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Ddesigning better enzymes: Insights from directed evolution

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Highlights

- Enzymes can be created by computational design and directed evolution
- Insights from directed evolution:
 - Active-site organization by H-bonding networks and evolved energy landscapes
 - Electrostatic redesign accelerates directed evolution
 - Dynamical networks integrate the protein scaffold into catalysis

Abstract

De novo enzymes can be created by computational design and directed evolution. Here, we review recent insights into the origins of catalytic power in evolved designer enzymes to pinpoint opportunities for next-generation designs: Evolution precisely organizes active sites, introduces catalytic H-bonding networks, invokes electrostatic catalysis, and creates dynamical networks embedding the active site in a reactive protein scaffold. Such insights foster our fundamental knowledge of enzyme catalysis and fuel the future design of tailor-made enzymes.

Introduction

Enzymes are nature's solutions to the challenges of catalysis, honed by billions of years of evolution [1]. Exploiting that catalytic power, e.g. for non-natural chemical transformations, offers tremendous practical opportunities. *De novo* enzymes have been created by computational design and directed evolution, but tailoring novel biocatalysts has so far been practically limited to simple model systems (Figure 1). Computational design can generate catalytic activity by equipping inactive protein scaffolds with binding pockets for 'theozymes' – theoretical minimal active sites comprising only the transition state and catalytic residues [2-8]. Such designs typically display only limited rate accelerations, but can be significantly improved by directed evolution [9]•[10-16]

allowing optimization of activity in iterative rounds of mutagenesis and screening [17]. Design and evolution has, in the best cases, produced catalysts with efficiencies approaching those of natural enzymes [9]•[11,13,16]. Though there have been notable successes, development of *de novo* enzymes is by no means routine, involving trial and error and time-consuming directed evolution. The evolutionary trajectories of successfully created enzymes provide invaluable insight for improving design procedures [18-20]. In-depth studies of evolving *de novo* enzymes will help in developing methods to reliably and rapidly design and optimize enzymes for mechanistically challenging and practically relevant transformations.

Figure 1

Novel enzymes by computational design and directed evolution. (a) The theozyme approach aims at the design of a binding pocket (grey) complementary to the TS (triangle) and capable of accommodating the catalytic residues in appropriate geometries for reaction (colored). The theozyme scheme was adapted from [10]. **(b)** Design can create incipient activity in inactive scaffolds, and evolution enhances catalysis to enzyme-like activities in the best cases. **(c)** *De novo* Kemp eliminases and retro-aldolases gained activity through evolutionary optimized **(d)** preorganization, electrostatics, and dynamics.

Improving enzymes design protocols requires thorough understanding of the activity enhancements brought about by directed evolution. Notably, tight transition state (TS) binding is not enough for efficient catalysis. What is important is differential TS binding: The enzyme must bind the TS more strongly than the substrate (Michaelis complex, GS). Focusing on the TS alone during design may lead to overdesign for either the GS or product-bound state, which increases the reaction barrier or induces product inhibition, respectively. We stress that catalytic origins are furthermore often intimately entangled [19,21]. Here, we nonetheless separate ‘static’ effects like preorganization and electrostatic catalysis from phenomena more obviously associated with protein dynamics such as dynamically correlated networks and entropic contributions (Figure 1d). We focus on Kemp eliminases¹ and retro-aldolases² (Figure 1c), which are well studied design benchmarks. By highlighting how evolution boosted irreversible C-H proton abstraction in Kemp eliminases and imine catalyzed aldol cleavage by retro-aldolases, we pinpoint opportunities for future enzyme design and optimization protocols.

Preorganization

Computational design protocols typically aim to create preorganized sites with high TS complementarity and precisely positioned catalytic residues (Figure 2a). Early design algorithms did not achieve the required precision, giving poor activity often attributed to misaligned catalytic residues, poorly packed active sites, and incorrectly formed H-bonding interactions [3-8]. Directed evolution has proved to be a powerful method for repairing such local flaws in the active site geometry [9]•[11-16]. The improved precision of state-of-the-art design algorithms, evident from the design of novel proteins [44] will similarly propel future enzyme design efforts. Additional preorganization can be gained by evolution of networks that can form e.g. catalytic dyads [9]•, tetrads [13], and oxyanion holes [24]•. Recently developed protocols for the design of hydrogen-bonding networks [45,46] may help to introduce such networks to boost activity.

