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Enzyme Engineering

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Abstract: Various approaches for creating enzymes with tailored activities are presented.

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Enzymes are essential to life as we know it. These proteins serve as catalysts for nearly all the chemical reactions that define cellular metabolism. Their enormous rate accelerations and exacting selectivities also make them extremely valuable outside the cell. As a consequence, enzymes are being used increasingly in research, industry and medicine.

Nevertheless, our understanding of these biological macromolecules lags far behind our understanding of small molecules. The properties of enzymes are determined by their precise three-dimensional structures, but we don't know the detailed rules that govern protein folding and our knowledge of structure–function relationships in proteins is at best incomplete. Not surprisingly, then, the design of enzymes from first principles remains an unrealized dream.

Study of biomacromolecules, like many of today's most interesting scientific problems, demands a multidisciplinary perspective. Enzyme engineering, in particular, has much to gain from an integration of chemistry, immunology, molecular biology and genetics. By exploiting these tools, we hope to better understand how enzymes work and evolve. We may also be able to provide researchers of the future with useful catalysts for diverse applications.

Semisynthetic Enzymes

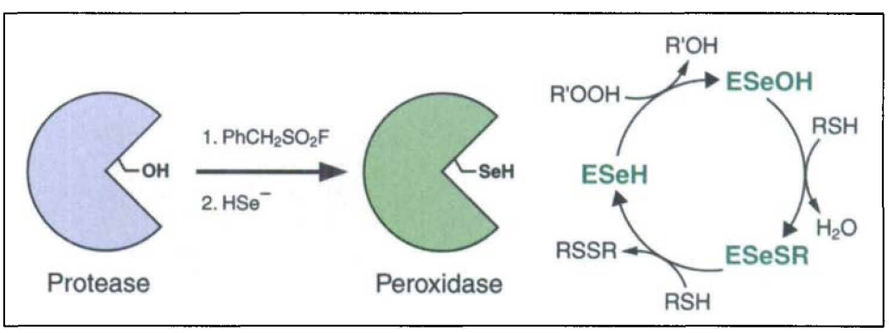
Although it is not yet practical to design enzymes from scratch, existing protein scaffolds can be readily modified with recombinant techniques, by site-selective chemical modification or even through chemical (semi)synthesis. In fact, site-directed mutagenesis has become an indispensable tool for studying enzyme mechanism and for altering enzyme selectivity. Fundamentally new activities can be conferred on proteins in this way, as well.

Conversion of a protease into a peroxidase is illustrative. As shown in Scheme 1, treating the serine protease subtilisin sequentially with phenylmethanesulfonyl fluoride and hydrogen selenide selectively converts Ser221 at the enzyme active site into a selenocysteine [1]. The resulting artificial selenoenzyme, selenosubtilisin, efficiently catalyzes the oxidation of thiols by alkyl hydroperoxides [2], mimicking the action of glutathione peroxidase, an important natural enzyme that protects cells from oxidative damage. In addition to its redox activity, selenosubtilisin also promotes the hydrolysis and aminolysis of activated esters [1]. Indeed, acyl transfer to amines is four orders of magnitude more efficient than with native subtilisin. This result, and the fact that the modified enzyme does not hydrolyze peptides, suggests that it could be a practical peptide ligase.

Kinetic analyses, site-directed mutagenesis, ^{1}H and ^{77}Se NMR spectroscopy, and crystallography are some of the techniques [2][3] that have provided detailed insight into the influence of active site microenvironment on the reactivity of the selenium prosthetic group in selenosubtilisin. By incorporating selenium into other proteins, it should be possible to modulate its reactivity and selectivity in a systematic fashion. Recent experiments show that selenoproteins can be prepared by selenocysteine-mediated chemical ligation of synthetic peptide fragments [4], which obviates the need for a reactive serine residue or special molecular biological methods and should provide ready access to such molecules.

