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Choreographing an enzyme's dance

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Abstract

While ground state structures combined with chemical tools and enzyme kinetics deliver useful information on possible chemical mechanisms of enzyme catalysis, they do not unravel the finely balanced energy inventory to explain the impressive rate enhancement of enzymes. For this goal, a complete description of enzyme catalysis in the form of an energy landscape is needed. Since the rate of catalysis is determined by the climb over a sequence of energy barriers, we focus here on the critical question of transition pathways. A combination of time-resolved NMR and simulation deliver a glimpse into how proteins can so efficiently move within the ensemble of the native conformations while avoiding unfolding during that journey. The loss of energy due to breakage of native contacts is compensated by non-native transient hydrogen bonds during the transition thereby “holding on” to the energy until the new native contacts form that define the alternate functional state. The use of kinetic isotope effects (KIE) to study the chemical step show that coordinated atomic fluctuations of the protein component dictate the probability of “correct” distance and orientation, due to its extreme sensitivity to distance. The examples here stress the point that highly choreographed conformational sampling together with chemical integrity is a prerequisite for efficient enzyme catalysis.

Introduction

The impressive catalytic power of enzymes has both fascinated scientists and created disputes among them. Despite remarkable information generated using chemical tools in the last 100 years, complemented more recently with structural and computational approaches, we cannot yet identify with a complete energy inventory how any enzyme works. A major obstacle lies in the fact that static ground state structures with fixed atomic distances do not give sufficient information needed to understand rate enhancements by enzymes. Catalysis occurs when the enzyme/substrate system moves between these ground state structures; the climb over the energy barriers (Fig. 1). The complete conformational transition pathways cannot be directly seen by atomistic experimental methods. All-atom molecular dynamics simulations in principle can deliver just that, however even the fastest enzyme known on earth has overall turnover times in the high microseconds, exceeding the current limit of these simulations [1].

Enzymes are highly sophisticated creatures with many, many atoms involved in their function. The secret of enzymes lies in their ability to partition energetic contributions among these atoms in a well-coordinated style just like the choreography of a beautiful dancer. Since not a single enzyme is yet understood on that level, we offer a personal perspective illustrating these

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principles with recent results particularly focusing on the question of transition pathways. We hope to provoke scientists to scrutinize enzymes with a battery of methods to expose the physical secrets of the enzyme world.

Dispute and confusion about “how enzymes work” [2,3] has been mainly created by ambiguity of language, which has lead to everything from simple misinterpretation to hilarious howlers [4,5]. Therefore, we first define enzyme catalysis through an unambiguous description in the form of an energy landscape [6,7]. Unlike the uncatalyzed chemical reaction, the enzyme-catalyzed reaction is composed of many steps, with the chemical step just being one of them (Fig. 1). The speed of the enzyme, k_{cat} , is the rate by which the enzyme converts substrate into product (start to finish), and is not the speed of the chemical step as is sometimes asserted [4, 5]. From Figure 1 it is apparent that for precise performance *all* steps need to be executed very well; a single inefficient step (e.g. a large barrier) will make the whole reaction slow. To re-emphasize, catalytic efficiency is manifested via the motions from substrate to substrate, which are equilibrium fluctuations. The challenge is to disentangle the entire energy landscape and link it to practical experimental methods.

The chemical step(s)

The steps in which the chemical structures of substrate(s) are converted into products(s) have traditionally been the major focus of enzymology. The all so familiar notion of arrows pushing electrons in the active site of enzymes has been enriched by x-ray and NMR structures, providing atomistic snapshots of ES- or EP-complexes. While in favorable cases, spectroscopy and other clever techniques allow one to measure the rates of the chemical step, none of the techniques unravel the pathways between the intermediates. Since these pathways can rationalize the observed reaction rates in proteins, we offer two examples of new approaches aimed at cracking the secrets of how enzymes accelerate particular chemical steps.

