De novo enzymes by computational design
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Computational enzyme design has emerged as a promising tool for generating made-to-order biocatalysts. In addition to improving the reliability of the design cycle, current efforts in this area are focusing on expanding the set of catalyzed reactions and investigating the structure and mechanism of individual designs. Although the activities of de novo enzymes are typically low, they can be significantly increased by directed evolution. Analysis of their evolutionary trajectories provides valuable feedback for the design algorithms and can enhance our understanding of natural protein evolution.

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Introduction
Tailoring enzymatic function, a long-standing goal in chemical biology, could provide significant benefits to both biomedicine and industrial biocatalysis. Although Pauling proposed the conceptual requirements for enzyme design more than 60 years ago [1], selective stabilization of transition states through design is difficult to realize in practice. The vastness of protein sequence space and incomplete understanding of enzyme structure–function relationships have hindered the development of general design strategies.

Catalytic antibody technology is one source of artificial biocatalysts [2]. Rather than crafting protein structure from scratch, clonal selection in the immune system is harnessed to create tailored protein binding pockets in response to stable transition state analogs or mechanism-based inhibitors. A wide range of chemical transformations has been catalyzed in this way, often with high selectivity, but the modest rate accelerations achieved by antibodies make them generally poor substitutes for natural enzymes.

Redesign of existing proteins, for example through semisynthesis, is another approach that has been explored toward novel enzymatic activities [3,4]. In this strategy, organic or organometallic cofactors are embedded in a protein scaffold to afford complexes in which the intrinsic reactivity of the cofactor is modulated by the steric and electronic properties of the protein. In a topical example, a biotinylated Rh(III) metal center bound to an engineered streptavidin was shown to catalyze C–H bond activation with enantiomeric ratios as high as 93:7 for the coupling of benzamides and alkenes (Figure 1a) [5**]. Due to participation of an engineered glutamate side chain in catalysis, reaction rates also increased by ca. 100 fold over the free complex.

Computational enzyme design represents a potentially wider-ranging approach to new enzymes [6]. Powerful computer programs such as the Rosetta software suite [7–9], developed in the laboratory of David Baker (University of Washington, Seattle, USA), and ORBIT [10,11] from Stephen Mayo (California Institute of Technology, Pasadena, USA) can facilitate the search of protein sequence space. Although applied only recently to enzyme design [10,12–14], in silico methods promise to address many of the limitations of other design technologies. Computer-based screening of protein scaffolds provides access to structures beyond the antibody fold, for instance, and active sites can now be sculpted around realistic transition state models for diverse reactions, including those lacking biological counterparts.

Initial successes
Computational enzyme design comprises several steps (Figure 2). First, a target reaction is chosen and an appropriate catalytic mechanism, including all requisite functional groups, defined. Second, an idealized active site that positions the catalytic residues so as to maximize transition state stabilization is modeled quantum mechanically. These structures, termed ‘theozymes’ [15], are docked into diverse protein scaffolds, and the resulting complexes are optimized in silico by a combinatorial search for amino acid substitutions that improve transition state binding [7,16–18]. Finally, the designs are filtered on the basis of their catalytic geometry, transition state binding energy, and shape complementarity between the designed pocket and the transition state. Synthesis, production, and characterization of the top ranking designs complete the procedure.
The first steps toward computationally designed enzymes were taken a little over a decade ago when the *E. coli* protein thioredoxin was successfully converted into a primitive esterase [10]. Introducing a histidine nucleophile into an engineered binding site on the protein surface yielded a catalyst that hydrolyzed *p*-nitrophenyl acetate (Figure 1b) with a 180-fold rate acceleration (*k*<sub>cat</sub>*k*<sub>uncat</sub>). Subsequently, artificial biocatalysts were generated for several additional reactions, including carbon–carbon bond cleavage via retroaldolization (*k*<sub>cat</sub>*k*<sub>uncat</sub> = 10<sup>3</sup> to 10<sup>4</sup>; Figure 1c) [14], proton transfer from carbon (*k*<sub>cat</sub>*k*<sub>uncat</sub> = 10<sup>7</sup> to 10<sup>10</sup>; Figure 1d) [12], and a Diels-Alder cycloaddition (*k*<sub>cat</sub>*k*<sub>uncat</sub> = 89 M; Figure 1e) [13].

