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Functional Roles of Enzyme Dynamics in Accelerating Active Site Chemistry: Emerging Techniques and Changing Concepts

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Abstract

With the growing acceptance of the contribution of protein conformational ensembles to enzyme catalysis and regulation, research in the field of protein dynamics has shifted toward an understanding of the atomistic properties of protein dynamical networks and the mechanisms and time scales that control such behavior. A full description of an enzymatic reaction coordinate is expected to extend beyond the active site and to include site-specific networks that communicate with the protein/water interface. Advances in experimental tools for the spatial resolution of thermal activation pathways are being complemented by biophysical methods for visualizing dynamics in real time. An emerging multidimensional model integrates the impacts of bound substrate/effector on the distribution of protein substates that are in rapid equilibration near room temperature with reaction-specific protein embedded heat transfer conduits.

Introduction

It is now widely accepted that functions of proteins are intimately dependent on both their structural and dynamical properties [1–8]. While X-ray crystallography remains a powerful tool to view the 3-D arrangement of atoms within a protein, traditional X-ray techniques provide averaged positions of atoms in a protein, leading to minimal information regarding protein conformational dynamics [9,10]. Room temperature X-ray structural analyses have introduced the ability to infer dynamical networks from the detection of alternate protein side chain conformations [11–14] and recent advances in time-resolved X-ray crystallography using free electron lasers (XFEL) [15,16] have opened a window for

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the direct capture of dynamical information and the visualization of reaction intermediates. Proteins typically populate numerous states [17–23], due to their inherent fluctuations that promote changes in sidechain positioning, backbone rotations, large conformational changes and domain shifts. A major goal (and challenge!) is *to discern the specific set of motions that influence biological function*, which in the case of enzyme catalysis produce a combination of high regio-, stereo- and reaction-specificity with rate accelerations as high as 10^{30}-fold [24]. Ongoing efforts in both computational and experimental methodology are rapidly advancing the "state of the art", with sophisticated models for the physical origins of enzyme catalysis emerging that go beyond historical views such as "enhanced transition state binding" [25–27].

Can Differences in Protein Structure be Detected for ES vs ETS Complexes?

Over many decades, the mainstream explanation for the huge rate accelerations of enzyme catalyzed reactions was enhanced transition state stabilization, as proposed by Pauling [26,27]. The theory attributes the catalytic power of enzymes to a higher affinity of the protein catalyst for the activated complex/transition state in relation to the ground state. Though this theory has contributed tremendously to inhibitor design, it does not provide a biophysical understanding of the trajectory of enzyme catalysis. Reaction barrier crossings are expected to occur on the femtosecond time scale, leaving open the question of what physical processes control the activation event that transforms a thermodynamically stable ES (enzyme-substrate) complex to a state near or at the top of the reaction barrier and results in sub-millisecond to millisecond timescale processes. Efforts at distinguishing protein structural differences between ES and ETS (enzyme-transition state) complexes have been pursued via X-ray crystallography, hydrogen deuterium exchange (HDX) and molecular dynamics (MD) simulations.

Early on, Bruice and co-workers performed picosecond MD simulations on ES and ETS structures of *Xanthobacter autotrophicus* haloalkane dehalogenase. No major structural rearrangement was observed at this timescale with the exception of an additional hydrogen bond in the ETS structure compared to ES complex that would account for a lowering the activation barrier by ca. 2 kcal/mol [28]. In a second study, the same group pursued MD simulations of catechol O-methyltransferase (COMT) for 1 ns in the presence of catechol, catecholate or the transition state to distinguish active-site structure differences. The calculated root-mean-squared deviation and positional fluctuations of the active-site residues within 10 Å of the methyl group of S-adenosyl-L-methionine (AdoMet) for the catecholate and transition-state simulations were found to be similar. The authors suggested that the catalytic power of COMT in going from ES to ETS arises from the ability of the active site to arrange the ground state into tightly packed conformers [29]. This view is supported by secondary kinetic isotope effect measurements and extended GPU-based QM/MM calculations for the reaction of COMT with dopamine, indicating a ground state electrostatic compaction that is maintained through contact with a second sphere tyrosine that resides behind the methyl-bearing sulfur of AdoMet [30–34]. A recent application of temperature dependent hydrogen deuterium exchange (TDHDX, see below) to COMT has uncovered two orthogonal and potentially dynamical networks that connect protein-water surfaces to the primary reaction coordinate [33].

