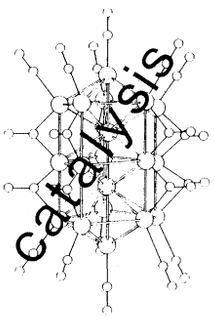
# Need a Catalyst? Design an Enzyme

## *By changing one or more amino acids in enzymes, it is possible to study their catalytic function and perhaps even to generate new catalysts*



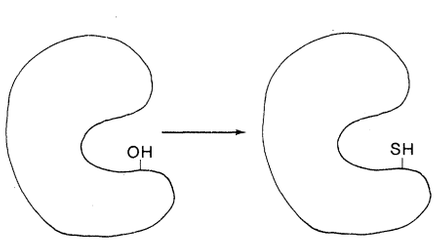

One way to alter the specificity of enzymes is to attach organic molecules or organometallic catalysts to their surface. This has produced some interesting changes in reactivity.* Potentially better results, however, should be obtainable by changing the amino acid sequence of the enzyme to alter its properties in a predictable manner. "In the past," says Kevin Ulmer of Genex Corporation, "everyone has viewed this as a dream. I think they will find it's not as far off as they think." Investigators in many laboratories are undertaking genetic manipulation of enzymes and are beginning to get some results. "We're not going to be able to do sophisticated things right off the bat," he adds, "but a lot of things are going to be possible."

Mutagenesis of proteins to change their activity is, of course, not new. The pharmaceutical industry, in particular, has a long history of initiating more or less random mutations in cellular enzymes and then screening the resulting cells to find improved production strains. During the past 40 years, for example, the bacterial production of penicillin has been improved approximately 10,000-fold. More recently, the Cetus Corporation has used such classical techniques to modify enzymes for the production of glycols: the optimum *p*H's of the three enzymes used in the process originally varied by 3 *p*H units, making it impossible to use all three in the same system. By mutation techniques, the optimum *p*H's have been altered so that all three enzymes can be used simultaneously. Genex has used similar techniques to improve the bacterial production of phenylalanine, an important constituent of the artificial sweetener aspartame.

The crucial difference in the new approach is that investigators are selecting specific amino acid residues they wish to replace and, at the same time, selecting the residues they wish to replace them with. Among other things, this approach requires a precise knowledge of the three-dimensional structure of the enzyme, knowledge that can be obtained only by x-ray crystallography. "Very soon," says Ulmer, "you'll see almost every major crystallographic group teamed up with a molecular biology group."

For the moment, most investigators—at least most academic investigators—are simply seeking more information about enzyme mechanisms and the dynamics of protein folding. For the near future, the major goals are simply to improve the stability of enzymes so that they can better tolerate the conditions that might be found in industrial fermentation (and thus last longer) and to improve their immobilizability so that less activity is lost when the enzyme is attached to a support. Longer term goals include changes in substrate specificity, changes in catalytic activity, reduction of molecular weight so that a desired enzyme might be produced more efficiently, and perhaps eventually the fusion of related enzymes so that a multienzymic process might be carried out on one protein.

The first controlled modifications of proteins were reported independently in 1966 by Daniel Koshland, Jr., and his colleagues at the University of California, Berkeley, and by Myron Bender and his associates at Northwestern University. They used chemical reactions to change the hydroxyl group of a serine residue at the active site of the proteolytic enzyme subtilisin into a sulfhydryl group. Conceptually, this was equivalent to replacing the serine with a cysteine. The resultant "thiol-subtilisin" did not possess proteinase activity; it was active only against highly activated substrates, such as nitrophenyl esters. These results were taken as confirmation of the postulate that the serine hydroxyl plays a key role in enzymatic catalysis. Since then, other investigators have used chemical techniques to replace a serine in the active site of trypsin with a cysteine residue and to replace a cysteine in the active site of papain with a serine residue. In both cases, the enzymes have lost their natural proteolytic activity.




