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Cooperativity and flexibility in enzyme evolution

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Enzymes are flexible catalysts, and there has been substantial discussion about the extent to which this flexibility contributes to their catalytic efficiency. What has been significantly less discussed is the extent to which this flexibility contributes to their *evolvability*. Despite this, recent years have seen an increasing number of both experimental and computational studies that demonstrate that cooperativity and flexibility play significant roles in enzyme innovation. This review covers key developments in the field that emphasize the importance of enzyme dynamics not just to the evolution of new enzyme function(s), but also as a property that can be harnessed in the design of new artificial enzymes.

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Introduction

The classical picture of enzymes has been that they are highly specific catalysts, with one structure correlating to one function [1]. This view was challenged, however, with the realization that many, if not even most, enzymes are catalytically promiscuous, and can catalyze one or more reactions in addition to their native activities [2••,3•,4]. As early as 1976, Jensen (and later O'Brien and Herschlag [3•]) surmised that this promiscuity provides a stepping stone for the evolution of enzyme function, allowing for greater flexibility to acquire novel activities. Indeed, the exponential increase in the number of publications on biocatalysis that occurred between the 1970s and the late 1980s was to a large extent linked to the realization that many enzymes were not as substrate-specific as previously thought, and thus to the emergence of the exploitation of protein promiscuity in biotechnological applications [5,6]. Finally, Tawfik and coworkers [7••,8••] presented an "avante garde" new view of proteins, in which they argued that one sequence can adopt both multiple structures and multiple functions, and that this flexibility forms the cornerstone of the evolution of new enzyme functions. That is, by harnessing conformational diversity and catalytic promiscuity, enzymes can vastly expand the functional diversity of a limited repertoire of sequences, and in this way allow for new functions to evolve in old scaffolds.

Recent years have seen an explosion of interest in this area, focusing on both the role of conformational dynamics in the evolution of enzyme function [7••,8••,9,10••,11,12••,13] as well as on how an enzyme's dynamical properties are altered along evolutionary trajectories [14–17]. Based on work by both ourselves [14,17–22] and others [7••,8••,10••,12••,15,23,24•,25], we propose a model for enzyme evolution that involves a tightrope balance between flexibility, rigidity, cooperativity, and modulation of active site polarity, that controls not only an enzyme's specificity, but also the evolution of new active sites with novel functionalities.

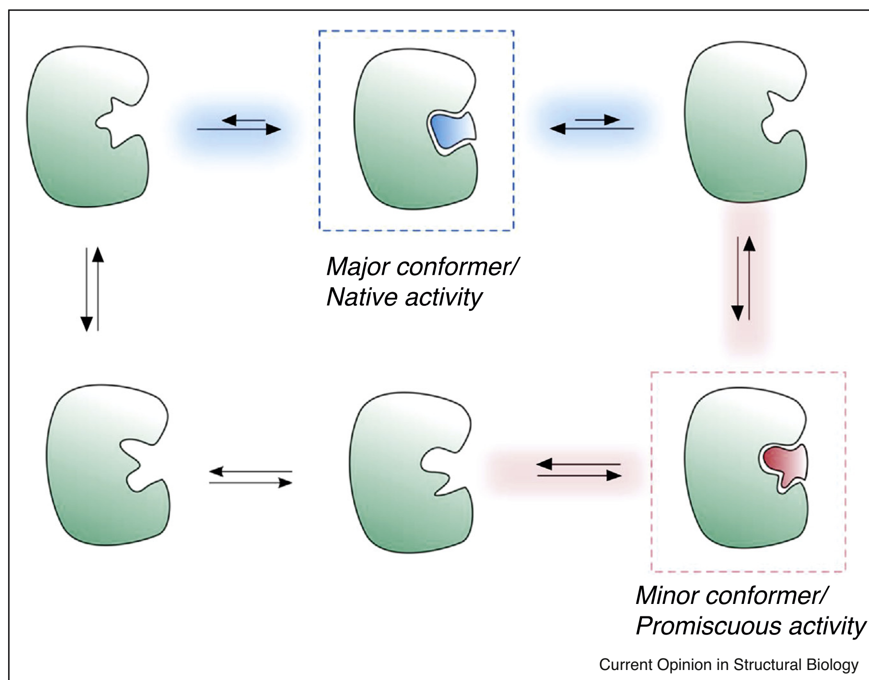
Conformational dynamics and the evolution of new enzyme functions