Catalytic Antibodies

Harnessing the microevolutionary processes of the mammalian immune system for catalysis represents a funda-



Scheme 1. Site-selective chemical conversion of a serine protease into a peroxidase

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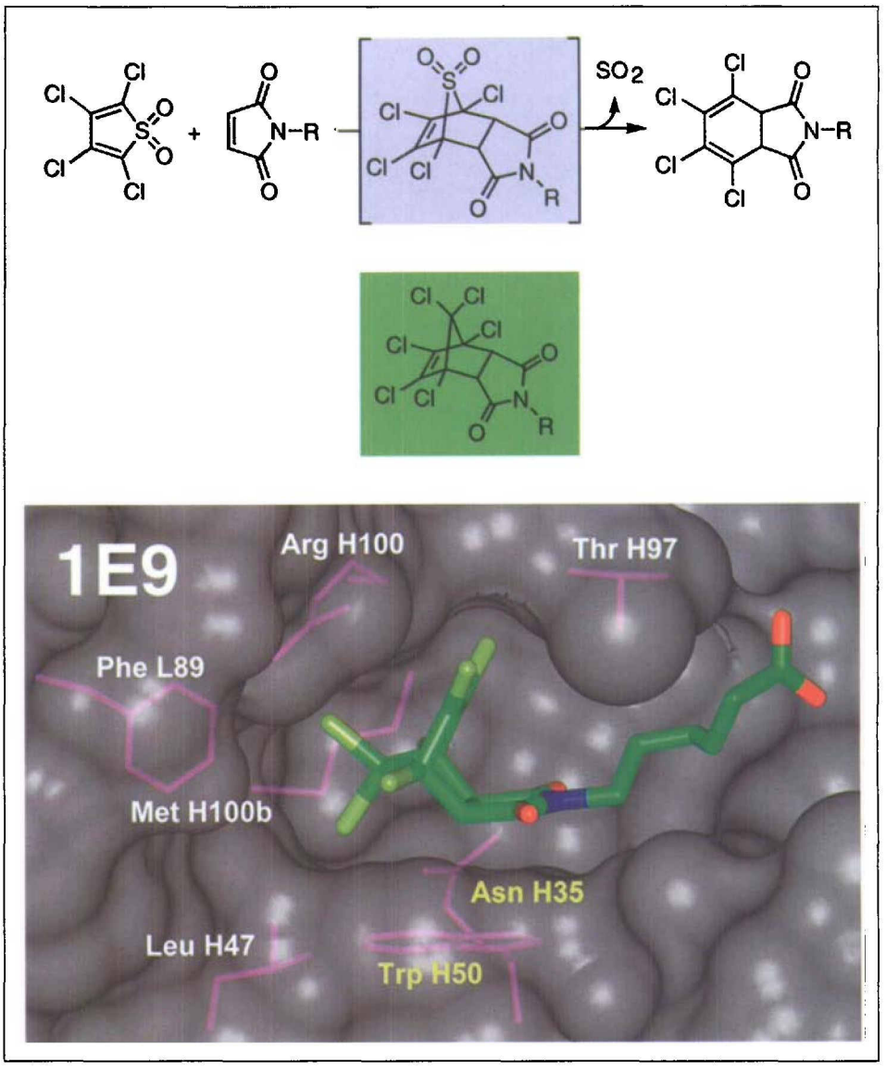
mentally different strategy to enzyme engineering. Antibodies elicited with suitably designed transition-state analogs have been found to catalyze a broad range of chemical reactions with high selectivity [5]. Using this approach, my laboratory has prepared antibodies that accelerate proton transfers, decarboxylations, and concerted pericyclic reactions, such as the Claisen rearrangement of chorismate to prephenate and a bimolecular Diels-Alder cycloaddition. Like natural enzymes, these catalysts exhibit substantial rate accelerations, substrate specificity, and regio- and stereoselectivity.

Catalytic antibodies can shed light on the fundamental catalytic mechanisms available to proteins [5], and we are carrying out detailed studies of representative antibody catalysts to better understand the roles of strain, proximity, and desolvation in enzyme catalysis. For instance, biochemical, computational and structural investigations [6] of antibody 1E9, which promotes the cycloaddition between tetrachlorothiophene dioxide and N-ethylmaleimide (Scheme 2), have revealed the relatively subtle mutational steps required for the evolution of both structural complementarity and catalytic efficiency.

Because their selectivity and mechanism of action are defined *a priori* by the structure of the antigen, catalytic antibodies can be generated for reactions that are difficult to carry out selectively with existing methods or for which natural enzymes do not exist [5]. Ongoing work in this area is devoted to expanding the repertoire of reactions amenable to antibody catalysis and to the elaboration of general strategies for augmenting catalytic efficiency.



Scheme 2. The abiological Diels-Alder reaction between tetrachlorothiophene dioxide and N-ethylmaleimide (top) is catalyzed by an antibody elicited with a stable hexachloronorbornene derivative (green box). The active site of the antibody is shown (bottom) with the transition state analog bound.

Evolutionary Strategies

Natural enzymes have been perfected over millions of years by the process of Darwinian evolution. Recursive cycles of mutation, selection and amplification are also ideally suited for creating and characterizing proteins in the laboratory.