Utilizing another well studied enzyme system, Herschlag and co-workers collected room temperature X-ray crystallography data for the *Pseudomonas putida* ketosteroid isomerase (KSI). From careful examination of the structures of both substrate and TS analogs, the aggregate conformational ensembles indicate limited change in active site positioning (on the 1- to 1.5-Å scale, note that the crystal structure resolution is from 1.1 to 2.5 Å) throughout the KSI reaction cycle [35].

Recently, the TDHDX methodology has also been used to analyze the behavior of the TIM barrel enzyme adenosine deaminase in the presence of either a ground state or bis-substrate/tight binding inhibitor [36]. Evaluation of HDXMS across a range of temperatures for apo- and ligand bound complexes showed almost identical impacts on ΔH° for local protein flexibility from two ligands that differ by ca. 10^6-fold in net affinity [36]. It appears that either an ES- or ETS- like structure has undergone a similar global restructuring of enzyme, in preparation for optimal active site chemistry.

The lack of strong evidence for structural differences when comparing protein complexes of substrate or transition state analogs is in contrast to the evidence for protein preorganization [37]. Direct detection of the preorganization of enzyme active sites that involves, for example, large protein conformational changes or loop closure, is readily accessible from X-ray crystallography or SAXS (Small-angle X-ray scattering [38]) and provides the first level of understanding in the generation of active site geometries that are optimized for catalysis.

Delineating the Functional Importance of Conformational Landscapes and Ensembles.

Proteins in solvent are “wiggling and jiggling”. This property of constant flux produces a wide range of distributed substates referred to as the conformational landscape, that are treated as a continuum of thermodynamic states at equilibrium. Fluctuations within a protein permit jumps between ensembles over relatively low energy barriers and lead to rapid sampling of heterogeneous landscapes [39–45]. Transitions between distinct conformations on different timescales can be coupled to facilitate enzyme catalysis (Figure 1). Although conformationally heterogeneous states of proteins are implicated in many important biological processes, including protein folding, signal transduction, allosteric regulation, and enzyme substrate turnover, detailed knowledge of how the individual substates interconvert among each other and impact function is less clear. One important issue has been the mechanism whereby a small ligand, protein effector or substrate alters the protein conformational landscape. While the role of induced fit held in traction in enzymology for many years [46], it is increasingly accepted that related protein conformations exist in both ligand free and native protein states [40], undergoing a shift in their distribution upon interaction with substrate or effector.

The introduction of such conformation ensembles and selection not only deepens our understanding of how enzymes perform their unparalleled catalysis but is relevant to the fields of protein design and directed evolution. In many cases, directed evolution yields beneficial mutations at remote positions that would not have been predicted from simple structural inspection. Studies show that enhanced catalytic efficacy can be ascribed to a shift in the populations of conformational substates towards those that are more catalytically

active and/or provide enhanced stabilization of catalytic intermediates [47–50]. The concept of rigidified active sites that are dependent on global protein conformational sampling was first apparent in a thermophilic alcohol dehydrogenase (ht-ADH), where the temperature dependence of the kinetic isotope was lost at elevated temperature, under conditions where enzyme had become more flexible and active [50]. Recent studies of directed evolution using a primitive form of dihydrofolate reductase (DHFR) illustrate this principle through the detection of kinetic evidence for a progressive restriction in substrate/cofactor positioning as enzyme variants arise with increased catalytic efficiency [50,51].