Enzymes are dynamical entities, that can change their conformation in many different ways, from local fluctuations of side chains, through to large scale loop and even domain motions [26]. These changes can be intimately linked to an enzyme's function: for example, many enzymes undergo conformational changes to attain catalytically active conformations [27•,28], allosteric regulation is critical to the function of many enzymes [29], and several proteins undergo order-disorder transitions to facilitate chemistry (see *e.g.* refs. [30–36]). These conformational transitions also facilitate catalytic promiscuity, allowing enzymes to adapt to bind substrates at the same (or sometimes even multiple) active site(s) [7••,8••,37], and fine-tuning these conformational ensembles can lead to the evolution of new functions (Figure 1) [8••]. To illustrate this point, we present a number of case studies where conformational dynamics clearly plays a critical role in different enzymes' functional evolution.

Dihydrofolate reductase

Dihydrofolate reductase is a monomeric catalyst of the NADPH-assisted conversion of dihydrofolate (DHF) to

Figure 1

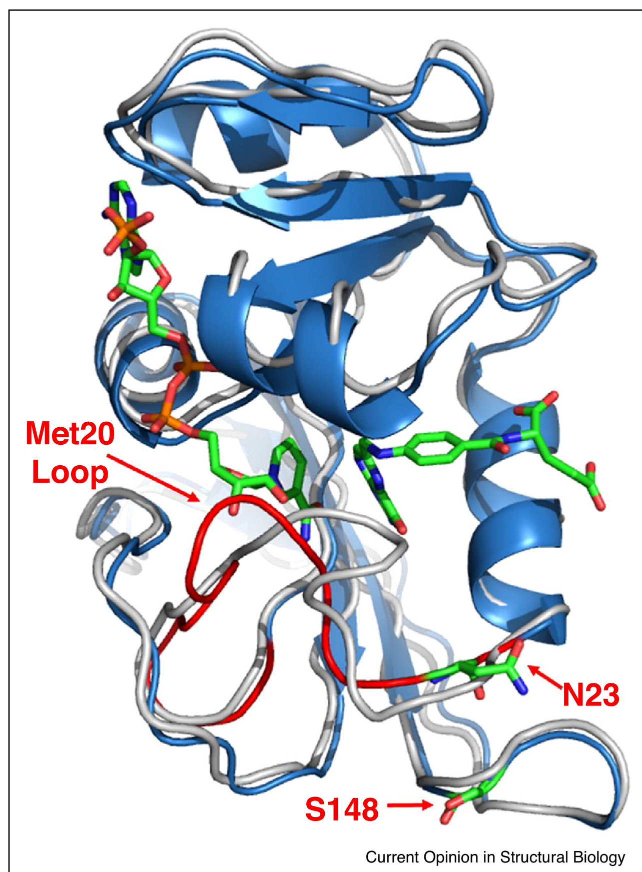


Schematic overview of the relationship between conformational dynamics and protein evolvability. In this model, proteins can interchange between multiple conformations, with the dominant conformation being considered to be the native state, which interacts with the native ligand (blue). Conformational fluctuations such as, for example, side chain or loop dynamics, can then lead to multiple alternative conformations which can either interact with the native ligand, or with promiscuous ligands (red). These alternative conformations may be only rarely sampled in the wild-type enzyme; however, mutations can gradually shift the balance of populations such that any of these alternate conformations becomes the dominant conformation in evolved enzymes, leading to a shift in activity. This figure is adapted from Ref. [8••]. Reproduced with permission from Ref. [8••].

tetrahydrofolate (THF) *via* hydride transfer [38]. This enzyme has a catalytically important and mobile active site loop (the Met20 loop, Figure 2) [39]. The unusual temperature-dependence of the kinetic isotope effects for the hydride transfer reaction catalyzed by this enzyme [40,41] have made DHFR a historically important model system for the study of tunneling and dynamical effects in enzyme catalysis [10••,16,24•,42–51].

