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Laboratory evolution of protein conformational dynamics

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This review focuses on recent work that has begun to establish specific functional roles for protein conformational dynamics, specifically how the conformational landscapes that proteins can sample can evolve under laboratory based evolutionary selection. We discuss recent technical advances in computational and biophysical chemistry, which have provided us with new ways to dissect evolutionary processes. Finally, we offer some perspectives on the emerging view of conformational dynamics and evolution, and the challenges that we face in rationally engineering conformational dynamics.

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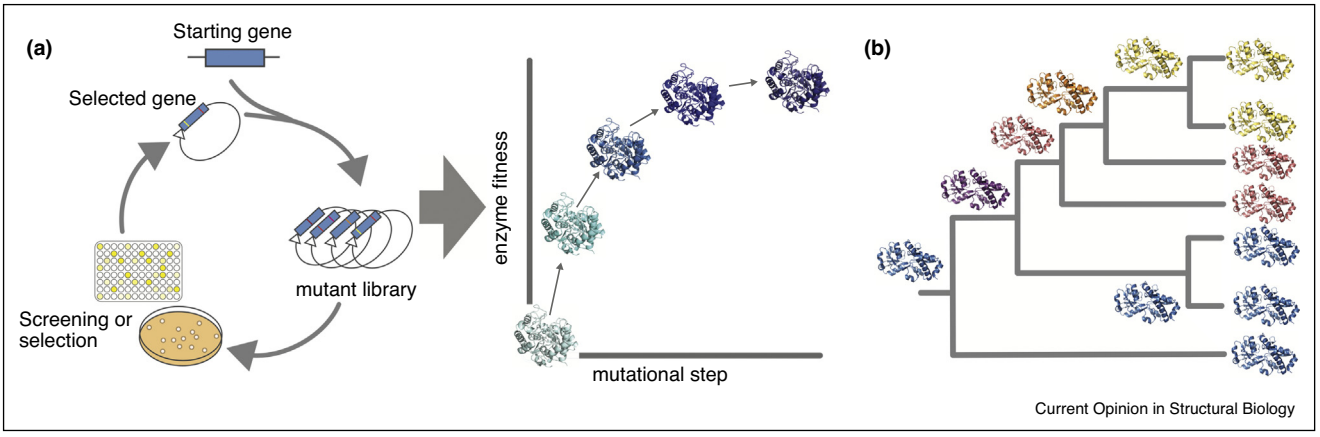
Introduction

The conformational dynamism of proteins is well established. Polypeptide chains are inherently flexible and undergo conformational change in solution at a variety of time scales. On the shortest of these timescales (fs–ps), bonds vibrate and side chains rotate. On longer time scales (ns–ms), macroscale motions can take place; loops 'open' and 'close', and domains can twist relative to each other or move on hinge-like regions. One important question in modern protein science asks how these conformations affect the function of the enzyme. The continued improvement in available biophysical techniques, including X-ray crystallography and NMR, in combination with advances in computational protein simulations, has allowed deeper analysis of protein motions. For example, the role of protein dynamics in substrate binding and product release is well studied [1–4], and cascades of conformational change are now known to underpin numerous biological functions [5]. There remains some controversy around the role of conformational dynamics in the catalytic step of enzymes; some works have proposed a role for conformational dynamics in the chemical step [6], while others suggest that experimental models have not yet conclusively demonstrated this link [7].

Given that protein structural dynamics clearly play important roles in several aspects of protein function, it is reasonable to assume that they must have evolved, or become optimized through selective pressure. Thus, one of the biggest questions relating to protein structural dynamics regards the role of molecular evolution, and how/if pathways for conformational change can be altered. During the evolution of new enzyme function, an enzyme active site must reorganize and adapt to a new substrate and/or new chemical reaction. It is generally, and reasonably, assumed that the adaptation of an enzyme to catalyze a new chemical reaction predominantly involves modification of the active site via mutation to better stabilize the transition state. However, the composition of active sites among homologous enzymes is often very similar, despite markedly different catalytic specificities [8], and laboratory (directed) evolution routinely demonstrates that remote mutations somehow have drastic effects on enzyme turnover rates or substrate preference [9–11]. These observations — in addition to our established understanding of allosteric communication between remote sites [12] — imply an important role for second/third/outer shell residues in modulating enzyme function, perhaps via control of protein structural dynamics/conformational sampling. The difficulty in studying the evolution of any trait, but especially structural dynamics, when relying on comparison between different extant proteins, is that we are comparing already highly evolved (and complex) states. To understand how something can change or evolve, it is much more informative to study the evolutionary process directly.