***Chemical modification***

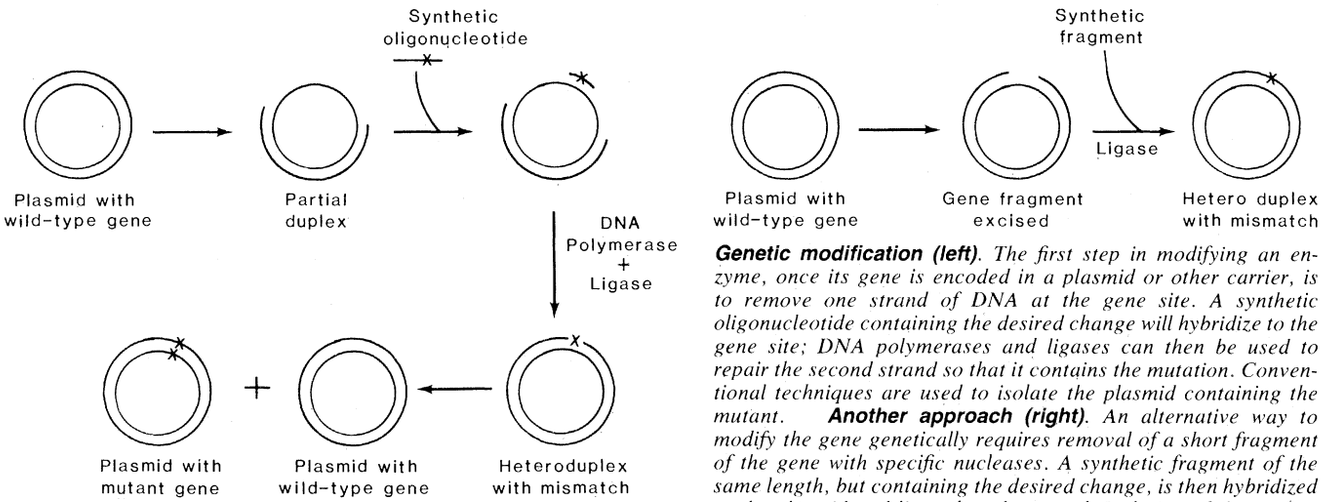
*The simplest way to modify an enzyme is to change the side chain of an amino acid by chemical means. This is not very selective, however, and often requires harsh conditions.*

The problem with chemical modification is that it is harsh and nonspecific. Unless great care is taken, an attempt to exchange a serine for a cysteine at the active site, for example, may result in exchanging all serines on the surface of the enzyme for cysteines—with unpredictable results. Many desired changes, furthermore, cannot be achieved easily with chemical techniques, and many residues that it might be desirable to change are on the interior of the enzyme and inaccessible to reagents. But advances in genetic engineering technology in the last 2 years, says Greg Winter of the Medical Research Council Laboratory of Molecular Biology in Cambridge, England, have made amino acid substitutions "almost routine."

There are two principal ways to carry out what has become known as "site-directed mutagenesis" of proteins. Both involve cloning the gene that codes for the protein and incorporating it into a suitable carrier, such as the plasmid pBR322 or the bacteriophage vector M13. One approach then requires synthesis of an oligodeoxynucleotide primer containing perhaps 15 or 20 nucleotides, a procedure that now takes only 8 to 10 hours. The desired mutation is encoded within this synthetic primer, typically by the change of only one deoxynucleotide, but occasionally with the change of two or more.

This sequence retains sufficient homology to the naturally occurring gene that it will bind (hybridize) to the appropriate position on the single-stranded template. DNA polymerases then use the primer to begin synthesis of a complementary copy of the plasmid or vector. The copy can be separated from the

*For a discussion of semisynthetic enzymes, see *Science*, 13 January, p. 154. Other articles in this series have appeared in the 1983 issues of 4 February, p. 474; 25 February, p. 944; 25 March, p. 1413; 6 May, p. 592; 3 June, p. 1032; 17 June, p. 1261; 22 July, p. 351; 30 September, p. 1358; and 14 October, p. 151.




***Genetic modification (left).*** *The first step in modifying an enzyme, once its gene is encoded in a plasmid or other carrier, is to remove one strand of DNA at the gene site. A synthetic oligonucleotide containing the desired change will hybridize to the gene site; DNA polymerases and ligases can then be used to repair the second strand so that it contains the mutation. Conventional techniques are used to isolate the plasmid containing the mutant.* ***Another approach (right).*** *An alternative way to modify the gene genetically requires removal of a short fragment of the gene with specific nucleases. A synthetic fragment of the same length, but containing the desired change, is then hybridized to the plasmid and ligated to the strand. Isolation of the mutant gene then proceeds as in the first case.*

original by various techniques and used to control the production of the mutant protein in an appropriate host.

