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Computational enzyme design: Transitioning from catalytic proteins to enzymes

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The widespread interest in enzymes stem from their ability to catalyze chemical reactions under mild and ecologically friendly conditions with unparalleled catalytic proficiencies. While thousands of naturally occurring enzymes have been identified and characterized, there are still numerous important applications for which there are no biological catalysts capable of performing the desired chemical transformation. In order to engineer enzymes for which there is no natural starting point, efforts using a combination of quantum chemistry and force-field based protein molecular modeling have led to the design of novel proteins capable of catalyzing chemical reactions not catalyzed by naturally occurring enzymes. Here we discuss the current status and potential avenues to pursue as the field of computational enzyme design moves forward.

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Introduction

Recent efforts in computational enzyme design have enabled the successful engineering of proteins with novel catalytic functions [1–9]. Here, we focus on computational design tools that have enabled novel reaction chemistries to be engineered into proteins. To achieve this, several different design methods have been successfully implemented [10–14], but all follow the general 'inside-out' approach first suggested by Houk, Chen, and Tantillo in 1998 [15].

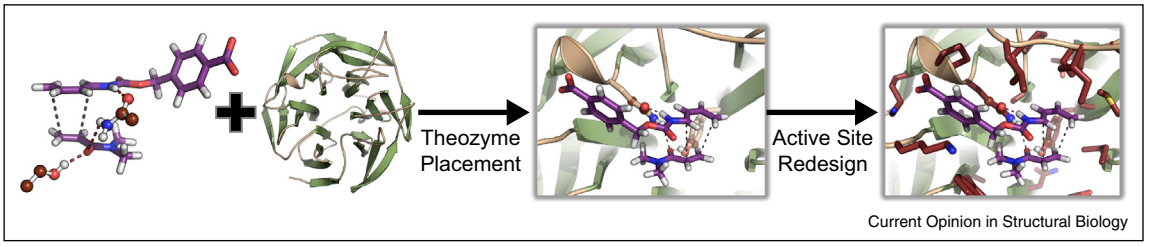
Briefly, the inside-out approach follows three steps (Figure 1). First, a 'theozyme' is computationally modeled in which functional groups are geometrically oriented around a transition state such that its predicted free energy is lowered, providing the basis for catalysis. Second, the transition state is placed into a native protein structure using a computational search procedure to identify residues in the structure where amino acids containing the appropriate functional groups can be introduced and interact with the transition state in the geometry defined in the theozyme. In these calculations, only the catalytic residues and the protein backbone are considered, and the native side chains are omitted as they will be redesigned in subsequent steps. Once the protein scaffold, catalytic residues, and relative spatial orientation of the transition state within the scaffold have been identified, non-catalytic residues in the pocket are designed. This provides additional interactions with the transition state to promote binding, as well as interactions with the catalytic residues to stabilize the catalytic competent conformation. The result of this simulation provides a remodeled protein that is predicted to bind and stabilize the desired transition state. A synthetic gene that encodes the designed protein is then constructed, protein produced, and catalytic activity experimentally evaluated.

In 2001 Mayo and Bolon successfully implemented this general procedure to engineer a novel esterase [4]. In 2008 Baker and colleagues demonstrated that inside-out design methods could be used to engineer several enzyme activities into multiple different protein scaffolds [5,8]. Since then over 30 proteins catalyzing a broad range of chemical reactions have been engineered and experimentally validated to have catalytic activity using these methods [1–3,6,7,9]. While this demonstrates a robust ability to rationally engineer enzyme activities into a protein scaffold, the designed enzymes only provide modest rate enhancements when compared to naturally occurring enzymes. Therefore, to guide the development of hypotheses about the difference between naturally occurring and designed enzymes, it is useful to compare their kinetic parameters.