The use of hydrogen kinetic isotope effect (KIE)s to probe dynamics influencing the chemical step

Hydrogen KIE's, which measure the dependence of an atom's isotopic identity on the rate of a chemical reaction, are extremely sensitive distance probes due to the strong distance dependence of hydrogen tunneling [8]. Hydrogen transfer from a donor to acceptor is a simple chemical reaction that only takes femtoseconds once the donor-acceptor comes within the reactive distance. However, experimentally measured rates in enzymes are in milliseconds. Clues for this big discrepancy came from ground state structures revealing that donor/acceptor distances are too far apart for the reaction to happen, and from KIE's suggesting that thermal fluctuations are responsible for the reaction. A primary deuterium KIE of 80 [9], together with unexpected KIE temperature dependences for mutants far away from the hydrogen transfer site, provided an experimental handle on the idea of probability of distance sampling for a reaction [8,10,11]. The experimental data on soybean lipoxygenase [10–12], alcohol dehydrogenase [13,14], dihydrofolate reductase [15–17] and other enzymes undoubtedly identify residues far away from the active site that can affect the probability of correct orbital overlap. Despite the elegance of this method, there are still many challenges in refining the interpretation of the abundance of data already produced to derive detailed energy landscapes for these complex macromolecules.

Transition state structures of PNP

The enzyme purine nucleoside phosphorylase (PNP) carries out a reaction that is more complicated in comparison to the hydrogen transfer reaction discussed above. The substitution chemistry involves the breakage of a carbon-nitrogen bond, dissociation of the leaving group, followed by a nucleophilic attack to the ribo-cation intermediate leading to the formation of a

new bond. Based on a very high degree of conservation between human and bovine PNP (100% identity for catalytic site residues and 87% sequence identity overall) it was expected that the transition state of both proteins would be the same [18,19]. Well-designed work by the Schramm lab using proton, carbon and nitrogen KIE's, however, suggests that human PNP has a fully dissociated S_N1 transition state, while bovine PNP's leaving group is only partially dissociated in the transition state [18–20]. The differences in the predicted transition states were verified with inhibitors designed based on the different KIE values; each inhibitor binds tightly and preferentially to either the bovine or human PNP. By comparing homologous proteins with identical active site residues, the authors were able to show that amino acids some distance away from the “site of chemistry” play a role in the reaction.

These two examples illustrate that conformational sampling (dynamics) is important for the chemical step. The average donor/acceptor distance of the reactants in the ground states is too far for the reaction to occur. Coordinated atomic fluctuations of the protein component dictate the probability of “correct” distance and orientation, which can be beautifully measured by KIE due to its extreme sensitivity to distance.

All microscopic steps of the catalytic turnover

By definition enzyme catalysis has to be a multi-step journey due to binding, chemistry and release. The enzymatic cycle is however much more complicated with more intermediate steps. Spectroscopic and chemical methods have been the main techniques used in the past for detecting individual microscopic steps. NMR has become a particularly powerful method to dissect microscopic steps (i) at steady state and (ii) steps of purely conformational changes of the protein component that do not involve chemical changes or the reactants. Notably, those conformational changes represent the rate-limiting step of overall turnover for a number of enzymes (Adk [21,22], CypA [23–25], DHFR [16,17] and others (see [1])) meaning that the chemical steps do not make up the bottleneck.

In addition to the rates of interconversion, conformational intermediates can, in favorable cases, be structurally determined, however, the conformational pathways from one state to another cannot. Given the range of complexity of conformational changes in proteins, it seems surprising how effortless these functional transitions (dynamics) take place while avoiding partial unfolding at the same time. Since one cannot “watch” the protein moving from one structure to another with any experimental technique, computational approaches have become essential for this question. The importance of the marriage between computation and experiment is highlighted below.

Transition pathways

Energetic correlation between the activation energy landscape and the folding landscape in the kinase NtrC

Nitrogen Regulatory Protein C (NtrC) is a bacterial signaling protein, which self-catalyzes the phosphorylation of an aspartate residue in its active site in response to low nitrogen levels. NMR relaxation experiments allowed a quantitative characterization of the energy landscape (Fig. 2) [26,27]. The power of these NMR dispersion methods lies in the ability to determine thermodynamic (relative populations of the interconverting species), kinetic (rates of interconversion) and structural information (in form of chemical shifts) at atomic resolution [28–30]. For NtrC, the NMR data revealed an allosteric model of activation in which a pre-existing equilibrium exists between the inactive and active conformations of the enzyme before phosphorylation [26]. Phosphorylation leads to a shift in this equilibrium from 14% to 99.9% active state through ground state stabilization of the active form, and does not induce a new

structure (Fig. 2A) [27]. We propose that such an allosteric mechanism could be a more general paradigm for kinases [26,27].

Importantly, we were able to unambiguously define the change in the functional energy landscape by linking this energy landscape to the energy landscape of unfolding (Fig. 2) thereby overcoming the well-known problem of a reference state for depicting absolute free energy changes in Φ -value analysis [31]. A key emerging question that arose from this model is: How can a protein be dynamic for function but prevent unfolding? The answer is rooted in the molecular pathways of the conformational transitions.