¹ HG3[22]••[23]•[24]•[7,11,25-27]; 1A53-2[9]•[28]••[7,27]; KE15[29]••[3]; KE59[3,16,30]; KE07[31]•[3,14,26,32-35]; KE70 [3,15,27,33-35]

² RA95[6,13,36-41]; RA110 [6,42]; RA22[4,43]

The evolution of precision can be directly evident from X-ray structures, but in-depth dissection of evolved designer enzymes reveals unexpected mechanisms of how evolution organizes the active site [22]••[23]•. Room-temperature crystallography and molecular dynamics (MD) ensemble refinement of Kemp eliminase HG3 and its evolutionary descendants revealed that evolution not only positions catalytic residues, but organizes the entire active site [22]••. Notably, only two mutations provided most of the improvements achieved during evolution of HG3, likely by synergistically introducing active-site organization. Computational design would similarly benefit from precise organization beyond the catalytic residues, e.g. by including the energy landscape of the TS ensemble. Recently, a combination of machine learning and design using Rosetta enabled the modelling of protein landscapes [47]•, and similar approaches may facilitate the selection of catalytically superior conformations during design. In another recent approach, ligand-binding proteins were designed with high precision by optimizing for the best ligand position relative to the ideal sidechain binding mode. This is different to the common strategy of optimizing sidechain orientations relative to the ligand, and fosters preorganization by direct optimization for intrinsically-preferred sidechain conformations [48]•. Design strategies involving conformational landscapes and preferred sidechain conformations may similarly foster precision in *de novo* enzymes.

Figure 2

Evolution of designer enzymes. Arrows indicate changes from designed to evolved catalysts (ligand: orange; catalytic residue: cyan; protein: green). **(a)** Evolution increases preorganization by precisely positioning catalytic residues and by introduction of complex catalytic devices (tetrad: RA95.5-8F [13], dyad: 1A53-2.9 [9]•, oxyanion hole: HG3.17 [24]•). Evolution also organizes the overall active site, as observed in the evolution of HG3 [22]••. **(b)** Electrostatic effects can be quantified by electric fields [21,49], and are signaled by pK_a perturbations [9]•[11,13,16,50] and Brønsted slopes [24]•[27,50,51]. Reorganization of the protein during the

reaction of the ligand is associated with an energy penalty (reorganization energy) that can be optimized by evolution [32,52]. Electrostatic interactions are often not ideally placed in designer enzymes [35]. Notably, optimizing these interactions by *in silico* screening for electrostatic effects facilitated directed evolution of KE15 [29]••. **(c, left)** Evolution affects the protein dynamics and induces a thermodynamic response of the scaffold to the TS that enhances catalysis. Evolution of a dynamical network in 1A53-2 allows the protein scaffold to adjust to the TS [28]••. The network gives rise to an activation heat capacity signaled by curved activity-temperature profiles, which indicates increased preorganization through selective TS rigidification. **(c, right)** Active site plasticity of designer enzymes allows evolution to stabilize reactive over unreactive substrate conformations [31]•[41], which can be facilitated by dynamical networks spanning the protein scaffold [41]. Dynamical networks can also affect entropic contributions to catalysis. Side chain entropy decreased during evolution of KE70, which concomitated with the introduction of a network (represented by mutual information hubs, green spheres [33]).