[5] Equal author contribution.

Figure 1



(a) The workflow of laboratory directed evolution involves the generation of a library of mutants of the gene of interest, and a screening or selection process to iteratively enhance a desired phenotype. **(b)** Ancestral protein reconstruction requires that the phylogenetic relationships between extant proteins are established, allowing ancestral sequences to be inferred and constructed.

Laboratory directed evolution and ancestral protein reconstruction

Laboratory directed evolution [Figure 1a] has been extensively used in an engineering context to produce many different proteins with a variety of improved functions, such as increased or novel catalytic activity [13], increased thermostability [14], and enhanced spectral properties [15]. However, directed evolution also provides great advantages over the study of natural homologs when it comes to the study of evolutionary processes. First, we focus on the evolution of (often) a single gene/protein of interest, rather than proteins that evolved in concert with the whole organism, which can involve complicated inter-gene epistatic relationships. Second, the high throughput screening of randomly generated mutants of a particular gene can result in the rapid enhancement of a desired phenotype over far fewer generations than is typical in natural systems, because directed evolution experiments allow for tight control of selection pressure, while natural evolutionary processes typically must balance several requirements to maximise the reproductive success of the organism. This results in significantly less neutral sequence variation, which can confound functional analysis. Finally, perhaps the most important advantage of laboratory evolution is that it allows for the study of as many intermediates along an evolutionary trajectory as desired, which can provide novel insights that cannot be gleaned through comparison of extant enzymes where only one current state can be assessed.

Ancestral protein reconstruction [Figure 1b] also seeks to remedy the shortcomings of studying extant proteins in isolation. Through the alignment of related sequences and the calculation of phylogenetic relationships between those sequences, points of diversification, or nodes, representing the predicted ancestors of extant proteins can be identified and probable sequences for these ancestral states inferred. This allows for the expression and characterization of these ancestral proteins that represent evolutionary intermediates, which can facilitate the study of evolutionary divergence [16].

Directed evolution and ancestral protein reconstruction have been instrumental in revealing fundamentally important molecular processes that underlie many protein functions. For example, our understanding of catalytic promiscuity has been substantially broadened through studying the evolution of substrate preference [17•,18]; we have gained insights into the complex relationships between thermostability and activity observed during the acquisition and optimization of new function [19,20]; and the constraints of epistasis on evolutionary trajectories can be more readily analysed thanks to the accessibility of evolutionary intermediates [21]. Most recently, attention has shifted to the study of how structural dynamics of proteins can change throughout an evolutionary trajectory, which has been facilitated by developments in computational structural biology and biophysical techniques.

Biophysical and computational analysis of protein structural dynamics

Studying the evolution of protein structural dynamics would not be possible without the use of computational and biophysical methodologies that allow structural dynamics to be dissected in different protein variants. To provide some context to the subsequent discussion of recent discoveries related to structural dynamics through evolutionary studies, we must first provide a brief

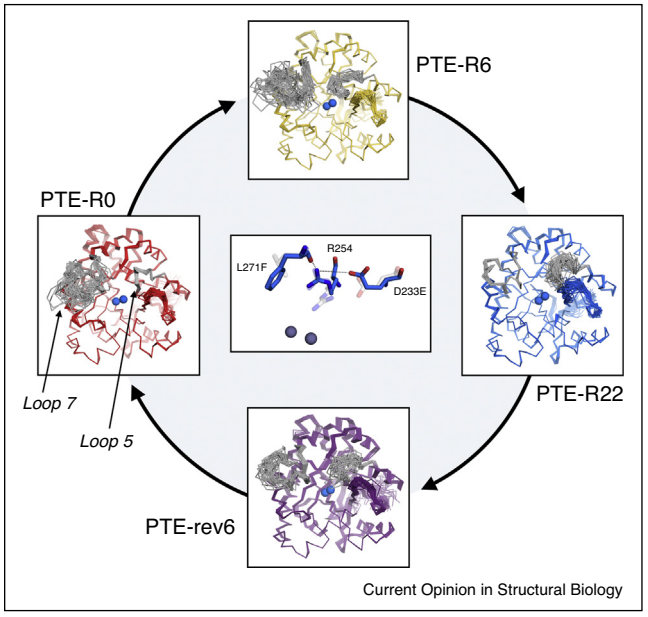
overview of some the key experimental approaches to the study of structural dynamics.