Computationally designed versus natural enzymes

In the simplest form, an enzyme functions by sequentially binding the substrate, then stabilizing the transition state to accelerate substrate to product conversion, after which product is released. The common kinetic measurements of K_M and k_{cat} report on the enzyme's approximate substrate binding affinity, and the rate of transformation from the bound enzyme-substrate complex to enzyme and free product, respectively. Combined with the uncatalyzed

Figure 1



Overview of inside-out computational enzyme design. Three general steps are followed: a theozyme model is constructed, and a set of proteins of known structure collected. Second, these protein scaffolds are searched for sites where amino acids can be built off the native backbone and interact with the transition state as defined in the theozyme. Finally, the non-catalytic residues in the pocket are redesigned to further refine substrate, and transition state binding ability.

rate (k_{uncat}), these kinetic constants can be used to quantitate enzyme catalytic proficiency (K_{tx}^{-1}) as defined by Wolfenden (Eq. (1)) [16].

$$\text{Catalytic proficiency}(\text{M}^{-1}) = K_{tx}^{-1} = \frac{(k_{cat}/K_{\text{M}})}{k_{uncat}} \qquad (1)$$

With these three parameters we can compare naturally occurring and computationally designed enzymes in terms of overall catalytic proficiency (K_{tx}^{-1}), substrate binding affinity (approximated by K_{M}), and catalytic rate enhancement ($k_{\text{cat}}/k_{\text{uncat}}$). It is our hope that through these analyses, we can gain insight into the structural and mechanistic features that should be the focus of future computational enzyme engineering efforts. Houk and colleagues have previously performed a related analysis comparing naturally occurring enzymes, catalytic antibodies, and other host–guest complexes to develop hypotheses about the mechanisms used by enzymes to achieve their unprecedented levels of catalysis [17].

As depicted in Figure 2a and Table 1 it can be observed that the catalytic proficiency of naturally occurring enzymes is often 11 orders of magnitude greater than the computationally engineered enzymes, and still 9 orders of magnitude after efforts to optimize the designed enzymes catalytic activities [1–3,7–9,18••,19,20,21••,22].

It is important to note that while these data include all computationally engineered enzymes reported to date, they only include 24 naturally occurring enzymes due to the limited number of measured k_{uncat} rates [23–28,29••,30]. It is therefore likely that the spectrum of naturally occurring enzyme catalytic proficiencies is much broader than depicted here. However an analysis of kinetic constants for a broader set of naturally occurring enzymes has previously been performed, and the average kinetic values observed are within an order of magnitude of the average kinetic values of the 24 enzymes used in this study [31•].

