we hypothesized that conformational fluctuations are important for function, the ability to identify the reaction promoting conformations and then modulating them through an external stimulus for better catalytic efficiency provides vital support for this model. Further preliminary model also provides evidence that the mechanical energy from the azo-benzene could also be transferred into the active-site (through the network of residues) allowing the enzyme to overcome the reaction at much faster rate. Moreover, the lack of increased enzyme activity during the control experiments also support the observations that not all motions are able to promote the reaction; only specific regions and specific motions have reaction promoting capability. Other groups have also used modulation of enzyme dynamics to develop enzyme engineering strategies.³⁴

VI. Summary

In this article, we have presented a biophysical model for how enzymes work. It has been acknowledged for a number of decades now that the enzymes function as a structural scaffold that bind and correctly orient the reaction participants and provide a chemical environment different from bulk solvent to facilitate catalysis. Emerging evidence, from a wide variety of experimental and computational techniques, has also revealed that enzymes play a more active role in catalyzing the reaction through internal protein motions and

conformational fluctuations. These conformational fluctuations occur over a wide range time-scales, including the time-scale of reaction, and allow the enzyme to access the region of conformational landscape that contains dynamical and structural features for promoting the reaction that is the functionally relevant transient state sub-states. It has also been suggested that the hierarchy of protein motions enable the coupling of the bulk and hydration-shell solvent with the catalyzed reaction. In our lab, this hypothesized model has already been used to design a hyper catalytic enzyme through conformational modulation through photo-activation, and preliminary results show ~30 fold increase in catalytic activity over the naturally occurring enzyme. Overall, the evidence discussed here indicated that the paradigm that structure encodes function is incomplete and it needs to be extended to enzyme *structure encodes dynamics*, and *structure-dynamics together encode function*.

A number of studies have questioned the role of protein motions, or dynamics, in catalysis. Part of the confusion comes from the lack of proper description/definition of protein dynamics. Much like all other objects, enzymes undergo a range of motions; however, this does not mean that any or all protein motions would promote the function. The issue is complicated by a number of computational (as well as some experimental) studies, which only provide explanations for already known observations but unfortunately fail independent validation and/or cannot make clear and testable predictions. Further, increasing number of literature reports have proposed existence of networks without providing clear evidence showing the effect of these networks on enzyme activity. Therefore, efforts dedicated to developing models that provide mechanistic insights and allow making predictions that can be independently validated and experimentally tested would help in resolving this debate. As an example, the study of enzyme in miscible organic solvents have already provided some insights that not all types of enzyme dynamics affects catalysis, however, it is a particular sub-set of motions (or conformational transitions into the functionally relevant sub-states) that alter enzyme rates.¹⁰³

Note, the aim of this article is not to claim that dynamics is the only contributor to enzyme catalysis. In addition to the well-known role of direct structural interactions, a number of other factors also contribute to catalysis through long range effects. These include electrostatics effects and solvent molecules (both external and internal). Suggestions have also been made that the energy released from binding of the substrate and/or factor could also provide energy required to overcome the activation energy barrier. These are all interesting mechanisms and possibly do make contributions. The exact contribution each factor makes would depend on the enzyme system and the type of chemistry being catalyzed. High quality models that allow quantitative estimates of contributions for each of these factors would also be extremely valuable.

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Figure 1: The inter-connection between protein structure, dynamics and function has significance for improving catalytic efficiency of enzymes.

The active-site region provides environment with optimal electrostatics to promote the catalyzed reaction. Several enzymes contain conserved networks of residues that connect surface regions to the active-site. These networks provide thermo-dynamical coupling between the hydration-shell and bulk solvent, and the catalyzed reaction. The discovery of these enzyme energy networks allows new strategies for increasing the catalytic efficiency through enhancing the energy flow through these networks and optimization through mutations.

Figure 2: Various types of internal protein motions.

(A) Fast motions. Each black dot indicates a unique conformation. (B) The conformational fluctuations correspond to large scale movements within protein domains or entire protein. These types of motions allow sampling large areas of conformational landscape that could span over multiple minima. (C) Structural changes are induced by events such as binding (or release) of other molecules. Shown here is the enzyme adenylate kinase in the apo form (left) and adenosine triphosphate (ATP) bound (right).

Figure 3: Identified network of promoting motions/vibrations in three different enzyme folds,⁴⁰ cyclophilin A (CypA), dihydrofolate reductase (DHFR) and ribonuclease A (RNaseA). The flexible surface loop regions with high flexibility show the presence of residues with long side-chains and are interconnected to the active-site through preserved hydrogen bonds. Experimental studies have confirmed presence of these networks.^{33, 61–67} *Reproduced from Ramanathan A et al. (2011), PLoS Biol 9(11): e1001193.*

Figure 4: A hypothesized model for the conformational and energetic coupling between enzyme and solvent.

Random motions of solvent molecules slave the motions of surface residues through collisions. These fast motions drive the conformational fluctuations of the interior regions of the protein. These intermediate motions eventually drive the slow conformational fluctuations, some of which promote the catalytic reaction. The hierarchy of protein motions enables the transfer of thermo-dynamical energy from the surface regions to the active-site.

Figure 5: Internal protein motions and conformational fluctuations allow sampling of conformational sub-states.

Protein motions at fast time-scales allow sampling within a sub-state, while fluctuations at long time-scales allow access to different sub-states. Some of these higher energy states may contain function promoting structure and dynamical features. Any factors that change the sampling of the higher energy conformational will change access to these functionally important states and therefore change the rate of the reaction.