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Molecular Modeling of Conformational Dynamics and its Role in Enzyme Evolution

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Abstract

With increasing computational power, biomolecular simulations have become an invaluable tool for understanding enzyme mechanisms and the origins of enzyme catalysis. More recently, computational studies have started to focus on understanding how enzyme activity itself evolves, both in terms of enhancing the native or new activities on existing enzyme scaffolds, or completely *de novo* on previously non-catalytic scaffolds. In this context, both experiment and molecular modeling provided strong evidence for an important role of conformational dynamics in the evolution of enzyme functions. This contribution will present a brief overview of the current state of the art for computationally exploring enzyme conformational dynamics in enzyme evolution, and, using several showcase studies, illustrate the ways molecular modeling can be used to shed light on how enzyme function evolves, at the most fundamental molecular level.

Keywords: computational enzymology • conformational diversity • enzyme design • enzyme evolution • enhanced-sampling approaches

Introduction

There has been substantial debate in recent years about the extent to which conformational dynamics plays a role in facilitating enzyme catalysis [1-11]. What is even less well understood, however, is the extent to which the fine tuning of enzymes' conformational ensembles acts as a driver for molecular innovation and the evolution of new catalytic functions. The "New View" of proteins, which was put forth by Tawfik and coworkers in the early 2000s [12,13] has found increasing experimental and computational support [14-16]. In brief, this model argues that a single protein sequence can adopt both multiple structures and functions, which can be separated by either local conformational fluctuations (such as side chain or loop dynamics), or the global conformations of the protein. This flexibility allows enzymes to vastly expand their functional diversity, as the incorporation of mutations can alter the conformational ensemble of a protein towards conformations that are only rarely sampled in the wild-type enzyme but allow it to bind new ligands or perform new chemistry (Figure 1). Being able to understand the role of conformational dynamics in enzyme evolution is important not only to expanding our fundamental biochemical understanding of how enzymes work, but also provides a new feature that can be manipulated in the design of novel enzymes with tailored properties for use as extracellular catalysts. We recently covered the increasing evidence of the important role of conformational diversity in enzyme evolution in a separate review [11], and refer interested readers to our previous work. In the present contribution, we will discuss the important role simulations are starting to play in unraveling the secrets of molecular evolution.

Examples of Relevant Methodologies

Molecular simulations have a long history of being used to address complex biological problems [17-21], and understanding the role of conformational flexibility in molecular evolution is no exception. That is, while traditionally, computational approaches to studying evolution have focused on the analysis of either sequence data or approaches based on the tools of structural bioinformatics [22-24], the increasing computational power now allows biomolecular simulation techniques to be used to study the evolution of protein function at the molecular level. This, of course, includes coupling hybrid QM/MM simulations [25-27] of enzyme function with overall conformational sampling using classical molecular dynamics (MD) [28-30] and enhanced-sampling approaches [31,32] such as different flavors of replica exchange simulations (e.g., temperature [33] or Hamiltonian [34]), umbrella sampling (US)

[35], or metadynamics [36]. In addition, long timescale MD simulations can be used to construct Markov state models (MSM) [37,38] in order to examine different potential conformational states sampled by the system. Finally, biomolecular simulations benefit, nevertheless, from structural bioinformatics and machine learning based approaches, and can in turn also be coupled to, for example, normal mode analysis [39,40], principle component analysis (PCA) [41-43], dynamic cross-correlation matrix (DCCM) analysis [44], mutual information analysis [45,46], or approaches to study flexibility such as the dynamic flexibility index (DFI) [47] or shortest-path map analysis [48], to name just a few (increasingly) commonly used examples. Our goal in this contribution is to highlight some showcase studies, using methods such as those listed above, to obtain valuable insights into the role conformational dynamics plays in determining both how new functions emerge on pre-existing scaffolds, as well as how enzyme function can emerge completely *de novo* on non-catalytic scaffolds.

Showcase Systems

Understanding the role of conformational dynamics in enzyme *evolution* is a relatively young field. Despite this, there have already been several seminal pieces of work from either computational or an experimental perspective, and, as has been discussed by both ourselves and others [48-53], there is increasing evidence that conformational dynamics at different timescales, whether at the local level of side chains in the active site or global motions across the whole enzyme, can be critical for allowing for the evolution of new enzyme functions. We emphasize here that, clearly, the degree of overall flexibility can vary across the evolutionary trajectory. Therefore, the fine-tuning of dynamics in the early stages of the evolution of new functions is not inconsistent with subsequently more rigid fully-evolved enzymes. This is a separate discussion from that of whether conformational dynamics is important to catalysis itself [11]. However, shifting and fine-tuning of conformational dynamics during the evolution of new functions can allow for an enzyme to sample new potentially catalytically productive conformations, while also binding new substrates, which would in turn lead to the ability to catalyze new chemical reactions.

