

brain injury is caused by the development of inflammation at the site of infarction. Proteasome inhibitors block inflammation by preventing proteasome-dependent activation of transcription factor NF- κ B. The study from the Crews group demonstrating neurotropic properties of proteasome inhibitors (Hines et al., 2008) suggests that an increase in NGF production can also contribute to this effect. This should stimulate the interest in continuation of clinical testing of proteasome inhibitors in stroke patients.

Finally, we would like to draw the readers' attention to the fact that all the inhibitors discussed in these articles are natural products, as are the well-known proteasome inhibitors lactacystin, epoxomicin, and salinosporamide A, and the less famous eponemycin, tyropeptin A (Momose et al., 2005), and TMC-95. If proteasome inhibitors are classified based on chemical mechanisms, by which they inhibit the proteasome, seven major classes can be distinguished (Figure 1). Classes represented by natural products outnumber those developed by organic synthesis.

Indeed, four of these classes (β -lactones, peptide epoxyketones, cyclic peptides, and macrocyclic peptide vinyl ketones) were discovered as natural products. Natural products are represented in the fifth class, peptide aldehydes, although these compounds (e.g., MG132) were initially developed by chemical synthesis. Only two classes of inhibitor (peptide boronates, e.g., bortezomib, and peptide vinyl sulfones) do not yet have natural products among them. Clearly, micro-organisms learned of the importance of the proteasome to their hosts long before scientists discovered this fascinating particle. We predict that this trend of discovery of proteasome inhibitors among natural products will continue, and hold hope that some of new inhibitors will open novel therapeutic applications for these compounds as the study by the Crews group suggests.

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Computational Design of Enzymes

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The de novo design of enzymes with activities not found in natural biocatalysts is a major challenge for molecular biology. Sophisticated computational methods have recently led to impressive progress in this exciting and rapidly evolving field (Röthlisberger et al., 2008; Jiang et al., 2008).

Natural evolution has yielded enzymes with well-defined active sites in which virtually all metabolic reactions are catalyzed with high efficiency and specificity. It has been a major goal of biochemistry for the past century to understand the chemical and molecular principles of these extremely precise and exquisite molecular machines. Recently, technical advances in molecular biology have led to a renaissance in enzymology by enabling researchers to modify at will the activities and stabilities of many

naturally occurring enzymes. This rapidly emerging field of “enzyme design” has provided new insights in the structure-function relationships of molecular biocatalysts. Moreover, these approaches have facilitated the generation of stabilized enzymes with increased turnover numbers and altered substrate- and stereo-selectivities to be used in industrial processes (Toscano et al., 2007).

Until now, the most impressive results in enzyme design have been obtained by

“directed evolution.” In this two-step approach random mutagenesis is used to create large enzyme repertoires, from which optimized variants are then isolated using either selection or screening techniques (Bloom et al., 2005). In contrast to directed evolution, the alternative approach of “rational” enzyme design requires a detailed knowledge of a specific enzyme structure and catalytic mechanism (Woycechowsky et al., 2007). Although occasionally successful, rational