The power of a Darwinian approach is illustrated by studies with chorismate mutase enzymes [7], which play an essential role in aromatic amino acid biosynthesis. We have engineered several strains of yeast and *Escherichia coli* that lack the genes for chorismate mutase [7]. Without the enzyme, these cell lines are unable to produce tyrosine and phenylalanine and hence cannot grow under selective conditions. However, we can reconstitute the biosynthetic pathway by supplying the cells with catalytically active polypeptides. This simple system can be used to evaluate up to a billion (10^9) different molecules simultaneously. Those that have chorismate mutase activity bring about cell growth, allowing their efficient selection and amplification from a background of predominantly inactive clones.

We have exploited this type of *in vivo* selection to probe the properties of natural chorismate mutases [7]. For example, combinatorial mutagenesis and selection experiments have shown that a cationic residue in the active site of the enzyme is a critical feature of catalysis [8], presumably because it stabilizes charge separation in the transition state for the chorismate rearrangement. This approach has also helped to clarify the role of a structurally unresolved segment of the *Bacillus subtilis* enzyme, to elucidate the chemical constraints on interhelical turns, and to identify the minimal functional domain of a catalytic antibody with modest chorismate mutase activity [9].

Because catalytic activity imposes stringent requirements on structure, selection experiments with large numbers of variants can provide statistically meaningful insights into the complex and often subtle interactions that influence folding, structure, and catalytic mechanism. Selection is also a useful adjunct to design, as shown by the successful conversion [10] of a homodimeric chorismate mutase into a highly active monomeric enzyme (Fig. 1).

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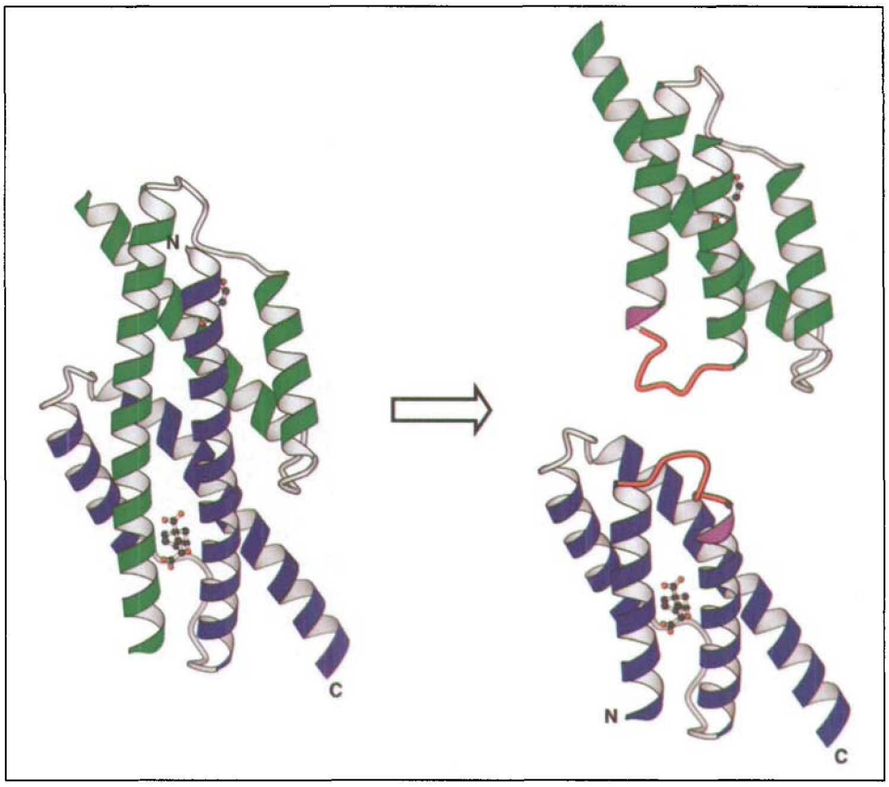

Fig. 1. Design, mutagenesis and *in vivo* selection were used to convert a homodimeric chorismate mutase into a highly active, monomeric enzyme.

Outlook

In principle, even relatively primitive catalysts can be optimized through multiple rounds of random mutagenesis and selection. For this reason, exploitation of evolutionary methods in combination with design may ultimately represent the most effective strategy for creating new enzymes. As chemists and biologists begin to tackle even larger and more complex assemblages, including supramolecular machines, such approaches may well be essential.

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