The transition landscape of NtrC

As rationalized above, molecular dynamics (MD) simulations with tricks to deal with the interconversion times relative to the sampling time are needed to “watch” the pathway. A large number of computational approaches have been developed to contend with this sampling problem, reviewed elsewhere [32–39]. We used Targeted Molecular Dynamics (TMD) [40] which uses a biasing potential towards the target, to predict pathways for the activation of NtrC [41]. The transition pathway is complicated with multiple steps, comprised by loop movements as well as a tilt, rotation, and helical register shift in the α -helix 4, due to the loss of two hydrogen bonds at the C-terminus of the helix when leaving the active state and the gaining of two hydrogen bonds at the N-terminus in the inactive state (Fig. 3A).

Interestingly, non-native, transient hydrogen bonds were seen in the TMD trajectories (Fig. 3A,B) seemingly compensating for the loss of backbone hydrogen bonds during the helical register shift [41]. This observation offered the opportunity to experimentally test the predicted pathway. Strikingly, mutations that disrupt these transient interactions led to a decrease in the rate of exchange between the inactive and active states as measured by NMR (Fig. 3C), corresponding to an increase in the transition barrier, but no change in the free energy difference between the active and inactive states [27].

Combination of simulation and time-resolved, atomistic NMR experiments delivered a glimpse into how proteins can so efficiently dance within the native state ensemble while avoiding unfolding during that journey. The loss of energy due to breakage of native contacts is compensated by non-native atomic interactions thereby lowering the energy barrier of interconversion and “holding on” to the energy until the new native contacts are formed that define the alternate functional state.

The experimentally verified structural configuration along the trajectory provides excellent starting points for future exploration of the entire multidimensional transition landscape (Fig. 3D) through unbiased simulations and a growing number of exciting computational pathway methods that promise to finally calculate entire free energy landscapes [42,43].

The enzyme needs it all

NMR and crystallographic results on the prolyl –cis/trans isomerase cyclophilin A (CypA) illustrate two fundamental principles: (i) that sampling of the conformations needed for catalysis is an intrinsic property of the enzyme that is simply harvested by the substrates and (ii) that all steps, chemical and conformational, need to be fine tuned for efficient rate acceleration (Fig. 4). Using NMR relaxation experiments, protein dynamics can be measured during turnover, and individual steps can be separated [23,25,44–46]. The fact that dynamics measured in the free enzyme is very similar to the enzyme dynamics during turnover led to the proposal of a pre-existing equilibrium being essential for catalytic turnover [23]. However, unlike the NtrC protein, the structure of the minor state for CypA has been missing. Intriguingly, the minor state can be seen directly in the electron density of an x-ray data set

collected at room temperature, one just has to look in the traditionally labeled “noise” of the data, below the 1σ level [24] (Fig. 4B). The interpretation of the diffraction data representing partial occupancy of the major and minor states and the hypothesis that interconversion between them is important for catalysis was buttressed by a designer mutant, S99T, located three β -strands away from the site of chemical action. The mutation indeed flipped the population as predicted, but surprisingly also made the conformational interconversion much slower. Strikingly, the overall turnover rate (k_{cat}) is also much slower although the chemistry (Fig. 4C) is kept intact. The fact that the chemically crippled enzyme (by removing the catalytic Arg in the active site) is reduced in k_{cat} (chemical step is rate limiting in R55K) by about the same amount as the dynamically crippled mutant, S99T (conformational change is rate limiting in S99T), stresses the point that both the chemical integrity and optimized conformational sampling is a prerequisite for efficient enzyme catalysis.

Conclusion

The performance of enzymes is just like the performance of dancers with basic common steps as foundation and open-ended sophistication and creativity needed for individual style and execution. Moreover, the choreography ultimately creates the flow of the entire dance. From the fascinating variety of enzymatic reactions and the impressive performances it is apparent that evolution created an amazing number of professional dancers. Right now, in the best scenarios, we may understand some of these basic steps, but are not even close to the sophistication that nature developed as evidenced by the difficulty of creating amateur enzymes [47,48]. The idea of choreography among all steps and efficient sampling of conformational states that guarantee fast overall enzymatic throughput is not part of the design strategy yet. The best way of improving our knowledge may be to learn how nature has evolved the professionals, or by studying enzymes evolved in the test tube through directed evolution [49–54] on the level of the energy landscape. We envision that exciting developments of more quantitative experimental methods, new computational approaches that are aimed at calculating free energy landscapes [42,43,55], and most importantly, a much tighter interaction between experiments and computation will take our current understanding of enzyme catalysis above the amateur level and hopefully produce some professionally dancing enzymes in the future.