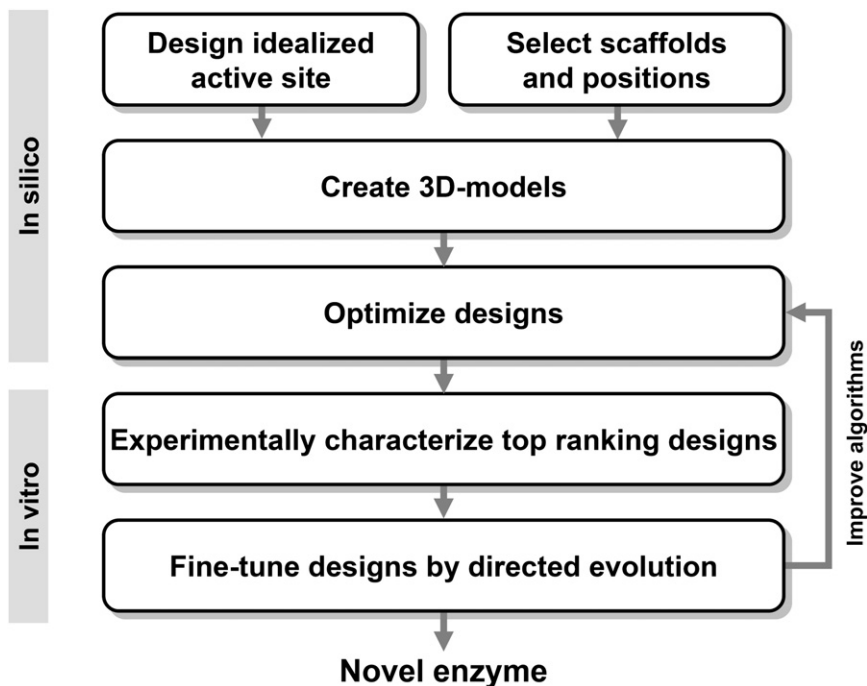


Figure 1. Computational Design and Optimization of Novel Enzyme Activities

A model for the proposed active site is created initially based upon the geometric constraints dictated by the expected transition state structure for the reaction to be catalyzed. Existing protein scaffolds that are compatible with the idealized active site are selected with RosettaMatch. The optimized designs are tested experimentally after synthesis of the genes required for expression of the designed enzymes. Those enzyme variants with catalytic activity are further enhanced via directed evolution. The information gathered from the directed evolution experiments can be incorporated into improvements within the initial design algorithms.

design approaches often fail, due to a limited understanding of the subtle interplay among amino acid side chains within an enzyme active site.

Two recently published papers by David Baker and colleagues suggest that this bottleneck toward the acquisition of tailored enzymes can be overcome by applying sophisticated computational methods (Röthlisberger et al., 2008; Jiang et al., 2008). The approach used by these authors is outlined in Figure 1.

In one example, computational methods were applied to the design of enzymes that will catalyze the Kemp elimination, which is a nonnatural model reaction during which proton abstraction from a carbon must be mediated by a general base (Röthlisberger et al., 2008). First, the authors designed two idealized active sites, which contained either an aspartate/glutamate, or a histidine-aspartate dyad as the catalytic base. For both sites, functional groups were then added to facilitate transition state (TS) stabilization, and their placement and orientation was optimized using quantum mechanical and classical

methods. In the next step, a large set of stable protein scaffolds with known X-ray structures was scanned to find backbone positions, which could harbor these idealized active sites. To this end, a generalized version of the program RosettaMatch (Zanghellini et al., 2006) was used, which is based on the RosettaDesign algorithm. RosettaDesign optimizes the packing of residues on a given backbone by combining side-chain orientations for the various amino acids deposited in a rotamer library. RosettaMatch assesses the quality of a design by means of a multiparameter scoring function, and ranks in a given backbone many different active site locations on the basis of catalytic geometry and TS energy. Out of the more than 10^5 initial Kemp elimination designs, 59 were experimentally tested. Eight of the designs, which contained between 10 and 20 residue exchanges, showed weak enzymatic activity with catalytic efficiencies (k_{cat}/K_M) between 6 and $160 \text{ M}^{-1}\text{s}^{-1}$, and rate accelerations ($k_{\text{cat}}/k_{\text{uncat}}$) of up to 2.5×10^5 fold. The X-ray structure of one of the variants was solved and the active site superim-

posed with a root mean square deviation of 0.95 \AA with the calculated active site model. Moreover, the back-mutation of the introduced bona fide catalytic bases in several designs abolished or strongly reduced catalytic activity, which suggests that the new enzymes catalyzed the Kemp elimination with the expected reaction mechanism. In order to further improve catalytic activity, seven rounds of directed evolution were performed with the enzyme for which the X-ray structure had been determined. The best variant isolated by this approach showed a >200-fold improvement in activity compared to the starting enzyme, reaching a respectable k_{cat}/K_M of $2600 \text{ M}^{-1}\text{s}^{-1}$. The eight additional exchanges were mainly clustered adjacent to the designed residues, suggesting that the beneficial effects of these new residues are due to subtle fine-tuning of the active site geometry.

