Directed Evolution

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Artificial Enzymes Made to Order: Combination of Computational Design and Directed Evolution**

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The classic theory of enzyme catalysis outlined by Haldane and Pauling suggests that weak interactions capable of stabilizing a transition state are responsible for the observed rate acceleration with enzymes.[1] Designing an artificial enzyme thus represents a daunting task, as it requires 1) a precise knowledge of the geometry of the transition state, 2) the tools to correctly position critical amino acid residues to stabilize this geometry within a protein host, and 3) a high-throughput screening assay to fine-tune the activity beyond what can be achieved by rational design.

This formidable challenge has recently been met by using either design[2,3] or directed evolution.[4,5] The latest study by Röthlisberger et al. relies on both computational design and directed evolution to yield an artificial enzyme for the base-catalyzed Kemp elimination (Figure 1).[6] This tour de force builds upon the remarkable progress made in the field of computational protein design.[7,8]

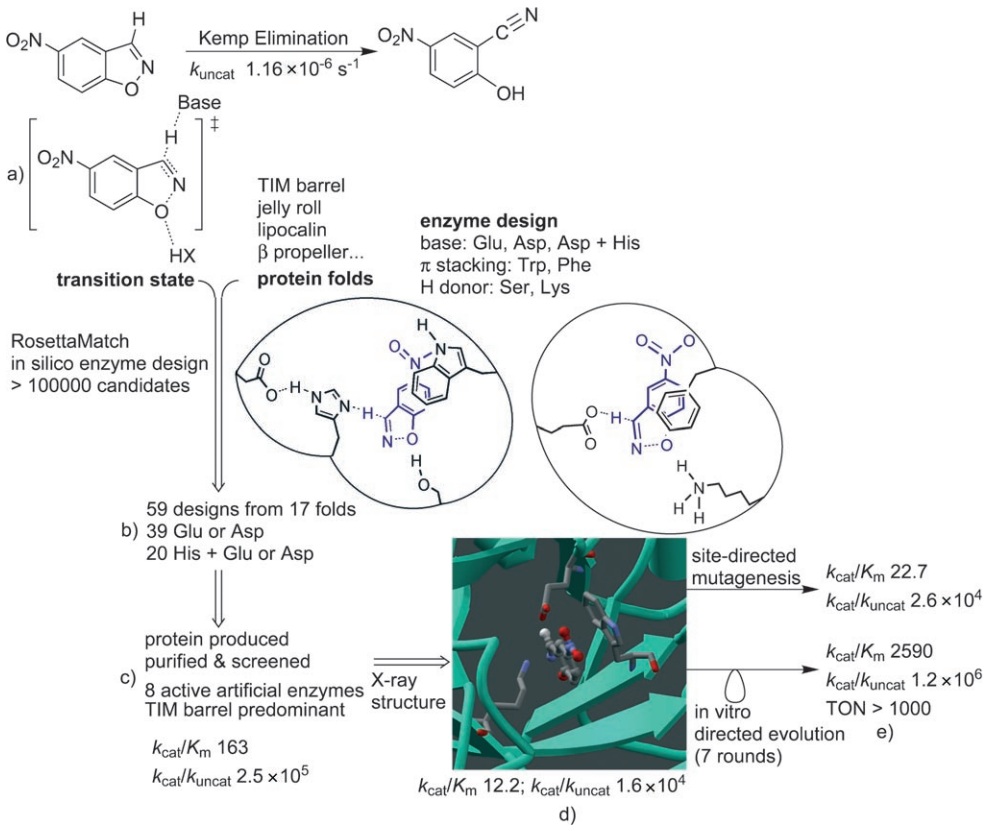

Figure 1. Computational design and directed evolution of an artificial enzyme for the Kemp elimination. The transition state for the Kemp elimination (a) was used to tailor an active site within a variety of protein folds by computational design (b). From this virtual screen, candidates were produced and tested, and eight active artificial enzymes were identified (c). The structural characterization of an active enzyme revealed a near perfect match with the computational design (the depicted computational design emphasizes the TIM barrel; the transition state is shown in a ball and stick representation, and the critical residues Trp50, Glu101, and Lys222 in a stick representation; d). Further optimization was achieved either by site-directed mutagenesis or by directed evolution (e). k_{cat}/K_m values are in $M^{-1}s^{-1}$.