**New challenges**

Encouraged by these initial successes, computational designers are turning toward increasingly sophisticated design challenges. Mimicking the active sites of natural

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**Figure 1**

Representative target reactions for designer enzymes.
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Figure 2

Computational enzyme design methodology. Once a target reaction is chosen, the structure of the rate-limiting transition state is calculated at a high level of quantum mechanical theory and catalytic groups are provided in silico to stabilize it. The resulting theozyme, which has all side-chains optimally oriented for catalysis but lacks a protein backbone to hold them in place, is then computationally docked into a protein scaffold from the Protein Data Bank (PDB). In order to improve packing, side-chains surrounding the active site are subsequently mutated using a computational search algorithm. Finally, the designs are ranked by a scoring function, and the most promising are produced and kinetically characterized in vitro. Although first-generation designs typically exhibit low catalytic efficiency, they can be optimized experimentally, for example by directed evolution.

hydrolytic enzymes is one example. In an attempt to extend the thioredoxin-based esterase designs [10], a more complex catalytic apparatus consisting of a nucleophilic cysteine–histidine dyad and backbone amides for oxoanion stabilization was targeted [19]. The resulting proteins were found to cleave activated ester substrates by a two-step acylation/deacylation mechanism as programmed. Cysteine acylation was rapid, with rate accelerations up to 4000-fold over background. However, turnover was limited by slow deacylation of the nucleophilic cysteine. Moreover, crystal structures of several designs revealed that the histidine had been misplaced, highlighting difficulties associated with correctly positioning multiple, mutually dependent residues with present-day design software.

Ironically, misplacement of a histidine in a computationally designed Zn(His)₄ structural motif opened up a metal coordination site, leading serendipitously to significant hydrolytic activity [20]. The artificial metalloprotein cleaves p-nitrophenyl acetate and p-nitrophenyl phosphate with $k_{cat}/K_M$ values of 630 M⁻¹ s⁻¹ and 14 M⁻¹ s⁻¹, respectively. This activity is in the same range as many de novo designs, showcasing the unique potential of metal ions. To capitalize on metal ion catalysis in deliberate fashion, a zinc-based adenosine deaminase was recently computationally redesigned to hydrolyze diethyl 7-hydroxycoumarinyl phosphate (Figure 1f) [21**]. Eight mutations engendered the new substrate specificity, enabling stabilization of a trigonal bipyramidal, instead of tetrahedral, transition state geometry. Though the phosphotriesterase activity of the redesigned catalyst is low ($k_{cat}/K_M = 4$ M⁻¹ s⁻¹), it could be improved some 2500-fold by only three rounds of directed evolution. A range of other metalloenzymes has been designed, among them ‘true’ de novo designs of the entire polypeptide backbone, auguring a bright future for this catalyst class [22].

Limitations

Despite evident progress, the palette of reactions catalyzed by computationally designed enzymes remains limited. Efforts to accelerate a number of relatively simple transformations have been unsuccessful. For example, despite much effort, de novo design of a triosephosphate isomerase (TIM; Figure 1g) is still an unsolved
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problem [8,23]. Also, attempts to mimic chorismate mutase (CM; Figure 1h), which promotes a biologically important Claisen rearrangement, have not yet yielded proteins with detectable activity (F Richter et al., unpublished data).

Though it is not entirely clear why CMs or TIMs are difficult to design, it is notable that many designable reactions, such as benzosoxazole deprotonation and retroaldolization, are susceptible to general acid/base or nucleophilic catalysis. Almost any base (e.g. triethylamine) will promote the Kemp elimination in an appropriate solvent [24], whereas aldol reactions are accelerated by small amine catalysts such as proline [25]. Not surprisingly, single basic or nucleophilic protein side chains, placed in a hydrophobic pocket and thus activated by medium effects, are also good catalysts. In fact, rudimentary rational design of such constructs can be remarkably successful [26–28]. An amine-containing β-peptidic foldamer, for example, was designed from simple chemical principles and shown to accelerate a retroaldol reaction by more than three orders of magnitude [27]. Symmetry too has been exploited to generate retroaldol catalysts with activities comparable to the first generation in silico designs [28]. In this context, combining rational design with minimal computation can be effective. An allosterically regulatable Kemp eliminase was engineered by computationally placing an aspartate or glutamate residue in the peptide binding cleft of the calcium binding protein calmodulin. The best variant had activity \( k_{\text{cat}}/K_M = 6 \text{ M}^{-1} \text{ s}^{-1} \) comparable to many Rosetta designs \( (k_{\text{cat}}/K_M = 10–160 \text{ M}^{-1} \text{ s}^{-1}) \), but required far less effort to generate [29].

What, then, are the benefits of computational design? Kipnis and Baker addressed this question by comparing computationally designed Kemp eliminases and aldolases in a TIM barrel scaffold with randomly generated libraries containing mutations at the rim of the barrel [30]. They found that the random libraries yielded a much smaller fraction of active clones (<1%), most of which exploited preexisting scaffold residues as functional groups. Nonetheless, the catalytic efficiency of the hits from the random libraries was similar to that of Rosetta designs in the same scaffold.