Interestingly, even though the human (*h*DHFR) and *E. coli* (*ec*DHFR) enzymes are highly structurally similar, they have significant differences in their sequences, and also their reaction kinetics and rate-limiting steps under physiological conditions [52–54]. To address these apparent discrepancies, Wright and coworkers used a combined structural biology, cell biology, bioinformatics and mutagenesis analysis to probe dynamical differences during the evolution of enzymes in the DHFR family [24•]. Based on this analysis, the authors were able to demonstrate subtle but significant differences in loop dynamics in the two enzymes, that were used to rationalize why *h*DHFR is unable to function efficiently in the environment of an *E. coli* cell. In particular, significant differences in the flexibility of the active site loop in the two enzymes, as exemplified by *h*DHFR lacking the critical closed-to-occluded conformational transition observed in *ec*DHFR, was argued to have a major impact on ligand flux, as well as the overall catalytic cycle, allowing evolution to fine tune the two different enzymes for two different types of cellular environment [24•]. Kohen and Klinman have similarly used DHFR as a model system to probe the evolutionary aspects of enzyme dynamics [10••], through examining evolutionary-dependent (coevolving) residues as well as the preservation of functional dynamics across broad spans of evolutionary time. Based on their analysis, they have argued that DHFR dynamics evolved with time in order to optimize the catalyzed reaction, and that there is a possible evolutionary conservation of functional dynamics at different timescales in the enzyme, which plays a regulatory role in both general biological function of this enzyme as well as in the enzyme-catalyzed reaction. Finally, based on combined isotope labeling and QM/MM studies, Alleman and coworkers have argued for a minimization of dynamical effects during the evolution of DHFR, in order to optimize a nearly-static, reaction-ready and electrostatically optimal ground state during the course of evolution [16].

Figure 2



Overlay of wild-type dihydrofolate reductase (DHFR) in the closed (blue, PDB ID: 1RX2 [39,104]) and open (gray, PDB ID: 1RX4 [39,104]) conformations of the catalytically important Met20 loop. The Met20 loop itself is highlighted in red on the closed conformation. The DHF-H^+ and NADPH ligands, and the sites of the N23 and S148 mutations are also indicated in the closed conformation. This figure was originally presented in Ref. [49]. Reproduced with permission from Ref. [49].