Often, a single technique is not sufficient to characterize structural dynamics on the wide range of timescales of interest in biological systems, and understanding the evolution of protein structural dynamics requires integrative structural biology [22]. X-ray crystallography is the most commonly used tool and is valuable because proteins often remain active in the crystalline state, which has similar solvent content (~30–80%) to the crowded environment of cells [23]. Advances in modelling anharmonic disorder using time-averaged ensemble refinement [24] and multi-conformer models [25] have proven to be particularly useful as they can allow less common ('hidden') conformational sub-states that exist among the billions of molecules in a crystal lattice to be modelled and studied. Likewise, the hunt for minor conformational sub-states has reignited interest in room temperature X-ray diffraction, which can eliminate the structural bias introduced by crystal cryo-cooling, which may 'freeze-out' higher energy sub-states [26,27]. Advances in NMR have also allowed detailed study of protein dynamics. Although often lacking the resolution of X-ray crystallography, NMR can characterize transient, low occupancy conformational sub-states, and the exchange kinetics between sub-states, with timescales ranging from picosecond-to-seconds [28]. While frequently limited to the study of small proteins, isotope labelling can extend the scope of NMR methods to study structural dynamics of larger proteins [29] and the synergistic use of NMR with X-ray crystallography can provide an integrated picture of protein dynamics [30]. Finally, computational simulations are now probing dynamic processes on increasing timescales [31] with improved accuracy and sampling [32]. As with NMR, combining computational simulations of molecular dynamics with empirically derived X-ray structures can provide a more detailed analysis of the conformational sampling of amino acid sidechains and loop regions that often have functional relevance [33]. Altogether, the numerous biophysical and computational techniques we now have at our disposal have given us the ability to better dissect the molecular and dynamic changes that occur through laboratory directed evolution and ancestral protein reconstruction, providing unique insight into the evolution of these complex traits.

Studying the evolution of protein dynamics through laboratory directed evolution and ancestral protein reconstruction

The application of laboratory directed evolution to bacterial phosphotriesterase (PTE), which had previously been shown to undergo functionally important conformational fluctuations that could be modulated by mutations [34], has resulted in several insights into the mechanisms of protein evolution, particularly with regards to dynamics

Figure 2



The directed evolution of PTE. Ensemble refinement of PTE variants R0, R6, R22 and rev6 illustrate the changing conformational dynamism of key loops (loop 5 and loop 7). R0 (PTE activity) possesses a large degree of flexibility in loop 7, while R22 (AE activity) possesses very little flexibility in loop 7 but enhanced dynamism in loop 5. Bifunctional 'midpoint' variants R6 and rev6 retain conformational flexibility in both regions, facilitating catalysis of both activities. The central panel illustrates the stabilization of the productive conformation of Arg254 (the first substitution fixed in the forward trajectory), by later round substitutions.

[Figure 2]. Tokuriki *et al.* exploited the promiscuous activity of PTE for arylester hydrolysis, and carried out a 22 generation directed evolution experiment towards arylesterase (AE) activity, followed by a 12 generation 'reversal', starting from the optimized arylesterase and selecting for native PTE activity [35,36••,37]. Over the course of the forward evolution, AE activity increased approximately 40 000-fold, and PTE activity dropped 40 000-fold. The reverse trajectory resulted in a 20 000-fold increase in PTE activity. There were two particularly notable characteristics of this evolutionary process: a minority of the mutations were located in the active site, despite the vast changes in activity, and the change in activity was noticeably gradual and smooth. Mutational analysis revealed that mutations were highly epistatic, with many of the remote mutations interacting with and modulating the effects of the active site mutations. The ability to capture every intermediate allowed us to extend beyond a simple description of mutation-activity-structure and probe what happened to the conformational landscape at each step. X-ray crystallographic data and molecular dynamics simulations of several intermediates revealed a pattern of shifting dynamics throughout the trajectory; large-scale motions of a 'lid' loop were

minimized through intramolecular hydrogen bonding networks, and the productive conformation of a key active site residue was enriched by a radiating pattern of substitutions [36••]. The midpoint of this trajectory, R6 (and Rev6 in the reverse direction), was of particular interest as it demonstrated high efficiency with both substrates. Crystallographic ensemble refinement of these midpoints revealed that their bifunctionality was due to their abilities to sample conformational sub-states similar to both the original and 'evolved' variants. These intermediates, only accessible through directed evolution experiments, highlight the role of conformational flexibility in the exploitation and evolution of promiscuous activities.