Electrostatics

Electrostatic interactions can directly stabilize the TS, and are central to the catalytic benefits of active site preorganization, providing the majority of rate enhancement in many enzymes (Figure 2b, [21,49]). Examining how substrate or catalytic residue pK_a affects activity can provide valuable insights into the active site electrostatics [24]•[27,50,51]. Raising the pK_a of a catalytic base increases its reactivity, and many designed Kemp eliminases gain activity by electrostatic desolvation of their general base, thus raising their pK_a . While pK_a perturbations can increase activity, they eventually lead to inactivation by changing the protonation state of catalytic residues. Directed evolution typically balances these effects, settling pK_a values around ~ 6 for Kemp eliminases and retro-aldolases evolved at neutral pH [9]•[11,13,16,50]. This effect is particularly pronounced in Kemp eliminases KE07 (+1.7 pK_a units through evolution), where the increase in pK_a resulted in destabilization of the general base that is part of the ground state (Michaelis complex) as a major driver for the additional rate acceleration [26,32]. Nonetheless, such pK_a perturbations can only provide rate accelerations of $\lesssim 10$ -fold for retro-aldolases and Kemp eliminases at neutral pH [50,51]. If higher activities are to be attained, differential TS stabilization is required, as observed in Kemp eliminases HG3.17 [26]. HG3.17 achieved such stabilization in part by evolution of the oxyanion-stabilizing Gln50. Its efficient stabilization of the TS oxyanion leaving-group is signaled by the shallow Brønsted slope of HG3.17, whereas replacement of the amide sidechain by solvent or hydrophobic residues decreased oxyanion-stabilization [24]•. Notably, X-ray structures and DFT modeling revealed a delicate balance of steric and electronic effects at the active site, with desolvation and a shortened catalytic interaction compensating for

the loss in electrostatic stabilization. This compensation underlines the need for design to accurately balance all catalytically relevant effects to maximize activity.

Electrostatic effects in catalysis can be quantified by analyzing simulations of reactions (e.g. by calculating effects on reaction barriers [25,27,40,49]), or indirectly by calculating or measuring electric fields exerted on ligands by the protein [29]••[21]. Though the theozyme approach includes the design of electrostatically complementary binding pockets, long-range interactions are generally not considered. Current designs thus often misalign the electric field from the protein and solvent for catalysis, as observed for Kemp eliminases KE07 and KE70 [35]. While evolution optimized electrostatic TS stabilization by the catalytic base, the overall protein field could apparently not be adjusted, because it would require an experimentally infeasible number of mutations. Aligning the active site with the scaffold's electric field during design could thus provide more efficient catalysts. Furthermore, guiding directed evolution by calculating how individual mutations affect catalytic electric fields led to the improvement of Kemp eliminase KE15 by 43 fold, while only requiring a minimal screening effort [29]••. Another electrostatic contribution that is optimized by evolution in several Kemp eliminase variants is the reorganization energy [27,32,52]. Solvent and protein reorganize to accommodate electrostatic and steric changes during the reaction [49,53]. In KE07, two mutations (N224D and I7D) synergistically reduced the reorganization energy, but only exerted a small effect on direct electrostatic TS stabilization [32]. Concluding, electrostatics seem key to successful design, and electrostatic differential TS stabilization should be explicitly considered. This can be achieved by orienting the active site to the scaffold's electric field [35] or reorganization energy [32,52], as well as the design of catalytic electrostatic interactions [29]••[54].

Dynamics

The protein scaffold dynamics can profoundly affect catalytic activity; not in 'driving reaction' – dynamical effects on rates, in the strict terms of TS theory, are generally very small [49,53] – but rather via conformational effects and by affecting thermodynamic properties that influence reactivity. Evolution alters the energy landscapes of the protein scaffold to improve preorganization and minimize entropic barriers in several designs

(Figure 2c [22]••[28]••[31]•[33,34,41]). Such effects require communication of the reactants with the scaffold, and may be related to the evolution of dynamically-coupled motions of catalytic residues and the TS [30,39]. Tight communication has been achieved in Kemp eliminase 1A53-2 by evolution of a network spanning not only the active site but also linking to distant parts of the protein [28]••. This network results in a protein framework that enhances organization of the transition state ensemble. Evolved changes in dynamics were signaled by the experimental observation of curvature in the activity-temperature profile of the evolved enzyme [9]•. Macromolecular rate theory predicts that this is due to an activation heat capacity, i.e. a change in protein rigidity in the TS ensemble compared to the GS [55]•. Selective rigidification and the underlying networks can be assessed by MD [28]••[55]•, but design is limited by computationally-intensive simulations. Their effects on active-site preorganization and flexibility can nonetheless be rapidly analyzed by MD, which promises to be a valuable addition to design.