β-Lactamases

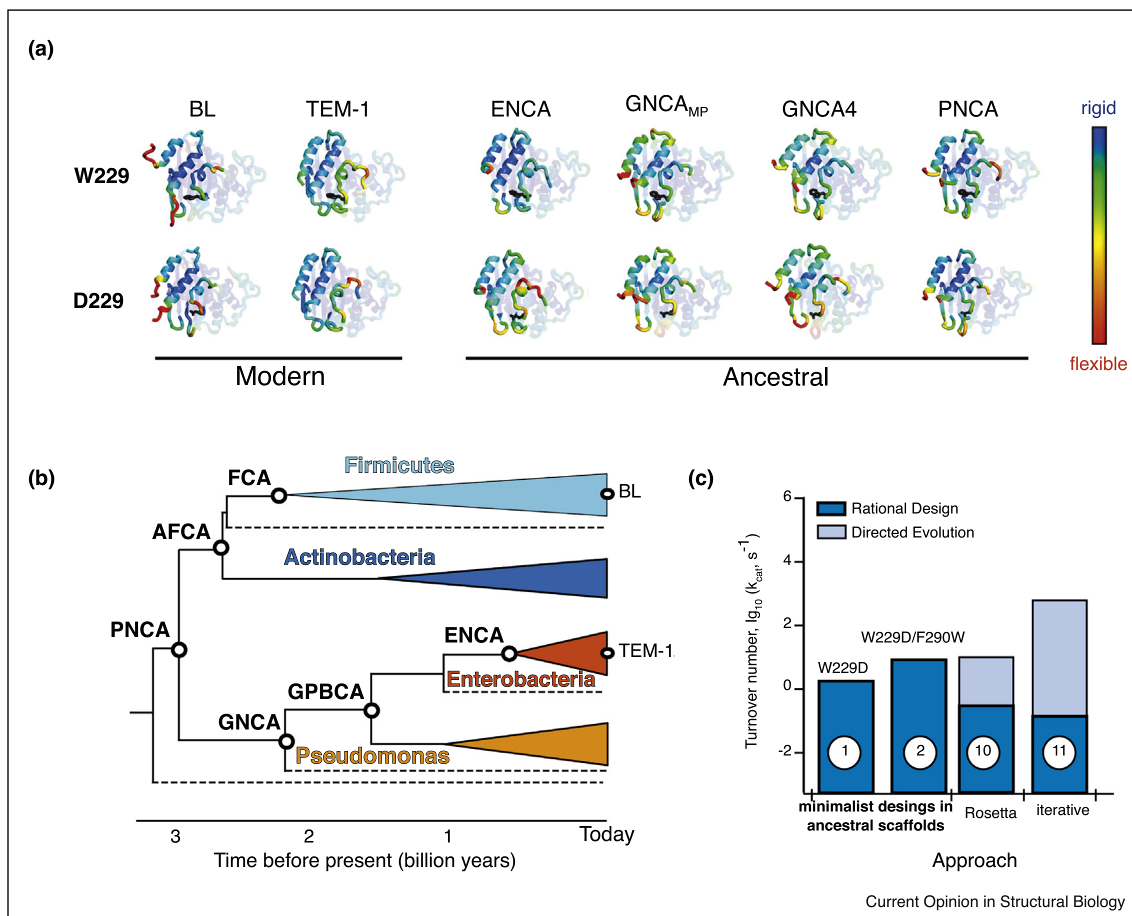
β-Lactamases are responsible for the primary mechanism of resistance towards lactam antibiotics [55]. Many cases of resistance that have been observed during the so-called antibiotic era are linked to mutant β-lactamases that have developed the ability to degrade new antibiotics [55]. However, β-lactamases are ancient enzymes that likely originated billions of years ago, and that are currently widespread throughout the bacterial domain of life [55]. The availability of a substantial number of sequences of lactamases belonging to the diversity of modern organisms has allowed researchers to derive plausible approximations to the sequences of ancestral lactamases [56] using bioinformatics procedures that have been systematically explored in the last ~20 years [57•]. The proteins encoded by reconstructed ancestral sequences corresponding to 2-3 billion year nodes were found to share the canonical lactamase fold. However, they departed from typical modern lactamases in terms of their stability and catalysis profiles. That is, they were highly stable, likely reflecting the thermophilic nature of early life [56]. Also, unlike the modern TEM-1 lactamase which is a penicillin specialist, these Precambrian lactamases were able to degrade a variety of lactam antibiotics, suggesting that they represented Jensen's generalist stage of evolution [2] (although other interpretations are also possible [56]). Computational studies [14] have supported that conformational flexibility, which allows the binding of antibiotics of different sizes and shapes, is responsible for such wide ancestral substrate scope. In addition, this flexibility can be harnessed to predict allosteric mutations that increase the activity of these enzymes, as shown using the CTX-M type extended spectrum β-lactamase, CTX-M9, as a model system [58]. Finally, very recently [17], resurrected ancestral lactamases have been used as scaffolds for the engineering of *de novo* active sites. Specifically, a minimalist design approach that was found to be unsuccessful on many different modern lactamases, was able to generate levels of *de novo* Kemp eliminase activity that was significantly higher than those reported in all previous rational design efforts, even after directed evolution (Figure 3). Molecular dynamics simulations, NMR relaxation studies and X-ray 3D-structure determination supported an essential role for ancestral conformational flexibility in the emergence of this completely new functionality. Overall, these [17] and other recent work [59,60•] support the potential of ancestral reconstruction in protein biotechnology.