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Figure 1.

Schematic of the Energy Landscape of Enzyme Catalysis. Catalysis of a chemical reaction by an enzyme is comprised by a number of steps: substrate binding ($1 \leftrightarrow 2$), protein conformational changes ($2 \leftrightarrow 3$) and ($4 \leftrightarrow 5$), the chemical step ($3 \leftrightarrow 4$), and the dissociation of product ($5 \leftrightarrow 6$). The energy landscape is defined by the individual free energy differences between the ground states (ΔG) and the individual activation free energies ($\Delta G^\ddagger$) (indicated for only one step) dictating the microscopic rate constants (k_1, k_{-1}, k_2, etc).

Figure 2.

Energetic relationship between the energy landscapes of activation and protein folding of the signaling protein NtrC. The energy landscape of the inactive (I) to active (A) conformational transition for non-phosphorylated (red), and the BeF_3^- -activated (black, BeF_3^- as phosphate mimic) receiver domain of NtrC is shown in A. The shift in the relative populations of I and A, as well as the rates of exchange between the two states (k_{IA} , k_{AI}) were determined through ^{15}N -CPMG NMR exchange experiments [27], which measure dynamics on the microsecond to millisecond timescale.

Verification of the depicted activation energy landscape (A) through the energetic connection to the unfolding energy landscape (B) represented by the horizontal dotted lines. The unfolding rates (k_U) were measured using stopped-flow fluorescence experiments. The difference in free energy calculated from the fluorescence experiments mirrored the difference in stability predicted from the NMR experiments on the conformational dynamics (bars representing the population average free energy of the I and A states) [27].

In contrast, mutations that remove the transient side chain S85-D86 or Y101-Q96 hydrogen bonds (see also Fig. 3) only affect the free energy of the transition landscape ($\ddagger$, blue) supporting the calculated transition pathway from TMD [41]. The structures of the inactive (1DC7) and active (1DC8) states and a snapshot from the TMD trajectory representing the transition region are shown highlighting the region undergoing conformational exchange by a color scale from blue to red to yellow with increasing root mean square deviation relative to the starting active conformation. (Modified from [27]).

Figure 3.

Transient non-native hydrogen bonds lower the energy of the transition landscape. (A) Snapshots of the active (starting point of TMD), inactive state (end point of TMD) and during the transition (1695 ps) are shown zooming in on the major switch region. In the active state, the C-terminal residues of helix 4, Q96 and Q95, are shown with their corresponding helical backbone hydrogen bonds (black arrow). In the inactive state, this last turn is lost and the hydrogen bonds are replaced by two new helical hydrogen bonds for S85 and H84 (black arrow) adding a turn at the N-terminal part of the helix resulting in the register shift. During the transition (middle panel), side-chain hydrogen bonds are formed between S85 and D86 (red arrow) and between Y101 and Q96 (blue arrow). (B) Corresponding time-trace of the distance between the hydroxyl hydrogen of S85 and the carboxylate oxygens of D86 (red) and Y101 and Q96 (blue) in the simulation [41]. (C) Experimental testing of the transition landscape: Removal of the predicted transient non-native H-bonds indeed decreases the rate of interconversion (S85D, Y101F) and can be restored (S85N) to the rate of WT. (D) Putative transition landscape for the conformational transition of NtrC highlighting the multi-stage pathway with meta-stable states, predicted from the TMD and additional simulations and supported by NMR experiments which show that the two transient hydrogen bonds between S85 and D86 and between Y101 and Q96 (A,B) are non-additive [27] (Modified from [27]).

Fig. 4.

Both conformational interconversion and chemical integrity are important for overall enzymatic turnover in CypA. (A) Residues with amide backbone conformational exchange measured by NMR relaxation dispersion are highlighted in red onto the x-ray structure of CypA ([23]); side-chains that differ between the major and minor states (B) are shown in stick representation in red. (B) The modeled major (red) and minor (orange) states of CypA are shown with the corresponding electron density map at 0.3 σ . Concerted side-chain rotations spanning from the active site through the β -barrel are responsible for the major/minor conformational equilibrium. (C) Schematic representation of the proposed role of Arg 55 accelerating the chemical step by lowering the double bond character of the prolyl peptide bond via hydrogen bonding to the free electron pair on the prolyl nitrogen.