In a second project, the authors designed enzymes for a chemically more demanding retro-aldol reaction, which requires the breaking of a carbon-carbon bond in a non-natural substrate (Jiang et al., 2008). Since this reaction proceeds in several steps, a composite active site had to be modeled, that would simultaneously accommodate multiple intermediates and TS. This challenge was met by modifying the enzyme design methodology. First, the various reaction intermediates and transition states were modeled in the context of a specific set of functional residues. These models were superimposed resulting in four alternative composite active site motifs, all of which comprised a nucleophilic lysine and general acid/base groups to catalyze various proton transfer steps. For each motif, large sets of discrete 3D models were generated by varying degrees of freedom of the composite TS, the orientation of catalytic site chains relative to the TS and the conformation of the side chains. RosettaMatch was then used to identify candidate catalytic site locations in a number of different scaffolds. Following a further optimization and an assessment of the models, 72 of them with 8–20 amino acid changes in 10 different scaffolds were experimentally tested. Altogether, 32 designs showed weak catalytic activity. Interestingly, the most active designs contained an explicitly modeled water molecule, which is assumed to be involved in catalysis by stabilizing a reaction intermediate and acting as a general base. The X-ray structure of

the best variant with eight residue exchanges largely confirmed the active site design. Nevertheless, it displayed only modest catalytic activity, with one molecule of product generated in 2 hr and an apparent catalytic efficiency (k_{cat}/K_M) of $0.74 \text{ M}^{-1}\text{s}^{-1}$.

From the large variety of protein scaffolds that were computationally scanned to harbor the Kemp elimination and the retro-aldol designs, only very few folds turned out to be suitable hosts for these reactions. Among them, the most prominent ones were two members of the $(\beta\alpha)_8$ - or TIM barrel family, which is the most frequent and versatile fold among naturally occurring enzymes (Gerlt and Raushel, 2003; Sterner and Höcker, 2005), and it is interesting to note that the computational design shows the same fold preference as found in nature.

Although the work reported here shows that computational enzyme design is feasible, the catalytic activities of the newly generated artificial enzymes are much lower than those evolved by nature. In this context it is interesting to compare the turnover numbers and catalytic efficiencies of the Kemp elimination and the retro-aldol designs with those of catalytic antibodies. For the Kemp elimination, the Hilvert group has isolated a catalytic antibody (34E4) with a k_{cat}/K_M of $5.4 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ and has also shown that this reaction can be catalyzed by

bovine serum albumin (BSA) with a catalytic efficiency of $2.4 \times 10^1 \text{ M}^{-1}\text{s}^{-1}$ (Hu et al., 2004). Thus, the adventitious catalytic activity of BSA with the Kemp substrate compares quite favorably with the best designed enzyme while the more optimized catalytic antibody outperformed the best designed enzyme even after seven additional rounds of random mutagenesis. Catalytic antibodies have also been characterized by the groups of Barbas and Lerner for the retro-aldol reaction. The 38C2 antibody catalyzed the cleavage of the test substrate with a k_{cat} of 1.0 min^{-1} and k_{cat}/K_M of $1.1 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ (List et al., 1998). Thus, the catalytic antibody is a better catalyst by 2–3 orders of magnitude. Notably absent from the discussion of the catalytic proficiency of the retro-aldolase is an assessment of the stereoselectivity for the target substrate. The target substrate has a chiral center at C-4 and it would have been of significant interest to learn whether or not the catalytically active proteins preferentially cleaved either the R- or S-enantiomers and whether this preference derived from their initial design elements. Thus, it remains to be seen whether de novo designed enzymes will be able to ever rival those optimized by Mother Nature. The results of the Kemp elimination reaction design suggest that the best strategy to reach this ambitious goal in the near term

would be to combine computational approaches with directed evolution.

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