In our own work, we have used MD simulations to probe conformational diversity along enzyme evolutionary trajectories in different contexts. For example, we recently combined ancestral sequence reconstruction [54], biochemical and biophysical characterization, structural and computational biology in order to engineer a *de novo* active site capable of catalyzing Kemp

elimination [55,56] (Figure 2A) in resurrected Precambrian β -lactamases [57]. We worked with selected systems that sampled a vast region of the sequence space of both ancestral and modern β -lactamases, going back up to ~ 3 billion years of evolutionary time (Figure 2B), in order to explore the structural and physico-chemical features that would allow for the emergence of new active sites. Our work demonstrated that it is possible to introduce a *de novo* active site with a novel biochemical function through a single hydrophobic-to-ionizable amino acid substitution, and that conformational flexibility can assist in the subsequent evolution of this new active site both by improving substrate- and transition state binding, and by increasing the sampling of potentially catalytically competent conformations of the active site [57]. We also showed that the subsequent loss of Kemp eliminase activity upon moving from Precambrian to modern β -lactamases is due to a loss of conformational diversity as the β -lactamases specialized for their modern functions (Figure 2C). This is in excellent agreement with previous computational work on the native β -lactamases activities of these systems [58], which combined MD simulations, clustering of the conformational dynamics using PCA, and assessing the contribution of each position to functionally related dynamics through DFI, and demonstrated that the evolution of conformational dynamics determines the conversion of the promiscuous Precambrian lactamases to specialized modern lactamases.

We note that these observations are in good agreement with a study of a laboratory recombined chimeric β -lactamase, cTEM17m, which is a chimera between the TEM1 β -lactamase and the PSE-4 β -lactamase, taking its active site from the latter enzyme [59]. While both enzymes are relatively rigid, the chimeric enzyme has been shown to demonstrate modified dynamics on the millisecond timescale (i.e., the timescale of the catalytic turnover), compared to its “parent” enzymes, suggesting that native-like conformational dynamics can be modified in engineered proteins [60]. Finally, the conformational diversity of these enzymes has also been used to predict the emergence of mutations that confer tolerance to antibiotics, both by using MSM to identify hidden conformational states that correlate with the ability to hydrolyze the antibiotic cefotaxime [61], as well as using MD simulations and machine learning to predict allosteric mutations that can increase the ability to hydrolyze β -lactam antibiotics [62].

Following from this, we have also recently explored the emergence of catalysis in a non-catalytic protein, by once again combining experiments with MD simulations in order to explore the emergence of chalcone isomerase (CHI) activity (a reaction that is key to plant flavonoid biosynthesis [63]) on a scaffold that initially showed no catalytic activity [64]. CHI

is believed to have evolved from a non-enzymatic ancestor related to fatty-acid binding proteins [65]. We traced the evolution of CHI activity from a protein previously lacking isomerase activity, identifying four founder mutations (each of which individually instated CHI activity), as well as a mutation, F133L, that is not phylogenetically traceable, but yet yields enantioselective CHI activity on this scaffold [64]. Combined X-ray crystallography, NMR, and MD analysis of the evolutionary trajectory revealed not just reshaping of the active site towards a productive substrate-binding mode, but also a shift in the conformational ensemble of a catalytically critical arginine, inherited from the ancestral fatty acid binding proteins [65], into a catalytically favorable conformation.