Catalytically promiscuous phosphatases

Phosphoryl transfer reactions are central to biology, and the enzymes that catalyze these reactions play an essential role in many life processes, including cellular signaling, energy production and protein synthesis [61–63]. Interestingly, many of these enzymes exhibit varying degrees of catalytic promiscuity, which makes them not only inherently important for understanding the mechanisms of phosphoryl transfer, but also makes them valuable model systems for studying the underlying principles of enzyme multifunctionality.

Among these enzymes, the alkaline phosphatase superfamily have long served as model systems for understanding catalytic promiscuity [64]. The members of this superfamily are metallohydrolases that can efficiently catalyze the cleavage of P-O, S-O and P-C bonds, and many members of this superfamily are highly promiscuous (including the ability to hydrolyze xenobiotic substrates) [64]. These enzymes have been extensively studied both experimentally [65–70,71••] and computationally [18,72–76]. In recent computational work [18], we demonstrated that the underlying feature driving promiscuity among the members of this superfamily is the electrostatic cooperativity of the key catalytic residues, which when combined

Figure 3



(a) Comparison of the backbone flexibilities of different modern and ancestral β-lactamases tested as scaffolds for the engineering of Kemp eliminase activity [17]. The backbone is colored according to root mean square deviations calculated from long-timescale molecular dynamics simulations, as described in Ref. [17]. BL and TEM-1 refer to the modern *Bacillus licheniformis* and TEM-1 β-lactamases. The ancestral β-lactamases are proteins encoded by reconstructed sequences corresponding to the common ancestors of *Enterobacteria* (ENCA), various Gram-negative bacteria (GNCA) and various Gam-positive and Gram-negative bacteria (PNCA). Only variants at the GNCA and PNCA nodes showed substantial Kemp eliminase activity upon minimalist active-site design, although activity at the GNCA proteins was significantly higher. **(b)** Schematic phylogenetic tree showing the nodes targeted for ancestral sequence reconstruction in Ref. [17]. The proteins encoded by the reconstructed sequences at these nodes, as well as 10 different modern β-lactamases, were used as scaffolds for *de novo* engineering of in ref. [17]. While all engineered ancestral proteins (with the exception of ENCA) showed significant Kemp eliminase activity, all the modern lactamases tested led to activity levels barely distinguishable from background. **(c)** Catalytic activities (k_{cat}) of rationally designed Kemp eliminases (dark blue) and improvements achieved through directed evolution (light blue). The numbers of mutational changes involved in the rational designs are shown. Values for the minimalist designs on ancestral scaffolds are taken from Ref. [17]. The value for design based on Rosetta is taken from Ref. [86] and the directed-evolution optimization was reported in Ref. [105]. The iterative design value is taken from Ref. [106] and the directed evolution was reported in Ref. [75]. In each case, we use the value for the best reported variant. This figure was adapted from Ref. [17]. Reproduced here with permission from Ref. [17].

with the very large active sites typically present among members of these superfamily, allows them to accommodate multiple chemically distinct substrates while retaining high activity towards their native substrates. That is, the enzyme's active site provides a subset of key residues to optimally stabilize the transition state for the native reaction, and at the same time this electrostatic preorganization is flexible enough to accommodate electrostatic requirements of various, chemically distinct substrates.