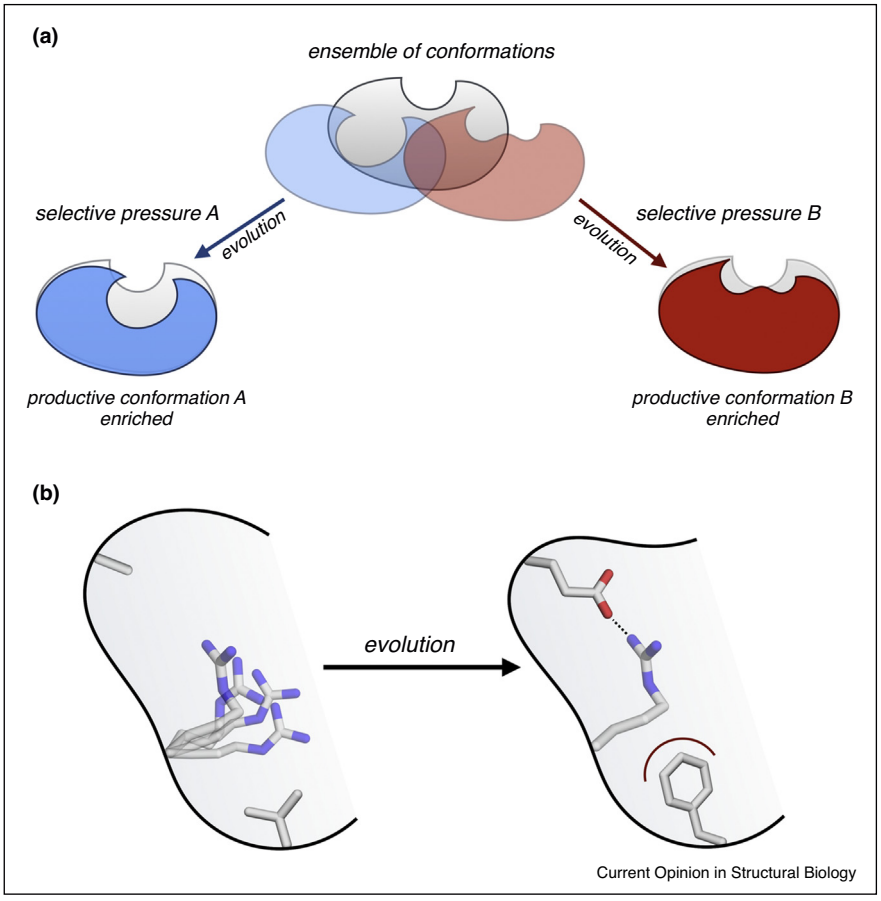
This pattern is observed in a different context in work by Biel *et al.* [38••] in which room-temperature X-ray crystallography was utilized to investigate the conformational heterogeneity of ubiquitin variants developed by Zhang *et al.* [39] a 'core' variant was designed for increased binding to deubiquitinase USP7, and then subjected to 'affinity maturation', in which random surface mutations were incorporated and variants screened for enhanced binding. Structures of the 'core' and 'affinity matured' variants were solved through room temperature X-ray crystallography, allowing for the elucidation of conformational heterogeneity in several regions, including the hinge-like β1β2 loop. The 'core' variant possessed substantial heterogeneity in this region, while the 'affinity matured' variant did not, due to the introduction of stabilizing interactions. The enrichment of a less-populated conformation through the rational selection of the 'core' mutations was proposed to disrupt the natural dynamics of protein, allowing the variant to sample a broader ensemble of sub-states (some of which are valuable in binding the target USP7), while the 'affinity maturation' froze out non-functional states, resulting in a stabilized, specialized variant. This mirrors the flexible, bifunctional PTE variants, which were then stabilized and specialized for their target activities.