Broom et al. pursued room-temperature X-ray crystallography to study changes in the conformational ensemble during directed evolution for the designed Kemp eliminase [52]. Catalytic residues are seen to become increasingly rigidified through improved packing leading to an active site that is better pre-organized to favor productive positioning of the substrate. Given the intrinsic reactivity of the substrate of the Kemp eliminase, its overall rate enhancement is small relative to native enzymes. Nonetheless, the authors achieved a variant, labelled HG4, that is >700-fold more active than its' predecessor. Crystallographic analysis of HG4 illustrated how mutations may shift the conformational ensemble toward an enrichment of catalytically competent substates [52]. The generation of new protein ensembles under the condition of directed evolution was also examined by Otten using the prototypic Kemp eliminase [53]. Through a combination of kinetic analyses, X-ray crystallography and NMR, these authors show how directed evolution gradually altered the conformational ensemble of the protein scaffold to shift to a narrow, highly active conformational ensemble and accelerate the catalytic efficiency by nearly nine orders of magnitude [53]. These experiments also uncover the complicating and off-pathway features that can arise in directed evolution, arguing for caution against overly simplistic molecular interpretations of its impact on enzyme function.

Is there a privileged path within the protein scaffold for the activation of enzyme active sites?

Historically, the protein matrix surrounding enzyme active sites was regarded primarily as a "scaffold" for the arrangement of active site catalytic residues that both generate a successful substrate binding site and provide catalytic rate enhancement. Many decades of active research in the fields of bioorganic and bioinorganic chemistry were focused on these active site elements, successfully producing small molecule mimics of the structural and catalytic elements of the active site [54,55], yet few models were able to approximate the enormous catalytic power of enzymes. In particular, the anisotropic topology of folded globular proteins points toward a role for catalytically privileged dynamical networks in the initiation of active site chemistry [56].

Approximately 20 years ago, Ranganathan et al. began this journey computationally, by analyzing the relatively small PDZ domain protein, comprised of 100 amino acids [57]. Their methodological approach used evolutionary data for protein families to define statistically based interactions between amino acid positions. In the case of the PDZ domain family, this analysis predicted a set of energetically coupled positions that included unexpected long-range interactions. Mutational studies confirmed the predictions, revealing

sets of interacting residues that generate connected pathways through a protein fold and could serve as the basis for efficient energy conduction. Application of this method was subsequently extended to three structurally and functionally distinct protein families, detecting structural motifs for allosteric communication [58].

The experimental detection of intrinsic and long range thermally activated dynamical networks also began to emerge ca. 20 years ago through an analysis of HDX (using mass spectrometry for detection, HDX-MS) in the thermophilic alcohol dehydrogenase (ht-ADH) [59,60]. Analysis of networks in the well-studied DHFR have similarly implicated selected regions of protein that interact with bound substrates and extend out to the protein/solvent interface [61,62]. Dyer and co-workers recently tested the importance of site-specific protein solvent surfaces through an analysis of changes in DHFR activity via the photochemical activation of a surface attached gold nanoparticle [63].

The combination of experimental and computational studies of dynamic networks in enzyme is having an increasing impact on the origins of catalysis. For example, Bunzel et al. performed molecular dynamics simulations for the designed Kemp eliminase [64]. In their MD simulations (out to 5 microsec), a decreased fluctuation in the ETS ensemble was observed relative to ES that was concluded to reflect a network of residues that centers on the bound ligand and spans the protein. Two mutations introduced during evolution were found to be directly involved in the proposed network to tune the dynamic and packing properties for enhanced catalysis.

In a methodological development, Markin et al. designed a high-throughput microfluidic platform to introduce more than 1000 mutations (either a glycine or valine substitution) throughout the structure of the phosphate-irrepressible alkaline phosphatase of *Flavobacterium* and to correlate their impact with catalysis [65]. They observed impactful mutants that extend from the active site to the enzyme surface, providing a map of the underlying protein architecture in dictating catalysis. It was seen that positions that give catalytic effects tend to cluster, and the pattern is asymmetric and complex. Deleterious effects distribute throughout the enzyme with many of the largest effects distant from the active site.