The importance of such electrostatic flexibility is further supported by comparison of the active site properties of different members of the superfamily, which show a correlation between larger active site volume and solvent accessible surface area (SASA), and a higher number of characterized activities for different key superfamily members [18]. This specific type of flexibility of the active site can be understood as a form of enzyme dynamics, in which large structural effects or conformational

diversity are not observed, but rather the local adaptation of active site residues allows the enzyme to facilitate the hydrolysis of various substrates. We note that this observation and its implications for the promiscuity observed in the AP superfamily has been indirectly supported through other studies revealing networks of cooperative residues coupled to the alkaline phosphatase activity [71••]. In addition, even when large changes in active site dynamics are not observed, electrostatic flexibility appears to be important in driving catalytic promiscuity, as exemplified by methyl parathion hydrolase (MPH) [20] and serum paraoxonase 1 (PON1) [21], both of which contain multiple catalytic backups in their active site that allow for multiple substrates to be hydrolyzed through either different mechanisms or interactions with different key residues. MPH also exhibits a different form of electrostatic flexibility, through promiscuity in the catalytic metal ions used, which not only allows for metal-dependent specificity patterns, but also the appearance of cryptic promiscuous activities with different metal ions [77••].

Finally, active site dynamics is also critical to the emergence of organophosphate hydrolase activity, often in enzymes that are either primarily lactonases or have evolved from lactonases [77••,78–80]. An illustrative example of this is provided by PON1, the active site of which is located in the central tunnel of a six-bladed β-propeller structure, and which is covered by a highly flexible loop that forms a lid that closes over the active site upon ligand binding [81]. In a recent study [21], we targeted a key tyrosine residue, Y71, positioned at the tip of the active site loop, and which is part of a catalytically crucial hydrogen bonding network along the central tunnel of the β-propeller [21]. We demonstrated that while mutating this residue clearly changes the loop dynamics irrespectively of which substrate is bound, the same mutations have differential impact on the lactonase and organophosphatase activities of this enzyme. This appears to be due to differential solvation of the PON1 active site with the two substrates bound, with the mutation of Y71 essentially flooding the active site compared to the wild-type when the organophosphate is bound (Figure 4), but not when the lactone is bound, thus having a much larger impact on the organophosphatase than the lactonase activity. We note that, structurally, most organophosphatases either have some form of active site loop [82,83], or deeply buried hydrophobic active sites [84], and it appears that harnessing the dynamical properties of these enzymes to generate solvent excluded active site cages appears to be crucial to the evolution of organophosphate hydrolase activity [21,85].