Ancestral protein reconstruction experiments can also provide insights into the role of dynamics in protein evolution, by filling in some of the previously mysterious gaps in the evolutionary trajectories to modern-day proteins. For example, the ancestral reconstruction of GFP-like proteins by Kim *et al.* led to the identification of long-range conformational effects responsible for the evolution of green-to-red photoconversion. Interestingly, these effects were not accompanied by any substantial rearrangement of the active site residues or changes to the protein backbone, highlighting the importance of conformational sampling of amino acid sidechains and the contribution of outer-shell regions [40]. The evolution of mesophilic enzymes as organisms adapted to a cooler Earth has also been investigated through ancestral protein reconstruction. Nguyen *et al.* reconstructed ancient adenylate kinases (Adk), and determined their thermostabilities and activities, which showed no evidence of activity/stability trade-offs occurring [19]. One ancestral Adk variant possessed substantial activity at low temperatures as well as significant thermostability. This is in-keeping with the hypothesis that the magnitude of thermal motions is not directly important to catalytic activity, and is in fact often 'frozen out' (a rigid, thermostable ancestor was still capable of enhancing reaction rates at low temperatures), but rather conformational flexibility may serve as a useful starting point for the evolution of novel functions.

Perhaps the clearest example of the value for ancestral protein reconstruction in the study of protein dynamics is in deciphering the role of protein dynamics in the emergence of promiscuous activities and the evolution of specialist enzymes. In a study of amino acid binding proteins (AABP), Clifton *et al.* constructed proteins that represented common ancestors of modern AABP subfamilies [17•]. One of these ancestors, AncQR, displayed strong L-arginine binding, and promiscuous L-glutamine binding. X-ray structures of AncQR in complex with either L-arginine or L-glutamine revealed conformational differences in the active sites of the two complexes, with residual electron density in the AncQR-Arg complex matching the conformation of the AncQR-Glu complex active site. This suggested that the adventitious sampling of alternative states by AncQR underlies its promiscuous binding of glutamine, with evolution then stabilizing the minor Gln-specific to produce contemporary Gln-specific proteins. Recent work by Risso *et al.* studied ancestrally reconstructed β-lactamases, revealing that incorporation of a rationally selected mutation into highly dynamic ancestral β-lactamases allowed promiscuous Kemp eliminase activity, while the same substitution in more rigid, modern β-lactamases had no effect [41•]. These studies again highlight the importance of conformation freedom in the acquisition of new functions; with numerous accessible conformations increasing the likelihood of sampling a productive conformation.

These studies reinforce the case for a model in which functional promiscuity and conformational dynamism are interlinked as proposed by Tawfik and Tokuriki [42], where rare, adventitious, conformational sub-states can become productive in changing environmental conditions and be selected for and enriched through evolution. This model, and the generally positive view of protein dynamics that exists in the literature, has limitations, however. High conformational freedom is associated with low structural stability, a factor already identified as being valuable in evolution. Indeed, work by Dellus-Gur *et al.* highlights that while conformational flexibility can be important in the evolution of new function, the introduction of too much dynamism or disorder can impede evolution through negative epistasis [43•]. Similar observations were made in the study of PTE and ubiquitin,

Figure 3



Evolution minimizes unnecessary motions. **(a)** An ensemble of conformations results in catalytic promiscuity. Selection for one of these activities results in the enrichment of the relevant conformation, and the 'freezing out' of non-productive motions. **(b)** Multiple conformations of a key residue are minimized through acquisition of substitutions that stabilize the productive conformation.