Temperature dependent hydrogen deuterium exchange (TDHDX) as an emerging tool to uncover site specific thermal activation pathways.

Enzyme catalysis is, with few exceptions [66–68], thermally activated. While most discussions of enzyme effectiveness have been historically focused on their free energy of activation [69–72], emerging studies are beginning to address intrinsic differences in the way that enzymes undergo thermal activation relative to small molecules in condensed phase [73,74]. In this context, a set of experimental protocols has been developed that focuses on the TDHDX. This methodology can be readily applied to a wide range of enzymes, under conditions that are similar to those used for biochemical studies of enzyme function [74,75].

For HDX-MS studies in general, the course of deuteron incorporation into backbone amides is monitored as a function of time, undergoing time dependent quenching of protein samples and their subsequent digestion into small peptides that are selected to represent the entire

protein sequence [76–78]. Single temperature HDX-MS is commonly used to investigate the extent of protein protection from bound ligands and/or protein/protein interactions, and also to study regions of proteins where no structural information is available (e.g., because of disordered structures that preclude positional X-ray structural data) or in protein-protein or protein-ligand interactions where structural effects propagate across long distances [79–81]. Though mapping deuteron uptake changes on a protein structure at a single temperature is extremely informative, these studies do not disclose the temperature dependent property of enzymes which, as noted above, is one of the key parameters underlying enzyme catalysis.

In the pursuit of TDHDX, the EX-2 regime is the targeted regime, and this can generally be realized through a screening of experimental conditions and confirmed from the patterns of mass spectrometric analysis. The general formulation for EX-2 is: $k_{HDX} = K_{op}\, k_{exc}$ where k_{exc} represents the intrinsic rate constant for hydrogen deuterium exchange within a transiently unfolded region of protein and K_{op} represents the equilibrium constant for local protein unfolding. From studies at a single temperature one obtains $\Delta G_{obs} = \Delta G° + \Delta G^{‡}$. The incorporation of temperature dependency into HDX allows a formal separation of the $\Delta H_{obs} = \Delta H° + \Delta H^{‡}$. As $\Delta H^{‡}$ has a well-established and fairly constant value ($\Delta H^{‡}$~17 kcal/mol) through peptide studies [12,82], $\Delta H°$ becomes the dominant new parameter that reports on temperature dependent changes in flexibility driven by native protein dynamics [74]. The introduction of a perturbation to the native folded protein structure further simplifies interpretation, with an observed $\Delta\Delta H_{obs} = \Delta H°(\text{variant}) - \Delta H°(\text{WT})$ that essentially eliminates the contribution of $\Delta H^{‡}$ from analyses.

A general TDHDX approach in the Klinman lab is to target hydrophobic side chains for site specific mutagenesis, with the goal of generating modest protein packing defects that impact the rate and especially the $\Delta H^{‡}$ of catalysis. Using the enzyme soybean lipoxygenase (SLO) as a prototype, TDHDX experiments on wild type and mutant forms of SLO have uncovered site specific trends in the enthalpic barriers for HDX-MS within two distal and solvent-exposed loops that correlate well with the enthalpic barriers for catalysis [74]. The remarkable specificity of the inferred thermal conduit in SLO has been replicated and expanded using room temperature X-ray studies that compare changes in the position of amino acid side chains between WT and function-altering mutants [83].