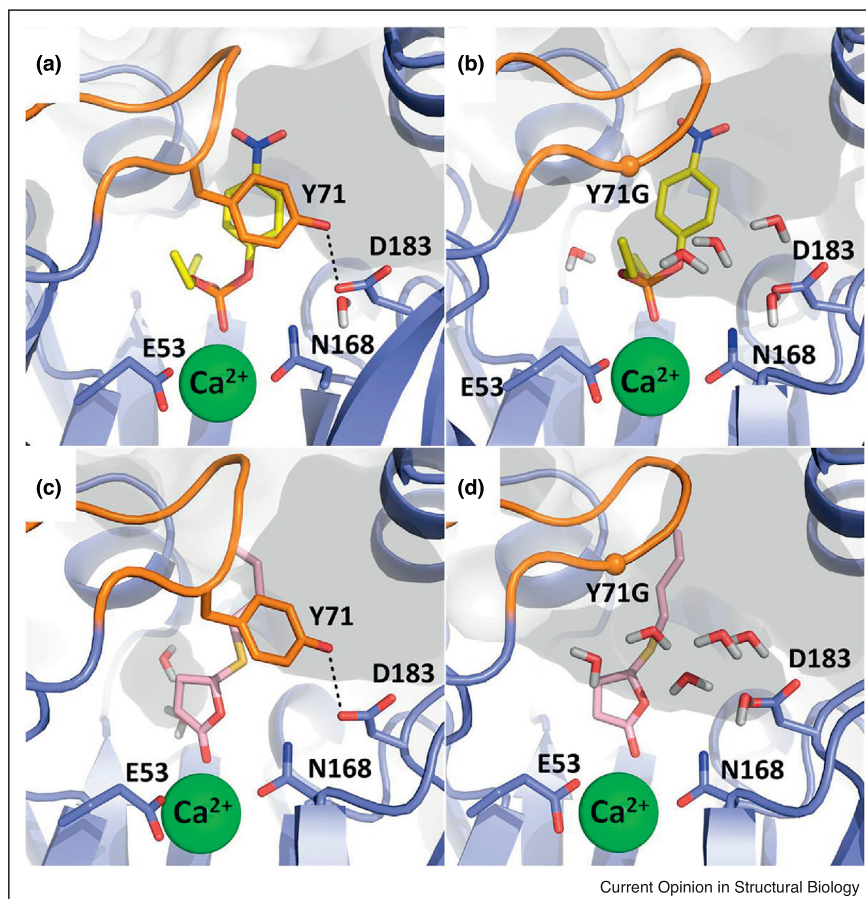
Other systems

While not all relevant systems can be discussed here exhaustively, we want to at least highlight a number of other relevant studies in conclusion of this section. In the context of our own work, we have examined the impact of conformational dynamics in the context of protein engineering for two key systems: 2-deoxyribose-5-phosphate aldolase (DERA) [19], and glucose oxidase (GoX) [22]. In both cases, a combination of experimental and computational work demonstrated that engineered mutations with significant impact on catalytic activity change both the global and local dynamics of the enzyme, in ways that can be correlated with the observed changes in activity. This agrees well also with work by Houk and coworkers, who have studied model systems such as Kemp elimination and transesterification (LovD) [15,25,86], and demonstrated the importance of mutations in altering global dynamics, active site shape, and solvent accessibility of the active site. Parisi has argued that protein conformational diversity modulates sequence divergence [87], and also correlates with the protein's evolutionary rate [88]. Vila and coworkers [89] have applied NMR spectroscopy to study the intrinsic conformational dynamics of a metallo-β-lactamase and identified three key variants through directed evolution. Through doing this, they have shown both that the micro-to-millisecond conformational dynamics of the enzyme is optimized during evolution, and that the effect of the introduced mutations is epistatic. This led the authors to suggest that conformational dynamics is an evolvable trait, and that proteins with more dynamic active sites are also inherently more evolvable (which is conceptually similar to our analysis of functional evolution in the alkaline phosphatase superfamily [18]). Finally, by following the evolution of a phosphotriesterase from *Pseudomonas diminuta* to an arylesterase, Jackson and coworkers were able to extract the role of protein dynamics in the evolution of new enzyme functions, arguing that changes in enzyme function can be achieved through the enrichment of pre-existing conformational sub-states [12••].

Semantic and conceptual considerations

To avoid semantic confusion, it is worth emphasizing here that protein flexibility and dynamics are often discussed in terms of the time scales associated with conformational motions. Motions in different time scales are in fact experimentally observed depending of the height of the free energy barriers separating the relevant protein substates, with picosecond-nanosecond motions reflecting local fluctuations and microsecond-second motions involving collective conformational changes. The latter "slow" motions have received much attention recently because of their potential role in enzyme catalytic cycles [90]. It is important to note, however, that discussions into the role of protein flexibility in enzyme evolution may or may not invoke a specific motion time scale. Thus, for instance, a native protein can be seen as an equilibrium ensemble of more or less related conformations and evolution towards a new enzyme function may be mediated by mutations that shift such equilibrium towards a given productive conformation (see also Figure 1). In this interpretation, flexibility