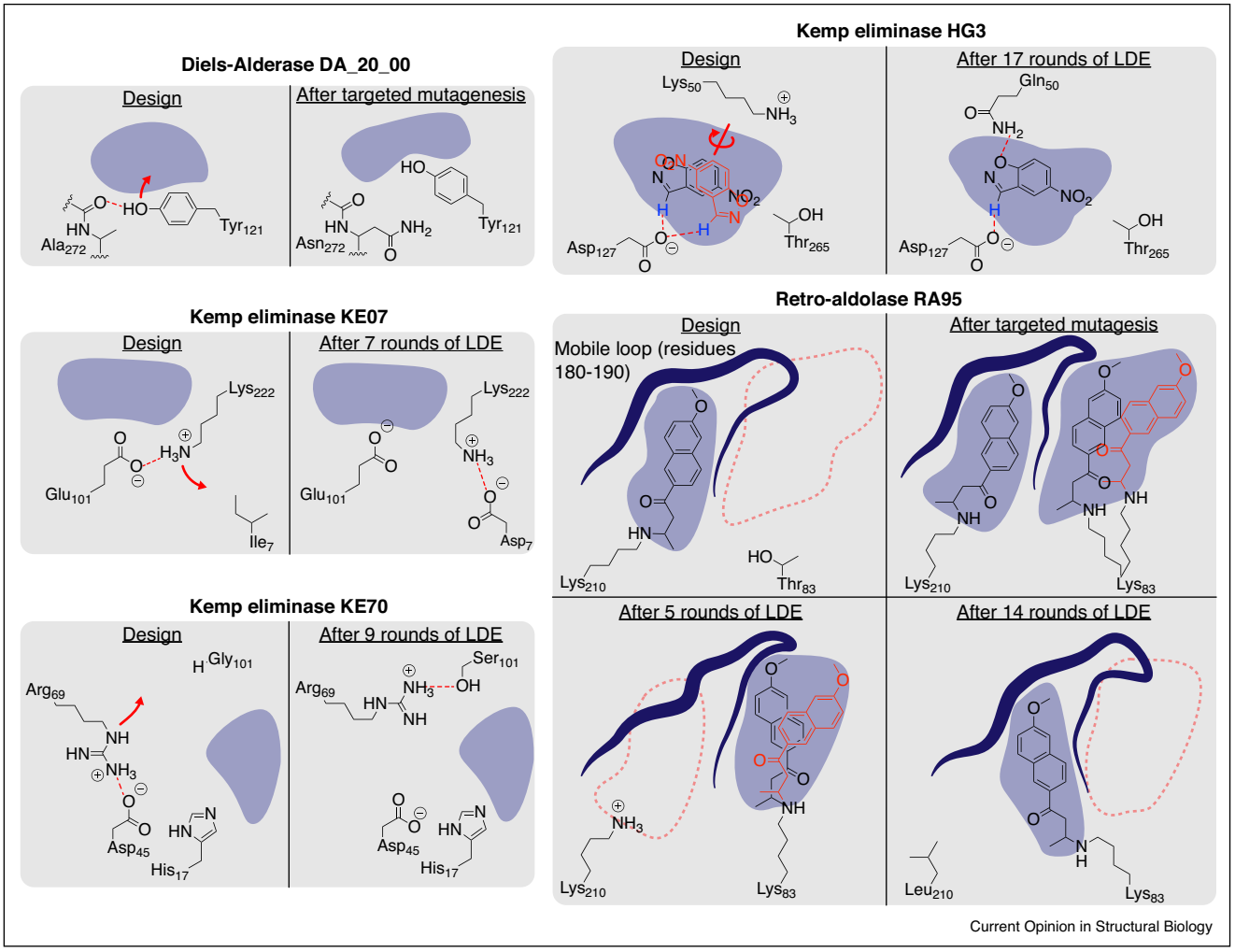
where it was clear that one of the strongest selective pressures was to *minimize* conformational dynamics, that is, rather than making the enzyme more dynamic, the optimization of the new activity involved stabilization of productive states. Thus, we should be cautious in terms of how we view the role of dynamics in molecular evolution and protein function — while they can make proteins more evolvable by allowing sampling of unique states, and some motions may be important for activity, proteins in general evolve to maximise the sampling of productive states (by minimizing the sampling of non-productive ones) [Figure 3].

The study of designed enzymes

The design of efficient enzymes continues to challenge our understanding of protein structure and function. Progress in protein design [44] has remarkably allowed the *de novo* creation of enzymes, although their substrate turnover (k_{cat}) values are typically several orders of magnitude lower than natural enzymes [45]. The low initial activity of some designed enzymes has meant that directed evolution is frequently, and successfully, used to optimize their activity. These studies are particularly important because they can reveal how designs are improved, and how subtle changes can turn a poor enzyme into an efficient one.