The TIM barrel family of proteins represents ca. 10% of structurally characterized enzyme structures and uses a conserved scaffold to catalyze five out of six of the known classes of enzyme reaction [84]. The TIM barrel scaffold has also served as a primary starting point for *de novo* enzyme design [85]. The diversity of the evolved chemical reactivity within TIM barrel enzymes provides an excellent platform for testing whether evolution of active site chemistry may be linked to the emergence of positionally selective thermal conduits. Thus far, several types of TIM barrel enzyme classes have been studied by TDHDX: a murine adenosine deaminase (mADA) [86], cf. Figure 3, and yeast enolase [87], together with the partial TIM barrel enzyme COMT discussed above [33]. In the case of mADA, Phe61, one of four side chains that generate a hydrophobic wall behind bound substrate was targeted for mutagenesis by insertion of a series of hydrophobic aliphatic side chains. In the case of catalysis these mutations produce a linear increase in $\Delta H^{‡}$ that correlates with changes in $\Delta H°$ obtained from TDHDX. Two thermal networks that reach from opposing

protein/solvent interfaces toward the active site zinc ion and substrate binding site have been identified [86]. In an analogous study, experiments were performed as a function of time, temperature and mutation on yeast enolase [87], uncovering a completely distinct and horseshoe shaped region where changes in protein flexibility from TDHDX correlate with changes in $\Delta H^{\ddagger}$ for enzyme catalysis. Once again, these reach from protein/solvent interfaces toward either the active site base or the metal cofactors that surround and activate bound substrate. The resulting data strongly implicate a general presence of thermal energy transfer pathways that are embedded within an enzyme's structure and are unique to each reaction catalyzed. We further propose that site specific thermal conduits result from co-evolutionary changes within the protein scaffold, to accompany the emergence of new active site structures and chemical reactivity.

Dynamical Probes for the Temporal Resolution of Protein Motions.

The TDHDX methodology discussed above is analogous to the majority of biophysical probes that generate time averaged structural information. It is generally assumed that the source of the thermal activation of enzyme reactivity comes from collisions of bulk water with a regime of biological water that includes protein bound and highly structured waters [88]. With the increasing body of evidence for protein networks that provide high site selectivity with regard to thermal transfer from solvent to the components of the active site, many questions arise. In particular, the time scales and mechanisms for communication between bulk solvent and enzyme active sites become of paramount importance. The time scale for enzymatic reactions is millisecond, making observed rates 10^{12}-fold slower than the actual (femtosecond) barrier crossings, leaving open a wide range of time dependent changes in protein structure that can be linked to catalytic activation.

In recent studies, Zaragoza et al. targeted the protein surface of SLO, through the introduction of site-specific cysteine residues that were modified through chemical attachment of a fluorescent molecule. Using Badan (6-bromoacetyl-2-dimethylaminonaphthalene) as the installed probe and focusing on time and temperature dependent Stokes shifts [89], initial experiments detected nanosecond transients that could be assigned to either bound water or free water undergoing rapid exchange with the bound water [88]. Quite significantly, incorporation of Badan at the primary loop identified by TDHDX for SLO leads to identical activation energies for the observed Stokes shifts and for catalytic turnover, while a control loop indicated no such correlation. Subsequently, five SLO variants within the network with different activation energies for catalysis were pursued, yielding an identity of Ea values for Stokes shifts to Ea values for active site catalysis [83]. These studies provide the first temporal resolution of nanosecond surface protein motions that reflect solvent-induced activation at a protein/water interface that is transmitted cooperatively over a distance of 15–30 Å to the buried active site [83]. Such behavior contrasts with the default role of distributed and rapidly equilibrating conformational landscapes as the dominant dynamical behavior impacting enzyme function (discussed above), leading to a new model for the essential role of protein dynamics in achieving optimal enzyme catalysis [83].

Integration of the dynamical properties of enzymes into *de novo* protein design.

Recent years have witnessed substantial progress in our ability to perform *de novo* design of proteins [90] and to introduce new functionalities into existing protein scaffolds [91,92]. Such protein design efforts have helped elucidate general principles of protein structure and test our understanding of the biophysical and functional mechanisms of naturally evolved proteins. However, computational protein design methods generally rely on the assumption of static conformations in order to handle the vast complexity of sequence space, generally providing relatively small overall rate accelerations. The success of design methods, especially in relation to functional proteins, is expected to benefit from explicit considerations of conformational heterogeneity and dynamics.