Inspection of the computed transtion state for this reaction intuitively suggests that stabilization may be achieved by the suitable positioning of: a) a basic residue to abstract the acidic proton, b) π-stacking interactions provided by an aromatic residue, and c) a hydrogen-bond donor to stabilize the developing negative charge on the phenolic oxygen atom.

The RosettaMatch computational algorithm was used to tailor an idealized active site from a broad range of protein

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folds. Starting from more than 100000 candidates, 59 structures were identified, which were then produced in bacteria, purified, and tested. Eight of these showed measurable catalytic activity.

While site-directed mutagenesis highlighted the importance of critical catalytic residues, it failed to improve the efficiency significantly. The authors performed directed evolution of a moderately active artificial enzyme, whose structure had been determined by X-ray crystallography, to afford a more than a 200-fold improvement in the k_{cat}/K_m value over that of the original design. During the computational design of the active site, 13 mutations were introduced into the input scaffold (pdb code: 1thf). The crystal structure of the artificial enzyme without coordinated substrate revealed modest positional shifts of the side chains compared to the designed structure, thus demonstrating the impressive predictive power of the RosettaMatch algorithm.

Up to eight additional mutations were inserted over the course of the seven rounds of the following directed evolution without directly affecting the designed positions. Several mutations were found in adjacent positions, thus suggesting that further optimization may be achieved by semirational approaches to fine-tune second coordination sphere interactions.[9,10]

Proton transfer is the simplest reaction in chemistry. It is a key step in many enzymatic transformations and achieving high catalytic efficiency is challenging, despite its apparent simplicity. In this context, the Kemp elimination offers an excellent opportunity to test our understanding of the key features required for efficient catalysis—catalytic antibodies,[11,12] serum albumins,[13] peptides, imprinted polymers, and natural carbon compounds have all been shown to catalyze this reaction.

The design and details of the active site of the artificial enzyme for the Kemp elimination of activated benzisoxazoles bear a striking resemblance to that of the catalytic antibodies (k_{cat}/K_m 2590 and 5500 $M^{-1}s^{-1}$, respectively).[14] The approach delineated by Röthlisberger et al. offers, however, several advantages over catalytic antibodies:

1) For an immune response, one or two specifically positioned residues suffice to produce nanomolar affinities typical of antigen–antibody binding. In contrast, any number of key residues can in principle be incorporated—albeit at the cost of a major computational effort—in the enzyme design to stabilize the transition state. This may allow more challenging reactions to be catalyzed.
2) For the computational design, the protein scaffold can be varied, whereas the catalytic antibodies are limited to immunoglobulins. The TIM-barrel scaffold (eight parallel β sheets flanked by eight α helices), which is widespread among natural enzymes, seems particularly suited, as many residues point towards the catalytic cavity at an ideal distance to stabilize a transition state.
3) The antigen binding fragment of immunoglobulins is challenging to express in bacteria, thus hampering the directed evolution of catalytic antibodies.[15] The in vitro evolution of the computational design was critical, and resulted in a greater than 200 fold improvment in the k_{cat}/K_m value over that of the initial design.
4) Rather than relying on a transition-state analogue to generate the active site of a catalytic antibody, the catalytic residues are ideally positioned to stabilize the true transition state.

The approach delineated by Röthlisberger et al. combines in silico design with the combinatorial diversity generated by directed evolution. Thus far, however, and compared to nature's catalysts, the artificial enzymes reported can only be regarded as primordial enzymes with modest activities. Now that compelling evidence has been found for this approach, attention may be focused on: a) improving the catalytic efficiency. For this purpose, the challenging issues of substrate binding and product release should be addressed. Although allowing the backbone scaffold to be flexible in the computational design may help to some extent, a deeper understanding of these phenomena is still lacking. b) Promoting more demanding transformations. Jiang et al. used the RosettaMatch alogorithm exclusively to design and characterize artificial retro-aldolase enzymes for a non-natural substrate.[2] It is possible that the modest activities achieved in this study (k_{cat}/K_m 0.74 $M^{-1}s^{-1}$; $k_{cat}/k_{uncat} > 2\times10^4$) could be significantly increased by applying a directed evolution protocol. To further broaden the scope of artificial enzymes, the introduction of prosthetic groups could be envisaged.

Overall, these recent breakthroughs[2,4–6] are landmarks in enzymology and augur well for the development of artificial enzymes into synthetically useful tools.

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