Computational algorithms evidently improve the likelihood of finding active enzymes, but the design protocols are clearly not robust. Poor predictability originates in the trade-off between speed and accuracy. Current algorithm force fields are relatively primitive; for instance, they fail to consider long-range electrostatic interactions and make the simplifying assumption that protein scaffolds are rigid and undergo no large conformational changes upon mutation. It is unsurprising that these programs are consequently unable to distinguish between active and inactive variants in silico, and that ‘shotgun’ approaches must be adopted wherein many designs are screened experimentally to identify a few catalytically competent variants.

Learning from experience

In an attempt to improve the efficacy of current design methods, the aldol reaction was recently revisited. In the original study [14], theoymes employing a water molecule as a proton shuttle performed better than designs based on more complex arrays of functional groups. Focusing subsequent design efforts on that configuration, success rates could be increased from 44 to 75\% by introducing an aspartate or glutamate for water positioning and by finer sampling of catalytic and binding pocket residues [31].

Weak initial activity can sometimes be increased by further computational design of a lead structure. For example, the low efficiency of a first-generation Diels-Alderase catalyst [13] was improved by subsequent computational remodeling of the protein backbone to minimize active site exposure to bulk solvent and thereby enhance interactions with the substrates. Because unrestrained backbone remodeling presents automated design algorithms with an almost intractable number of combinatorial possibilities, human expertise was sought from the foldit community, an online network of computer players who fold and design proteins as a part of a computer game. ’Crowdsourcing’ in this case yielded a novel helix-loop-helix motif that enhanced Diels-Alderase activity 18-fold by increasing catalyst affinity for the diene and dienophile [32].

Failed designs offer the chance to begin again more intelligently. Rather than discard the large fraction of inactive proteins that emerge from a typical design campaign, detailed biophysical and structural analysis can help rationalize failures in mechanistic terms and suggest strategies for improvement. Such an approach was used to rescue an unsuccessful Kemp eliminase, which was reengineered to give one of the fastest de novo enzymes for this reaction [33]. The redesigned catalyst exhibited higher kinetic parameters \( (k_{\text{cat}} = 1.7 \text{ s}^{-1}; k_{\text{cat}}/K_M = 710 \text{ M}^{-1} \text{ s}^{-1}) \) than the best Rosetta design selected from a screen of 59 mostly inactive proteins \( (k_{\text{cat}} = 0.29 \text{ s}^{-1}; k_{\text{cat}}/K_M = 160 \text{ M}^{-1} \text{ s}^{-1}) \) [12].

How to proceed

While rescuing or improving initial designs may represent a thrifty rebound process, the ultimate goal remains to engineer more efficient enzymes from the start. Assuming that \( ab \text{ initio} \) theoyme geometries are reasonably accurate [34,35], problems often arise from imperfect realization of the idealized active site in available protein scaffolds. The use of implicit solvent models and neglect of long-range electrostatic interactions are particularly crude approximations made by the design software.
Improved force fields [36,37], coupled with increased computing power, can be expected to minimize these problems. Backbone flexibility [38] and the highly dynamic nature of protein structures [39] must also be considered in the design process. Finally, since many reactions proceed via multiple transition states, as in the instances of aldol and ester cleavage, multistate design [40] could also be implemented.

In principle, classical molecular dynamics (MD) and combined quantum mechanics/molecular mechanics (QM/MM) simulations should facilitate the search for suitable active enzymes. Although MD simulations are too time-consuming at present to be brought into the design routine, they have been successfully utilized in scoring and analysis at a late stage of the process [33**,39,41–43]. Indeed, active and inactive Kemp eliminases are distinguishable by differences determined in MD simulations in key angle and distance relationships in the respective enzyme–substrate complexes [43]. In the future, more reliable scoring functions may reduce the number of proteins that have to be experimentally characterized. As computational power improves, it is probable that slow but physically more accurate energy functions in MD programs will also complement faster knowledge-based, empirically oriented design algorithms used today.

**Coupling design and experiment**

While improved algorithms have the potential to move the enzyme design field forward, true enzyme-like activities are likely to remain out of reach for *in silico* design, at least for the near future. To generate practically useful catalysts, experimental optimization methods such as directed evolution must therefore be applied. In principle, any assayable property of a protein is optimizable by multiple rounds of mutagenesis and screening [44]. First-generation computationally designed enzymes have proven to be particularly amenable to this approach as demonstrated by 10²–10³-fold increases in activity for several retroaldolases [31*]. Kemp eliminases [45–47], and a phosphotriesterase [21**]. Generation of an improved Kemp eliminase with a $k_{cat}/K_M$ of 570 000 M⁻¹ s⁻¹ is notable in this context (Figure 3) [45**].