A common trend in evolutionary trajectories is that the resulting mutations are not necessarily found in or nearby the active site. Of particular interest to protein design, propagated effects can occur via long distance side chain reorganization. Jackson et al. analyzed interaction networks in the evolutionary trajectory of a phosphotriesterase from *Pseudomonas diminuta*, showing that while the atomic positions changed very little, the interaction network changed substantially. In particular, one loop gained more interactions, resulting in a loss of mobility, while removal of interactions to another loop caused increased mobility [93]. We note that while progress is being made, we remain a long way from being able to routinely improve protein function by manipulating conformational sampling. Going forward, a deeper understanding about enzyme catalysis is still required, especially in terms of how enzymes generate and utilize site selective networks that are in contact with solvent and transmit thermal activation to initiate active site chemistry.

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the atomic positions changed very little, the interaction network changed substantially. Those interaction networks play an important role in enhanced catalysis.

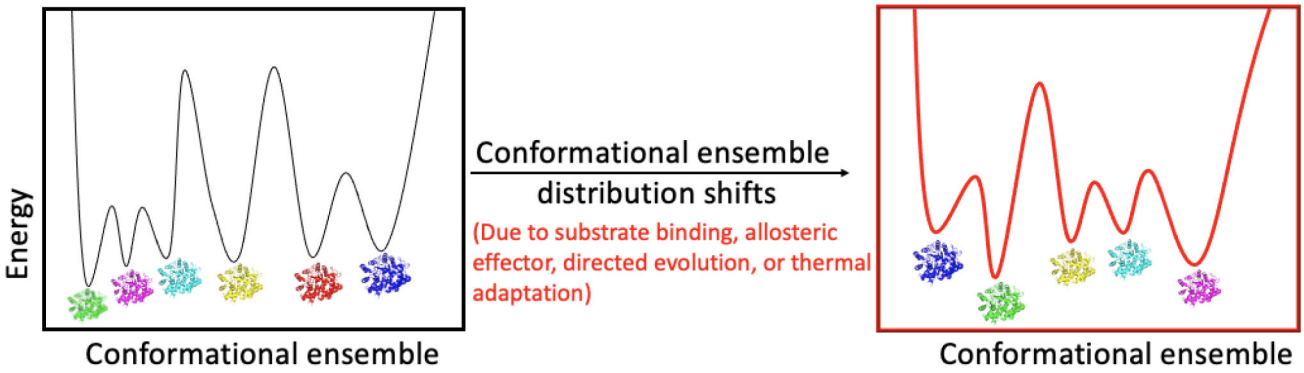

Figure 1.

Conformational selection occurs in many biological processes including substrate binding, allosteric activation, directed evolution and thermal adaptation. Conformational ensemble redistribution has been detected in many systems (dihydrofolate reductase (DHFR) [50], Kemp eliminase [52,53], and tryptophan synthase [49]) using different techniques including X-ray crystallography, NMR, and computational simulations. For the above example, the impact of perturbation is shown to alter both the number of accessible protein sub-states and their relative populations (where green represents the most stable state).