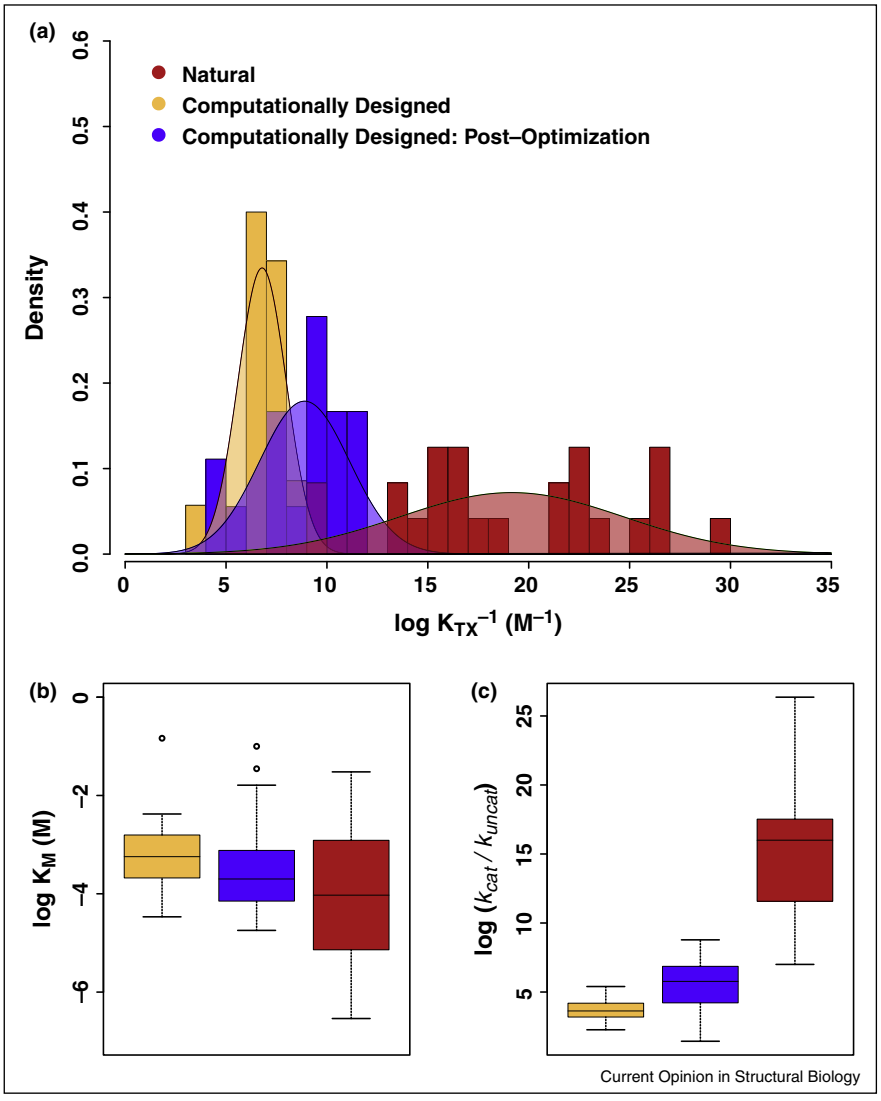
On the basis of this analysis, one immediately wonders what the root cause is of the vast discrepancies (up to 11-orders of magnitude) between naturally occurring and designed enzymes. While the catalytic proficiencies of natural and engineered enzymes provide an overall benchmark of catalysis, a comparison of substrate binding affinity and rate enhancement can provide direction in identifying the structural and mechanistic features missing from the engineered enzymes. In Figure 2b and c, computationally designed and natural enzymes are compared in these terms. Interestingly, the naturally occurring and computationally engineered enzymes have essentially equivalent substrate binding affinity, suggesting that the structural features engineered for

Table 1

Kinetic constants of designed, optimized, and natural enzymes. The median (minimum, maximum) values of the kinetic constants for the enzymes in Figure 2

	k_{cat} (s^{-1})	K_{M} (M)	K_{tx}^{-1} (M^{-1})	$k_{\text{cat}}/k_{\text{uncat}}$
Computationally designed	1.5×10^{-4}	5.7×10^{-4}	8.3×10^{6}	4.2×10^{3}
	(3.2×10^{-6}, 0.3)	(3.4×10^{-5}, 1.5×10^{-1})	(7.6×10^{3}, 1.1×10^{9})	(1.8×10^{2}, 2.6×10^{5})
Computationally designed:	1.5×10^{-2}	2.0×10^{-4}	4.7×10^{9}	5.9×10^{5}
post-optimization	(8.3×10^{-6}, 7.0×10^{2})	(1.8×10^{-5}, 3.5×10^{-2})	(3.1×10^{4}, 4.9×10^{11})	(27.0, 6.0×10^{8})
Natural	1.1×10^{2}	9.7×10^{-5}	5.2×10^{18}	1.0×10^{16}
	(6.9, 1.0×10^{6})	(2.9×10^{-7}, 3.0×10^{-2})	(2.5×10^{9}, 1.2×10^{29})	(1.0×10^{7}, 2.3×10^{26})