Houk and coworkers have used MD simulations to perform extensive studies of conformational dynamics in the active sites of both designed and evolved enzymes [66,67]. As one example, this group performed μ s timescale MD simulations of the enzyme LovD from *Aspergillus terreus* [66], which can be used to generate the statin drug Lovastatin [68] (LVA, which is in turn used to treat or prevent dyslipidemia [69] and cardiovascular disease [70]). While wild-type LovD can efficiently synthesize LVA, it exhibits poor activity towards a related statin drug, Simvastatin (SVA), which differs from LVA by only one methyl group [71]. However, 9 rounds of directed evolution yielded an engineered LovD, LovD9, with a 1000-fold increased efficiency towards SVA, *via* the introduction of 29 beneficial mutations [66]. Interestingly, crystal structures of snapshots across this evolutionary trajectory showed that despite the increased activity, the key active site residues remained largely unperturbed. The long-timescale MD simulations demonstrated that, across the evolutionary trajectory, the introduction of the new mutations act to stabilize the conformational diversity of key catalytically important residues. This increased the amount of time the active site spent in a catalytically productive conformation, leading to the observed increases in catalytic efficiency.

MD simulations and structural bioinformatics can provide insights for the role of conformational dynamics in enzyme evolution, as in our recent study of glucose oxidase (GOx) from *Aspergillus niger* (Figure 3A) [52]. GOx is an oxidoreductase commonly used for manufacturing blood sugar meters [72], and with potential applications in biofuel cells to power small implantable devices [73-75]. The significant industrial value of this enzyme has, therefore, led to a high number of directed evolution studies with the aim to engineer various features, such as the possibility for the direct electron transfer [76] or to minimize the influence of oxygen [77]. Using DCCM analysis, we noted that, over an evolutionary trajectory which increased catalytic activity, the motion of residues around the active site became more

correlated, which created a tighter active site with stronger enzyme-substrate contacts [52]. However, the key feature of GOx is the floppy catalytic H516 residue [78,79], which can rotate between catalytic and noncatalytic conformations. As short unbiased MD simulations were not satisfactory to statistically describe this H516 flipping (we typically observed up to one flip per 100 ns of simulation time), we turned to enhanced-sampling MD simulations. In particular, US-MD simulations suggested that, while the two conformations are equally populated in the wild-type enzyme, the additional five beneficial mutations observed in the experimental engineering study [80] drastically increased the energy of the noncatalytic conformation (Figure 3B), as well as also increasing the barrier for the rotation between the two states [52]. Due to the high computational cost of US-MD, we switched to Hamiltonian replica exchange molecular dynamics (HREX-MD) to investigate the changes over the evolutionary trajectory. The enhanced sampling of H516 lead to significant differences between the GOx variants over the evolutionary trajectory. That is, the addition of the first three experimentally studied mutations [80] caused a slight increase in the catalytic conformation population, while as the consequence of the final two experimentally studied mutations, the noncatalytic conformation was almost completely lost (Figure 3C) [52].

We applied a similar set of methodologies to study 2-deoxyribose-5-phosphate aldolase (DERA), an important natural enzyme able to form new C–C bonds [81,82] in the pentose phosphate pathway [83]. DERA is a TIM-barrel enzyme that has an absolute dependency on phosphorylated substrates. In an initial computational/experimental study, we used classical MD simulations, coupled with DCCM analysis and experimental measurements of the temperature dependence of the kinetic parameters, to rationalize the detrimental effect of mutations in the non-canonical phosphate binding site, in the context of disruption to correlated motions in the enzyme [84]. Specifically, when comparing the wild-type enzyme with a catalytically detrimental S239P variant, we observed that the overall level of motion was similar between the WT and mutant enzymes, apart from helix VIII, which interacts with the phosphate group of the bound substrate through the helix dipole, and which was significantly rigidified in the mutant enzyme. We also observed that the overall motions of the protein are highly correlated in the wild-type enzyme (corresponding to “breathing” motions in the TIM-barrel), while the rigidification of the helix VIII broke the correlations almost completely, resulting in a high energetic penalty for substrate binding and catalysis. Furthermore, DERA has an intrinsically disordered C-terminal tail which has been proposed to protrude into the active site, due to the fact that the mutation of the C-terminal Y259 to phenylalanine reduces the reaction

rate of this enzyme by two orders of magnitude [85]. Due to its high flexibility, the C-terminal tail of DERA could not be crystallized. We, therefore, used a combination of HREX-MD simulations and NMR to obtain a structure for the closed state DERA, in which the flexible C-terminal tail of the enzyme enters the active site, and discuss its implications for catalysis [86]. Our study showed that such a conformation is a transient one only, and due to its low population, it would be sampled extremely rarely in unbiased MD simulations.