The low initial activity of designed enzymes can in some cases be traced to excessive active-site dynamics, which allows catalytic residues to depart from their designed conformations and sample non-productive states [Figure 4]. For example, in a designed Diels–Alderase [46], computational simulations suggested that the catalytic tyrosine was sampling a non-productive conformation due to a hydrogen bond with the backbone of a nearby alanine. This prompted the substitution of the alanine with a bulkier asparagine, which shifted the tyrosine to the designed conformation, and improved substrate activation [46]. In the designed KE07 Kemp eliminase [47], the catalytic glutamate was adversely affected by a salt bridge to a nearby lysine. Directed evolution corrected this mistake by introducing a new salt-bridge which

Figure 4



Overview of the role of flexibility in the optimization of designed enzymes. **Left three panels**: examples of designed enzymes where amino acid side-chain flexibility enabled catalytic residues to sample non-productive states. Targeted mutagenesis/laboratory directed evolution (LDE) selected productive states. **Right top panel**: an alternative substrate binding mode was selected and optimized in the laboratory directed evolution of the HG3 kemp eliminase. **Right bottom panel**: loop mobility was essential for the emergence and subsequent fine-tuning of a new binding site in the RA95 retro-aldolase.

shifted the lysine away from the catalytic glutamate, thereby increasing the basicity of the glutamate [48]. A similar correction was identified in the optimization of the KE70 Kemp eliminase [49] in which directed evolution removed a deleterious interaction between the histidine-aspartic acid catalytic dyad and a nearby arginine.

As observed in ancestral reconstruction, active site flexibility has allowed alternative substrate binding modes, which has promoted evolution [Figure 4]. In the designed HG3 Kemp eliminase, the substrate was flipped by 90° relative to the design [50]. Although this new conformation precluded productive interaction with the designed hydrogen bond donor, directed evolution introduced a new hydrogen bond donor via a lysine to glutamine substitution [51]. This new catalytic residue could only function in the alternative substrate binding mode, and was critical to the ~230-fold increase in k_{cat}. Flexibility was also essential in the optimization of the RA95 retro-aldolase [52], where loop mobility was exploited to create a new substrate binding pocket with a new catalytic lysine [53,54]. In subsequent rounds of directed evolution, the original substrate binding pocket was re-created, however the new catalytic lysine was retained [55•].

These examples of directed evolution of designed proteins mirror previous examples from directed evolution of natural proteins, illustrating that enzyme evolvability and dynamism are linked [56], but also that excessive

dynamics result in low efficiency that must be rescued through the gradual elimination of non-productive sampling.

Engineering protein dynamics

As our understanding of protein dynamics, and how protein dynamics can evolve to enhance protein function, develops, it is natural for attention to turn to the rational design and implementation of protein structural dynamics in an engineering setting. There are two related aspects of structural dynamics that could potentially be engineered: the conformational sampling of different states, and the rates at which different regions move. There have already been many studies in which rational changes to protein structure been shown to alter protein dynamics [36••,57,58]. However, we are still some way from being able to routinely improve protein function by manipulating conformational sampling, although there have been some notable achievements towards this ambitious goal [59•,60,61,62], and most protein design approaches do incorporate optimization of second-shell residues to constrain sampling of the active site [63–65]. Arguably, the biggest obstacle is the need to first understand the conformational landscape of the protein to be engineered, which is itself a major task. Interestingly, in the case of enzyme loop dynamics, it has been demonstrated that changes to the millisecond-timescale dynamics of various loop-motions in some β-lactamase variants did not have a substantial effect on activity, highlighting the dependence of enzymatic turnover rates on the rate limiting step, which is often chemical [58,66]. Similar results have been obtained from computational studies of adenylate kinase [67], suggesting that the rates of certain motions are unlikely to affect turnover rates until they approach the rate of the rate-limiting step, which in many cases is the chemical (bond making/breaking) step.

Conclusions

The examples described in this short review exemplify the profound insight that we can gain into complex processes, such as the evolution of protein dynamics, through observing intermediates in evolutionary processes. In this sense, laboratory evolution and ancestral protein reconstruction have been instrumental in building our understanding of how protein dynamics impact function, and how the conformational sampling of different sub-states can be subtly manipulated, in particular by remote outer-shell mutations, which have for a long time remained relatively mysterious. Two clear trends emerge from these studies: first, it is clear that structural dynamism allows sampling of states that could confer new activities; second, beyond this initial capture of promiscuous activity, evolution seems to progressively eliminate non-productive sampling, in a sense reducing non-essential dynamics.

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