Figure 4



Comparison of the active sites of serum paraoxonase 1 (PON1) in complex with **(a,b)** paraoxon and **(c,d)** thiobutyl-γ-butyric lactone (TBBL), in the Michaelis complexes of wild-type and Y71G RePON1, respectively. The shaded area shows the solvent-accessible area, and water molecules within 6 Å of the reacting atoms are shown explicitly. The Y71G mutation has a negative impact on the paraoxonase activity of this enzyme, while minimally affecting the lactonase activity [21]. As can be seen here, in the wild-type enzyme, the Michaelis complex with paraoxon is almost completely solvent excluded in the vicinity of the reacting atoms, whereas the Y71G mutation substantially increases the solvent exposure of the active site. In contrast, in the Michaelis complex with TBBL, even the wild-type is already solvent-exposed, and thus the relative impact of this mutation is much smaller. This figure was originally presented in Ref. [21]. Reproduced with permission from Ref. [21]. The original article is available at http://pubs.acs.org/doi/abs/10.1021/jacs.6b10801. For further permission requests, please contact the American Chemical Society.

(conformational diversity) is key to the evolutionary process but does not necessarily appear explicitly in the description of the evolved enzyme. In other words, a mechanism of functional evolution based on conformational flexibility/diversity is not inconsistent with a “rigid” evolved enzyme that populates several closely related conformations, which are capable of efficiently catalyzing the new function. Still, such pre-adaptation need not be complete, and a remaining degree of flexibility may allow for local cooperative rearrangments to occur in response to different substrates.

Finally, it is sometimes stated that the marginal stability of many natural proteins guarantees the degree of flexibility necessary for function. However, there exist analyses that support that marginal protein stability may not be an adaptation for enzyme function, but the result of the existence of a stability threshold together with the fact that the number of available protein sequences decreases with increasing protein stability [91–93]. Indeed, as reviewed in ref. [94], experimental and computational studies on several protein systems support that high stability and enhanced conformational flexibility are not necessarily incompatible.

Overview and conclusions

While there has been substantial research effort invested into probing the role of enzyme dynamics in *catalysis* [26,95–100], significantly less effort has been put into understanding the role of such dynamics in enzyme *evolution*. Already in 2003, James and Tawfik presented this “new view” of the role of conformational dynamics in protein evolution [7••]. This hypothesis has been further supported by the demonstration that most enzymes have

evolved to only be moderately efficient [101•], in part due to diminishing returns and tradeoffs which constrain enzymes from reaching their maximum catalytic potential [102]. In addition, futile encounters and enzyme floppiness have significant impact in modulating an enzyme's reaction rate [103•]. As the field grows, an increasing number of studies have shown that enzyme flexibility, whether as electrostatic flexibility at the local side chain level (as in the case of the promiscuous phosphatases presented here), or at the level of correlated motions across the whole enzyme, appear to play a substantial role in allowing for the evolution of new enzyme functions. It is clear, therefore, that flexible scaffolds may be useful as starting points for protein engineering, thus opening new avenues for biocatalysis. Ancestral reconstruction targeting very ancient proteins (plausibly, Jensen's primordial generalists) or pre-duplication phylogenetic nodes may provide a convenient route to such flexible scaffolds. Finally, as with all biology, this flexibility is in conflict with the specificity and precision in the position of key active site residues required for efficient catalysis, and it's a tight interplay between these features that allows for new functions to evolve in either native or *de novo* active sites uncovered during evolution. While there have been seminal experimental papers in this area, as highlighted in this review, computation has struggled to keep up with experiment, in no small part due to the large computational cost associated with performing the extensive simulations needed to understand the link between structural, functional and mechanistic changes across an enzyme's evolutionary trajectory. However, advances in structural bioinformatics, as well as new approaches for enhanced conformational sampling and modeling of chemical reactivity, together with constant improvements in experimental and structural biology methods, are changing the landscape in this area. Taken together, interdisciplinary studies such as those presented here will allow us to obtain, for the first time, not just a complete molecular picture of how protein function evolves, but also learn how to manipulate the evolution of protein dynamics for the design of artificial enzymes with tailored properties.

Acknowledgments

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- •• of outstanding interest

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