As a final example, there has been recent work on computationally designing a series of retroaldolases (RA) that can break the C–C bond in 4-hydroxy-4-(6-methoxy-2-naphthyl)-2-butanone substrate (methodol) [87,88]. One of these designs, RA95, was further subjected to 19 rounds of directed evolution to increase its specific activity [89,90]. The evolution of RA95 was the topic of an important contribution on how MD simulations can be applied to study population shifts of conformational substates, a recent work by Osuna *et al.* [48]. Based on long timescale MD simulations coupled with the shortest-path map analysis, the authors pinpoint how mutations at 23 different residue positions, even far from the active, cause shifts in the population of different enzyme conformations. PCA was further able to separate between active and inactive enzyme conformations, which could be translated into the distance between the reactive substrate atoms and the catalytic machinery. Finally, the use of the shortest-path map analysis enabled the identification of residues that are important for the conformational shifts, which can be, in principle, used for the enzyme redesign purposes [48].

Conclusion and Future Perspectives

Understanding protein evolution remains a “hot” topic, due to both its importance to our understanding of fundamental biochemistry, and the implications of this understanding for the design of new enzymes with tailored biochemical properties [91-93]. What is becoming increasingly clear is that conformational dynamics plays an important role in the acquisition of new enzyme functions, and the present contribution highlights some examples of where computational studies have been able to, *ex post*, rationalize the impact of different mutations along an evolutionary trajectory on the conformational dynamics of an enzyme, and how it can be linked to changes in the catalytic activity. As computational power increases and specialized computational algorithms become more refined, molecular simulation will play an ever-increasing role in the design of new enzyme functions [94-96]. Our ultimate goal is to harness conformational dynamics, in a predictive way, in computational enzyme design. As we have

shown in the present contribution, while that goal still remains out of reach, the community is rapidly heading in that direction, making this a particularly exciting moment for such a fast-paced field.

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Conflict of Interest

The authors declare no conflicts of interest.

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Figure 1. Conformational diversity and sampling during the evolution of enzyme function. While a hypothetical wild-type enzyme samples two states with significantly different free energies (i.e., major and minor), mutations accumulated over the evolutionary trajectory, even if located far from the active site, can influence the conformational ensemble of the enzyme's active site. In that way, evolution arranges that, what was originally the structure of a minor state in the wild-type enzyme, becomes the energetically more favorable major state in the evolved variant. Such changes in conformational dynamics and sampling can have an impact on, for example, increasing catalytic activity, shifting reaction selectivity, or enabling enzymes to bind diverse substrates and catalyze novel chemistries.

Figure 2. Kemp eliminase activity can be engineered *de novo* into resurrected Precambrian β -lactamases. (A) The Kemp elimination reaction of 5-nitrobenzoxazole. (B) The phylogenetic tree for β -lactamases (class A) [97], where the ancestral nodes correspond to the common ancestors of Firmicutes (FCA), Actinobacteria and Firmicutes (AFCA), Enterobacteria (ENCA), Gammaproteobacteria (GPBCA), various Gram-negative bacteria (GNCA) and various Gram-positive and Gram-negative bacteria (PNCA). (C) A comparison of the backbone flexibility of different ancestral and modern β -lactamases tested for Kemp eliminase activity in ref. [57], predominantly corresponding to enzyme along the line of descent leading to the modern Enterobacteria. Shown here are the tertiary structures of the selected β -lactamases, colored by C_α RMSF values (in Å) as calculated from 3 x 100 ns MD simulations per system, using the same naming acronyms as in panel (B). The top row illustrates the wild-type enzymes while the structures in the bottom row carry an additional W229D mutation (numbering according to TEM-1 β -lactamase), that was introduced in order to provide a general base that could confer Kemp eliminase activity to these enzymes. This figure was assembled from panels originally shown in ref. [57], and reproduced with permission from ref. [57].

Figure 3. The evolution of the active site dynamics in glucose oxidase. (A) The active site geometry of *A. niger* GOx, featuring E412, H516, and H559 as the catalytic triad, together with the flavin adenine dinucleotide (FAD) cofactor. (B) US-MD simulations of wild-type GOx and its pentamutant indicate that both the catalytic and noncatalytic conformations of the catalytic base, H516, are equally populated in the wild-type enzyme, while the catalytic conformation is largely stabilized in the mutant enzyme. (C) HREX-MD simulations over the evolutionary

trajectory indicate an incremental stabilization of the catalytic conformation of H516. The figure is modified with permission from figures originally published in ref. [52]. Copyright 2017 American Chemical Society.