Figure 2



Comparison of natural and computationally designed enzymes. **(a)** Comparison of catalytic proficiency (K_{tx}^{-1}). Computationally designed (gold) represent the activities of designed enzymes prior to any efforts to further optimize function. Post-optimization (blue) represent the final, and most active, designs after either directed evolution or further rational design efforts were performed. The natural enzymes (red) constitute a set of twenty-four enzymes for which k_{cat}, K_M, and k_{uncat} are known. **(b)** Comparison of the approximated substrate-binding affinities (K_M). **(c)** Comparison of the catalytic rate enhancements (k_{cat}/k_{uncat}).

binding are similar to those found in nature. However, as readily observed in Figure 2c, the vast majority of difference in catalytic proficiency appears to lie in rate enhancement, which is directly dependent on the k_{cat}.

Possible paths forward

There are likely several structural and mechanistic factors that contribute to the 11-order of magnitude discrepancy between the rate enhancement of naturally occurring and computationally designed enzymes. In addition to selective transition state stabilization, it maybe important to consider ground state destabilization, active site gating, and enzyme dynamics. Each will be briefly discussed in terms of recent evidence in regard to catalysis, and their potential structural and mechanistic impacts in future computational enzyme design efforts.

Transition state binding

One possible explanation for the rate enhancement discrepancies between naturally occurring and engineered enzymes is that the engineered binding pocket's shape and electrostatic complementarity to the transition state are suboptimal. One way to assess the importance of this feature is by separating catalysis from binding of the

transition state. For example, many binding studies of natural enzymes to small molecules that closely mimic the expected transition state have been reported. Considering that the binding affinity of a real transition state to a protein, a transient species, cannot be directly determined, the K_i of transition state analogs are often used to estimate transition state binding affinity (i.e. K_{tx}). For example, in the case of acetylcholine esterase, the transition state analog *m*-(*N*,*N*,*N*-trimethylammonio)-2,2,2-trifluoroacetophenone has been reported to have a K_i of 10^{-14} M, which is approaching the calculated K_{tx} of 10^{-17} M [32]. This is consistent with the majority of this enzyme's ability to catalyze the reaction being derived from its ability to tightly bind the transition state. Meanwhile, the computationally designed enzyme with largest rate enhancement to date (the kemp eliminase HG3.17 with a k_{cat}/k_{uncat} of $>10^8$) binds a transition state analog closely related to the expected transition state with a K_i of $\sim10^{-6}$ M, but has a calculated K_{tx} of $\sim10^{-11}$ M [18••]. The relatively weak binding of the transition state analog implies that the designed enzymes ability to catalyze the reaction may not be achieved through the stabilization of the desired transition state, and that significant improvements in transition state binding affinity can still be made.

Two avenues likely to improve transition state binding of computationally designed enzymes are through the use of continuum electrostatic calculations or tighter integration of quantum mechanics simulations. The use of continuum electrostatics was demonstrated to be potentially useful for designing protein–protein binding interfaces, in which the addition of a Poisson–Boltzmann based electrostatics term to the existing energy function significantly improved the ability to computationally recapitulate experimental mutagenesis data [33]. In addition, using quantum mechanics/molecular mechanics based methods, the catalytic proficiency of kemp eliminases can be recapitulated using the empirical valence bond approach [34•,35]. While the computational expense of this type of simulation currently prevents its use during design simulations, this is a promising approach for ranking designs prior to experimental characterization.