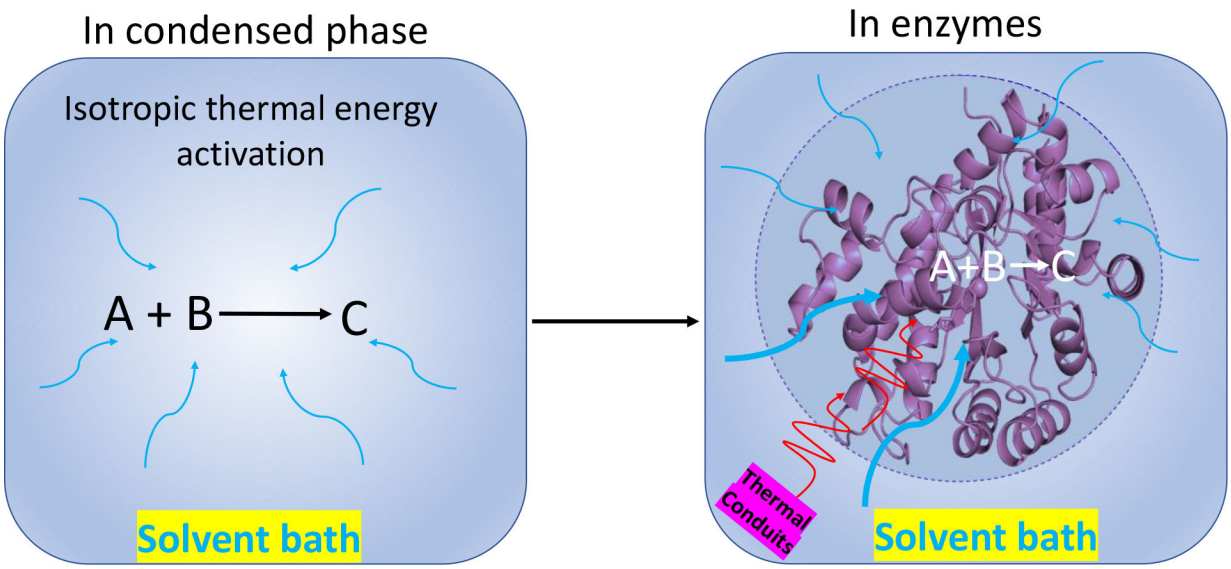

Figure 2.

Collision theory states that for a chemical reaction to occur in condensed phase, the reacting particles must collide with one another. The rate of the reaction depends on the frequency and direction of collisions. For such temperature dependent reactions, thermal energy is expected from all directions in the solvent bath via isotropic thermal energy transfer (Left). As a comparison, enzymes are anisotropic structures, with active sites for chemical reactivity that are generally protected from direct collisions with solvent, implicating the protein scaffold as the basis for controlled heat transfer from the solvent bath to an enzyme active site (Right). Mechanistically, it is likely that enzymes will have evolved throughout time to construct privileged thermal conduits for efficient conduction of heat from the solvent to the active site that facilitate catalysis.