**Figure 3**

(a) Kemp elimination theozyme: A theozyme containing a carboxylate base, an aromatic side-chain for substrate binding, and a hydrogen bond donor to stabilize developing negative charge in the transition state was used as the template for design. (b) The crystal structure of the first-generation enzyme (blue) complexed with the transition state analog 5,7-dichlorobenzotriazole shows good qualitative agreement with the computational design model (green). However, the ligand in the crystal structure (orange) is flipped relative to the designed orientation (pink). (c) The catalytic efficiency of the starting enzyme (R1) relative to the nonenzymic acetate-promoted reaction was increased by more than two orders of magnitude over thirteen rounds of mutagenesis and screening (R2–R13). (d) The structure of the best evolved variant R13 (blue) cocrystallized with benzotriazole (orange) shows conformational changes of active site residues, enhanced desolvation of the catalytic glutamate, and a water molecule in hydrogen bonding distance to the transition state analog.
Detailed characterization of computationally designed enzymes and their evolutionary descendants can help to identify the limitations of current design models. For instance, mechanistic investigations of the retroaldolase RA60 suggested that binding of substrate in a hydrophobic pocket proximal to a reactive lysine largely accounted for the observed 10^4-fold rate acceleration [48], in accord with design. However, residues supposed to position a water molecule for proton shuttling did not show the expected sensitivity to mutation, suggesting that this design feature did not work as intended. Illuminating how favorable mutations increase the activity of these catalysts might show how such problems can be corrected.

Structural analysis promises to play an increasingly important role in this context. For example, the crystal structure of another retroaldolase, RA34, in complex with a 1,3-diketone inhibitor confirmed the general features of the design model, while highlighting a few important differences [49]. The mechanism-based inhibitor covalently modifies the enzyme by forming a Schiff base intermediate with the catalytic lysine as intended, but the density assigned to the ligand exhibited considerable positional heterogeneity within the pocket. Different binding modes arise — at least in part — because the lysine adopts more than one conformation. Additionally, although the substrate naphthyl group binds in the appropriate hydrophobic pocket, it is rotated and translated relative to its position in the model. Water-mediated interactions at the active site are also more extensive than predicted. Together, these observations strongly suggest that obtaining more active catalysts will require improved control over substrate binding and better preorganization of the active site.

Several Kemp eliminases and their evolutionary descendants were similarly subjected to structural and biochemical analysis (Figure 3). Co-crystallization with substituted benzotriazoles, which are close structural analogs of benzisoxazoles, revealed that the designed active site residues mediate catalysis and binding but, surprisingly, not in the predicted mode. The small ligand was found to adopt unanticipated, and sometimes even unproductive, binding orientations [33**,45**,47]. Loose positioning and ineffective deactivation of the catalytic base were identified as additional problems. During evolutionary optimization, the floppy starting pockets were often reshaped, resulting in tighter substrate binding and improved alignment with respect to the base. In addition, depending on the design, the efficacy of the base was enhanced either by fine-tuning its electrostatic microenvironment [47] or minimizing its interactions with water molecules from bulk solvent [45**]. Creating active sites with tighter, unambiguous substrate binding modes directly by design is therefore desirable, particularly as the best evolved variants generally derive from the most active starting points.

Conclusion
Recent years have seen continuous evolution of computational design procedures, yet the activities of the resulting artificial enzymes are more typical of catalytic antibodies than natural biocatalysts. As computational algorithms are further fine-tuned and more powerful computer hardware becomes available, the number and activity of protein catalysts designed de novo will certainly increase [50]. In contrast to catalytic antibodies, computationally designed enzymes have proven to be highly evolvable [45**]. In the future, the powerful combination of theory and experiment may afford deeper insights into the tenets underlying the remarkable catalytic efficiencies of natural enzymes as well as practical catalysts for streamlined reaction sequences [51] and other applications.

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References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest

De novo enzymes by computational design

Kries, Bloemberg and Hilvert


This paper explores the computational design of artificial esterases using catalytic Cys-His dyads for nucleophilic catalysis and backbone amides for oxygen stabilization. Although efficient acylation of the active site cysteine was achieved, slow hydrolysis of the acyl-enzyme intermediate limited overall catalytic efficiency, most likely due to misplacement of the catalytic histidine.


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The catalytic efficiency of a designed Kemp eliminase was enhanced 2000-fold over 13 evolutionary cycles. Extensive biochemical, structural and molecular dynamics simulations of selected variants along the evolutionary trajectory helped rationalize this improvement in molecular terms.


The crystal structure of a computationally designed retro-aldolase in complex with a covalently bound inhibitor shows qualitative agreement with the design model. However, inaccuracies in placing the ligand and active site residues highlight limitations of current design algorithms.