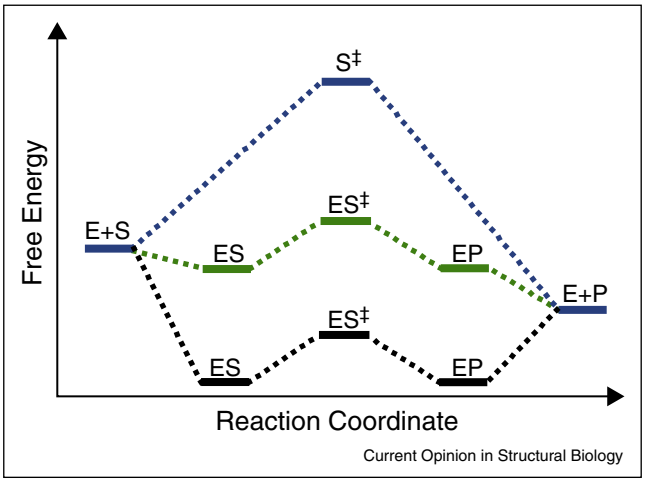
Another potentially important, but likely more difficult, challenge is to improve the overall shape complementary between the transition state and protein. Shape complementarity of the native protein pocket to the transition state is already a significant consideration in computational enzyme design algorithms. However, the native protein has not faced evolutionary selective pressure for the engineered function, yet changes throughout the protein sequence are likely required to fine tune the shape of the pocket for the desired transition state. Current design algorithms generally only modify the first shell of residues surrounding the ligand. Meanwhile, residues outside of this first shell are maintained as their native amino acid identities during the design. One possibility for engineering a pocket with an ideal shape for the transition state could be through *de novo* protein design. Here the entire sequence would be tuned to produce a structure optimal for theozyme binding. The significant advances made recently in *de novo* protein design suggest that the concept of designing a *de novo* protein with catalytic function is a possibility [36••].

An alternative to *de novo* protein design is the *de novo* design of fragments of the protein near the active site. This strategy has recently been employed to enhance the catalytic proficiency of a designed Diels-Alderase enzyme, and utilized the assistance of online gamers [21••]. In this case the gamers designed a helix–turn–helix motif, which had the effects of burying the ligand within the protein, and enhancing shape complementarity between the transition state and protein. The engineered structural motif was experimentally verified to be highly accurate, and enhanced catalytic proficiency ~20-fold. While this demonstrates that structural redesign of a protein active site can improve catalytic proficiency, the gain was found to arise entirely from changes to the K_M and not k_{cat}, which raises the issues of selective transition state binding and ground state destabilization.

Ground state destabilization

In order to achieve catalysis an enzyme must not only bind the transition state, but must also do so selectively relative to the substrate (Figure 3, black versus green path) [37]. In fact, there are many documented examples in which enzymes actually destabilize the substrate in

Figure 3



Hypothetical energy diagram. The blue path represents the uncatalyzed reaction, the green path represents a hypothetical reaction pathway of a proficient enzymes. The black path represents a hypothetical pathway of an enzyme without selective transition state stabilization, nor ground state destabilization of either substrate or product. In this pathway product release would be rate limiting.

order to help minimize the energy gap between the enzyme-substrate (ES) and enzyme-transition state ($ES^{‡}$) complexes [38,39,40••]. For example, binding isotope effects have been used to probe substrate distortion in orotate phosphoribosyltransferases [40••]. In this case, the sp^3 geometry at the anomeric carbon is strained in order to resemble the sp^2 geometry of the carbon in the transition state, which enhances catalysis 240-fold.

These differences in binding energies are not currently evaluated in enzyme design simulations. However, recent tools have been developed to employ multi-state design strategies [41••]. To be implemented for enzyme design a minimum of four states need to be considered (for the simplest possible mechanism): E + S, ES, $ES^{‡}$, and EP (the enzyme–product complex). A delicate balance must be achieved between substrate binding, a requisite for enzyme catalysis, and ground state destabilization of the substrates reaction center. In addition, it is critical to consider the EP state since product release is commonly a rate-limiting step in naturally occurring enzymes [42]. This occurs if the EP state is too stable, in which case the largest energy barrier encountered during catalysis is from product release (Figure 3, black path). To employ a multistate design strategy balancing all these factors it would be optimal to use experimentally measured free energy profiles of native enzyme catalyzed reactions to train design algorithms. Unfortunately, there are only a limited number of experimentally determined full free energy profiles of enzyme catalyzed reactions [43••]. Therefore, efforts to better understand relative energy levels between the E + S, ES, $ES^{‡}$, and EP for many different enzymes will be essential to calibrate the relative energy differences in multi-state computational enzyme design efforts.