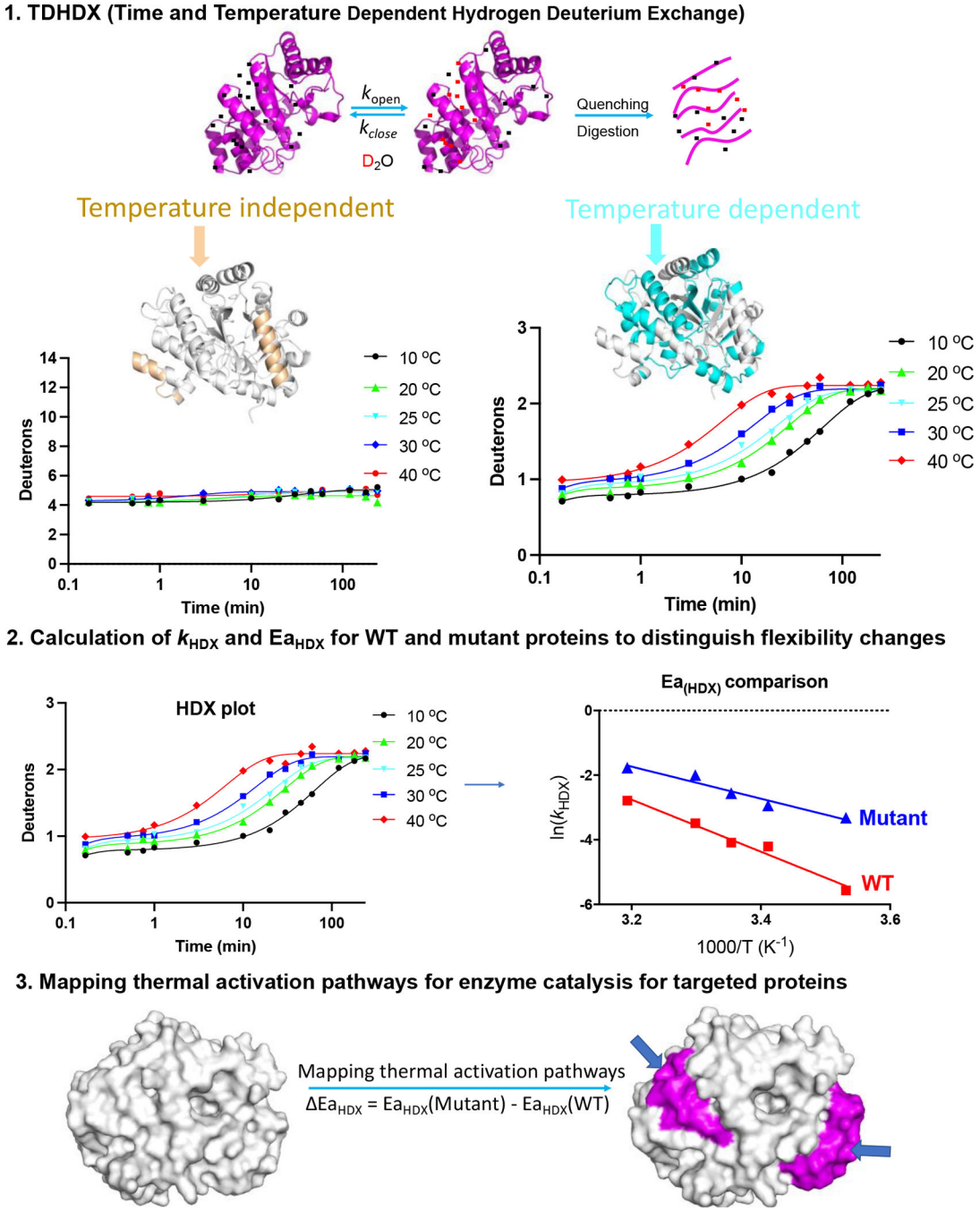

Figure 3.

1. Procedures for uncovering thermal transfer pathways using temperature dependent hydrogen deuterium exchange coupled to mass spectrometry (TDHDX) using data from mADA as an example. (1) HDX samples are digested into small peptides and analyzed by mass spectrometry to detect deuteron incorporation. Most peptides will manifest at least 2 types of patterns: temperature independent plots (left panel, mapped in gold for the two peptides of this type in mADA) with the example shown undergoing rapid exchange for 4/14 amides and no further detectable change in deuteron uptake throughout the experimental

period; and temperature dependent plots (right panel, mapped in cyan for the 10 peptides of this type in mADA) and where temperature dependency is apparent throughout the time course. 2**.** Peptides with apparent temperature dependent trends are fitted to a three-exponential equation (see Ref [86,87] for details) providing rate constants for the different regimes of HDX exchange. Using weighted average rate constants, Arrhenius plots can be generated to calculate the observed activation energy for HDX (Ea_{HDX}) for each peptide. Accordingly, corresponding parameters are analyzed for mutants of interest and compared to the WT protein behavior. 3**.** using ΔEa_{HDX} as a proxy, regions within a protein that show functionally relevant changes in protein flexibility are mapped onto the protein structure, to reveal possible thermal energy transfer pathways for efficient enzyme catalysis. *Emerging models for enzyme catalysis invoke a redistribution of protein substates upon binding of substrate (Figure 1) that acts in concert with efficient heat transfer via embedded thermal conduit (Figure 2) to enhance the probability of reaction barrier crossings.*