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Reengineering enzymes

Stefan Lutz

Department of Chemistry, Emory University, 1515 Dickey Drive, Atlanta, GA 30084, USA
sal2@emory.edu

The versatility and proficiency of enzymes makes them promising catalysts for the laboratory and industrial synthesis of organic molecules, yet natural biocatalysts often do not meet the scientists and engineers' expectation in regards to stability, selectivity and specificity. In this issue of *Science*, two different approaches show how enzymes can be engineered to make them part of the organic chemist's toolkit. Using a first-principles approach, Siegel *et al.* (1) applied the Rosetta design software to create an enzyme that performs the stereoselective, intermolecular Diels-Alder cycloaddition, a reaction of great value to organic synthetic chemistry. In contrast, Savile *et al.* (2) used structure-based rational design and directed evolution to reprogram the substrate specificity and stability of a native transaminase to replace a rhodium-based hydrogenation catalyst in the production of an anti-diabetes compound, sitagliptin.

The enzyme design reported by Siegel *et al.* is the latest example, of synthetic biocatalysts for Diels-Alder cycloadditions (3-5). In this reaction, a conjugated diene (a molecule with two adjacent double bonds) and a dienophile (that donates one double bond) form a cyclic product via simultaneous formation of two new chemical bonds. The Diels-Alder reaction enjoys great popularity among chemists for the rapid and stereoselective assembly of complex molecules and natural products (6, 7), yet there are only two confirmed examples of naturally occurring Diels-Alderase (DAs), and both catalyze only intramolecular cycloadditions (8, 9).

Tackling the challenge to create a synthetic DA for intermolecular cycloaddition, Siegel *et al.* began with a computational model of the transition state, which sets the geometrical arrangement of the two substrates in the enzyme active site. The substrates' electronic properties were modulated by placing hydrogen-donating and accepting amino acid side chains near the dienophile and diene, respectively, to lower the energy barrier to reaction (see the figure, panel A). Based on this core design, quantum-mechanical simulations then generated an ensemble of $\sim 10^{19}$ so-called theozymes, which varied the two assisting amino acid side chains and their orientation relative to the model of the transition state.

In a massive computational undertaking, the RosettaMatch algorithm next matched all of the theozyme conformers against a library of 207 protein scaffolds (known protein folds) to identify approximately 10^6 theozymes that fit into one or more of the scaffolds. The authors subsequently used a combination of RosettaDesign software and scientific intuition to optimize the active site geometry of selected candidates and to eliminate questionable designs. Of the remaining 84 candidates that they synthesized by protein expression, two designs, DA_20_10 and DA_42_10, showed small but significant increases in DA activity relative to the uncatalyzed reaction. After additional fine-tuning of selected residues in the active sites by site-specific mutagenesis, these enzymes matched the performance of catalytic antibodies for this reaction, and did so with excellent stereoselectivity and for multiple reaction cycles.

Built on first-principles, these enzyme designs not only test our understanding of protein structure and function but also allow prediction and exploration of individual mutations on

the overall performance of the biocatalyst. In DA_20_10, each of 15 amino acid changes contributed to the new catalytic function, and the effects of intricate design differences, such as the choice of a glutamine over a glutamate as hydrogen-acceptor, were predicted accurately. The work by Siegel *et al.* is a milestone in enzyme design, extending the application of the Rosetta algorithm for de novo biocatalyst design beyond reactions that break bonds in a single substrate, such as the Kemp elimination and retro-aldolase reaction (10, 11). Nevertheless, the rates of these DAs are still slow compared with most native enzymes. The discrepancies in catalytic performance between native and synthetic biocatalysts suggest there are major contributing factors to enzymatic activity that have not been considered in our current designs. For example, protein dynamics and the functional contribution of correlated motion to catalysis in enzymes remain an intriguing but controversial factor yet to be explored in design approaches (12, 13).

Savile *et al.* describe a more pragmatic approach to the tailoring of a biocatalyst. Undesirable process conditions and problems with product quality during the asymmetric rhodium-catalyzed hydrogenation step in the production of the antidiabetes compound, sitagliptin, spurred their exploration of a transaminases substitute. Transaminases are enzymes that catalyze the enantioselective transfer of an amino group from a donor substrate via a pyridoxamine intermediate onto the carbonyl group of an acceptor substrate. Savile *et al.* envisioned a direct transamination of pro-sitagliptin ketone (see the figure, panel B). This approach would shorten the process by one step and improve product quality by taking advantage of the high stereo and enantioselectivity of the enzyme.

However, initial screening of native transaminases quickly established the absence of any detectable enzymatic activity for bulky substrates such as pro-sitagliptin ketone (the immediate precursor to the final product). Furthermore, process conditions demand high substrate concentrations, which can be achieved with the poorly water-soluble pro-sitagliptin ketone only by adding organic solvent and raising temperatures, either of which diminishes enzyme lifetime.

Savile *et al.* used structure-based rational design followed by directed evolution to alter the substrate specificity and improve the stability of ATA-117, a transaminase from *Arthrobacter sp.* (14). They first used docking studies between a homology model of ATA-117 and pro-sitagliptin ketone to identify potential steric conflicts and unfavorable interactions in the enzyme active site that prevent substrate binding in native enzymes. Guided by the computational model, the binding pocket for the substrate's triazolo piperazine moiety could be optimized via site-saturation mutagenesis of a single position while substitutions in three positions expanded the binding pocket for the trifluorophenyl group. Although the catalytic activity of the resulting ATA-117 variant for pro-sitagliptin ketone was rather low, the establishment of detectable transaminase activity for the target substrate at this stage was critical to the subsequent directed evolution protocol (16, 17).

They next applied ten rounds of directed evolution, using a combination of site-saturation and random mutagenesis, as well as DNA shuffling, to select transaminase variants with the highest catalytic activity while raising the fraction of organic solvent to 50%, the reaction temperature to 40°C, and the pro-sitagliptin ketone concentrations to 200 g/l. The best enzyme variant not only met these selection criteria but performed the desired transamination with >99.9% enantiomeric excess. Its 27 amino acid substitutions were found not only in the enzyme active site, but also in a second hot-spot at the protein-protein interface of the ATA-117 homodimer, where they likely help to stabilize the protein. Overall, the work by Savile *et al.* is an impressive demonstration of current enzyme engineering methods to tailor enzymes to specific requirements and consequently the potential of biocatalysis to revolutionize chemical processes in industry.

The reports by Siegel *et al.* and Savile *et al.* are a testimony to our current understanding of enzymes. While the successful design of DAs illustrates the advances (and limitations) in our knowledge of biocatalysis fundamentals, the customized transaminase shows our technical capabilities to effectively manipulate existing enzymes. Together, our current design and directed evolution methods offer the possibility to create new enzymes to meet specific reaction requirements for a wide range of synthetic, industrial, and therapeutic applications.

Acknowledgments

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Figure.

Tailoring enzymes to specific reactions. (A) Savile et al. reprogrammed the substrate specificity of a transaminase to perform a reaction in the industrial production of an antidiabetes drug, sitagliptin. Mutational hot spots shown in red are in the active site, and those shown in yellow stabilize binding of the dimeric form of the active enzyme. (B) Siegel et al. designed an enzyme to perform the Diels-Alder reaction between two molecules based on first principles. They computer-generated a large ensemble of structures in which two key amino acids in the active site (depicted in red and blue) are bound to the transition state. These structures were fitted to a protein scaffold, and target enzymes were then designed and synthesized.