Designer enzymes often have highly variable active sites that may facilitate evolutionary selection of the ideal ligand binding mode. Alternative binding modes are observed in X-ray structures of many designer enzymes, showing variable substrate orientations [11,31,42] and catalytic residue positions [13], or populating entirely different pockets [13]. Evolutionary sampling has been elucidated in Kemp eliminase KE07 by interrogating the active site environment using tryptophan fluorescence, crystallography, and MD simulation [31]•. Evolution of KE07 acted on various sidechain rotamers and alternative hydrogen bonding interactions to stabilize a catalytically superior competent binding mode. Similar conformational selection has been observed in retro-aldolase RA95[13]. MD simulations revealed sampling of various states with catalytically competent distances of the covalent Schiff-base intermediate to the tyrosine general base. Notably, the effect of remote mutations during evolution must be communicated to the active site by some means, and MD simulations of the RA95 family indicates that this involves correlated networks spanning the protein [41]. Sampling various conformations is likely particularly relevant in multistep reactions such as the aldol-cleavage [43], where evolution acts on the rate of individual steps to increase overall catalytic efficiency [36]. Multistate design may be necessary to target more complex (multistep) reactions [56], and recent successes in the design of proteins with

divergent folds [57]• indicate that computation attained the necessary precision to accurately target multiple states during enzyme design.

Dynamical networks can furthermore affect the entropic contributions to catalysis. Evolution of TS networks in the ensemble of Kemp eliminates KE07 and KE70, as indicated by mutual information hubs representing core vertices of the network, is anticipated to decrease the entropic reaction barrier [33]. Solvation is also expected to affect entropy, mirroring its catalytic role in active site electrostatics as discussed earlier [34]. Evolution of Kemp eliminates KE07 and KE70 either removed bound low entropy water molecules or changed the entropy of bound water molecules to decrease the entropic penalty associated to substrate binding. Targeting dynamical networks and entropic contributions by design maybe beneficial, albeit computationally expensive. However, their effects on active site preorganization and flexibility can readily be observed in MD, providing an opportunity for indirect optimization by quick post-design MD analysis.

Figure 3

Designing better enzymes. *De novo* enzymes design will benefit from targeting preorganization, electrostatic catalysis, and dynamical effects. Molecular simulations can contribute in all of these. Such integrated design procedures will complement directed evolution, by providing better starting point designs, analyzing evolutionary trajectories and potentially by suggesting engineering routes out of evolutionary ‘dead ends’. The theozyme scheme was adapted from [10].

Perspective

Retrospective analyses of the changes wrought by evolution on designer enzymes show that improved *de novo* enzyme design protocols require: (1) enhanced precision, particularly through creating extended catalytic hydrogen bonding networks and control of protein energy landscapes; (2) explicit accounting for electrostatics and differential TS stabilization in catalysis; (3) harness the scaffold for catalysis, including effects of dynamical networks (Figure 3). Practical additions to established design approaches will furthermore require a balance of speed and accuracy to explore sequence space efficiently. MD simulations have the potential to contribute in all of these areas, e.g. in quantifying molecular properties such as ligand affinity [58], active site flexibility, and protein electrostatics [29]••[54]. Empirical valence bond [27,59] and quantum mechanics/molecular mechanics methods [28]••[25,40] can furthermore be used to accurately reproduce (and predict) catalytic activity, and identify chemical mechanisms and TS structures. Emerging technologies such as interactive MD simulation in virtual reality will help to bring human intuition and knowledge to bear on the problem in new ways [60]•. Catalyst design may also benefit from information gained through machine learning analysis of evolutionary trajectories [61], and can be supported by complementary strategies involving reactive cofactors [62], non-canonical amino acids [63], and phylogenetic-guided design [64]•.

Directed evolution of designer enzymes provides novel insights into the physical origins of catalysis. Incorporating these insights in refined design methods promises a next generation of made-to-order biocatalysts that will successfully target complex reactions with enzyme-like efficiencies.

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