Active site gating

Another feature demonstrated to be important for catalytic efficiency is the path into and out of the active site [44–47]. For example, lipase B from *Candida antarctica* was circularly permutated such that no mutations were introduced into the protein sequence, but the placement of new N and C termini converted the narrow tunnel leading to the active site into a broad crevice, and increased k_{cat} 175-fold [45]. While there are several computational algorithms developed to consider the path from solvent to the active site, none have been used in the inside-out design efforts [48•]. Currently, they would have to be implemented as a post-design filter, but efforts to integrate these types of calculations into the design process as sequence space is being searched could improve the pathways into and out of the active site.

Dynamics

One of the most highly debated mechanisms of catalysis in enzymology today is in the role of dynamics. Concepts range from small vibrations that lead to rate-promoting motions [49•] to large changes in molecular structure where each conformation is uniquely optimal for binding either the S, $S^{‡}$, or P [50]. Furthermore, it has been proposed that the excited states between each conformation resemble the ground state of the next, which allows for the enzyme to systematically proceed through catalytic cycles [51]. While explicit dynamic motions to promote catalysis have not been investigated for computational enzyme design, the analysis of movements that would hinder catalysis has been studied. For example, molecular dynamics simulations were used in the case of the computational design of a Diels-Alderase to identify potential unfavorable movement of the active site residues [9]. A catalytic residue was observed to move during the simulation, which led to the design of mutations predicted to buttress the catalytic residues, thereby preorganizing their conformation for a productive trajectory. The mutant enzyme was observed to have enhanced catalytic activity. This concept of examining structural stability (i.e. side chain conformation and ligand placement) through a molecular dynamics simulation was then demonstrated to be a generalizable method for identifying functional versus non-functional designs [52]. Since dynamics appears useful for performing a negative selection to filter non-functional designs, it is not unreasonable to think it can play a role in positive selection to help improve designs.

Conclusion

Using a basic model of transition state theory (i.e. transition state stabilization as the sole criterion for design of catalytic ability) alone has enabled the design of enzymes, when coupled with directed evolution, to achieve catalytic proficiencies $>10^9$. As an optimist, one can look at this as a glass half full on an energy scale, as roughly half the energy for catalysis commonly achieved by naturally occurring enzymes ($\sim 10^{18}$) has been realized. In other words, we have gone from zero to nine, and now we need to go from nine to eighteen (Figure 1a and Table 1).

To achieve this last, but significant half, it is clear that the primary focus of basic science in enzyme design should be for improvements in k_{cat} (Figure 1c and Table 1). However, to move beyond the basic transition state theory currently implemented in enzyme design, a better understanding of both the structural and mechanistic determinates of catalysis for naturally occurring enzymes is still needed. In addition, there are many clear areas in which improvements can be made in current computational design algorithms. Specifically, tighter integration of continuum electrostatic and quantum mechanics, access of the active site to the substrate, and molecular dynamics into the design process should all be considered as the field progresses.

A critical feature of designed enzymes presented in this study is that the engineered enzymes have binding

constants (approximated by K_M) on par with naturally occurring enzymes (Figure 1b and Table 1). Therefore if the catalytic machinery is already in place, the use of computational tools to re-engineer substrate specificity is ready for the applied sciences. Several efforts using computational enzyme design methods to reengineer substrate specificity have already been successful, resulting in novel biological catalysts with significant potential for clinical and industrial use [53•,54•,55•].

At just a dozen years old, the field of computational enzyme design is still in its infancy. With continued efforts, the ability to design proficient enzymes with a specific set of desired properties should be within reach. However the fundamental methods and theories being developed now will likely hold true in future generations.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

Citations [1–9] encompass all of the experimentally validated computationally designed enzymes, engineered through the inside-out method, that have been reported to date. All are of outstanding interest.

Conflict of interest

None declared.

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