Alternatively, a segment of the cloned natural gene containing the site where mutagenesis is desired can be excised enzymatically and replaced with a synthetic segment that contains the desired changes. These techniques are well known and, typically, the only significant problem is coaxing the host to produce the mutant protein in adequate quantities.

One of the first successful examples of this approach was reported last October by a team headed by Winter and Alan Fersht of the Imperial College of Science and Technology in London. They cloned the gene for the tyrosyl-tRNA synthetase of *Bacillus stearothermophilus* and inserted it into bacteriophage M13; this enzyme catalyzes the aminoacylation of tyrosine tRNA (the RNA that binds to a DNA template to insert a tyrosine into a growing amino acid chain) with tyrosine by a two-step mechanism in which tyrosine is activated by adenosine triphosphate (ATP) to form tyrosyladenylate, which is then transferred to its tRNA.

X-ray studies by David Blow of Imperial College showed that cysteine-35 of the enzyme makes contact with the 3′ hydroxyl of the tyrosyladenylate sugar, and thus presumably assists its bonding to the enzyme. They replaced this cysteine with a serine residue. This substitution reduced the catalytic activity of the enzyme by about 70 percent, primarily by lowering the strength of tyrosyladenylate binding.

This finding was somewhat surprising because hydroxyl groups normally form stronger hydrogen bonds than sulfhydryl groups. The optimum O–H····O bond distance for a hydrogen bond, however, is at least 0.04 nanometer shorter than the corresponding S–H····O distance, and presumably the long hydrogen bond from serine-35 to the substrate is therefore weak. To test this hypothesis, Winter and Fersht replaced cysteine-35 with a glycine residue which cannot form a hydrogen bond. This mutant protein has rate and binding constants for tyrosyladenylate virtually identical with the serine-35 mutant, suggesting that the net bonding contribution from serine-35 is zero. The group suggests, however, that the serine-35 does make a weak hydrogen bond to the substrate; in the absence of substrate, however, the residue makes a strong hydrogen bond to a water molecule. When the enzyme binds substrate, then, this strong hydrogen bond with water is replaced by a weak one to substrate.

The group will shortly report on some other modifications to the enzyme. Threonine-51 is involved in what Winter calls "a rather long hydrogen bond with the tyrosyl adenylate, and sometimes a weak hydrogen bond can be worse than no hydrogen bond at all." To test this, the threonine was converted to an alanine, which cannot hydrogen bond; this improved binding of the substrate by about a factor of 2. "We had also noticed," says Winter, "that the corresponding enzyme from *Escherichia coli* has a proline residue at position 51. That seems a rather strange place for the proline, since it would disrupt the α-helix in that segment." When threonine-51 in the *B. stearothermophilus* enzyme was converted to proline, it did, in fact, disrupt the backbone of the enzyme. But this disruption probably had the effect of increasing interaction of the substrate with histidine-48, and the binding of the substrate was improved 100-fold.

A team headed by John Richards of the California Institute of Technology has made several changes in the active site of β-lactamase, the enzyme that cleaves the lactam ring of penicillin and its derivatives and thereby provides bacteria with resistance to the antibiotic. The enzyme contains a serine-threonine dyad at positions 70 and 71. The hydroxyl group of serine-70 is presumed to be acylated during cleavage of the lactam ring. The role of threonine-71 is not clear, but its importance has been demonstrated by the prior isolation of a mutant, probably with an isoleucine at that position, which is catalytically inactive.

Richards and his colleagues initially interchanged the serine and threonine residues to test the role of serine-70. A bacteria containing this doubly mutated enzyme showed no β-lactamase activity. They subsequently converted only serine-70 to a threonine and again found that bacteria containing the enzyme had no β-lactamase activity; this is not surprising because the conversion has the effect of adding a methyl group to the serine side chain, thereby hindering access to the hydroxyl. A mutant bacteria in which threonine-70 was converted to serine has a low level of activity.

Richards, David Botstein of the Massachusetts Institute of Technology, and Patricia Foster of the Harvard School of Public Health have also made a mutant with arginine at position 70; this mutant, as expected, is catalytically inactive. The significance of this substitution lies not in the amino acid inserted, however, but in its codon. The codon for arginine is CGC (C, cytosine; G, guanine; T, thymidine; A, adenine) and it can under-

go mutation in two ways to produce an enzyme with catalytic activity. In a process called transition, the first C is changed to a T to give the codon for cysteine (TGC); in a process called transversion, the first C is converted to A to give the codon for serine (AGC). This mutant thus serves as a probe to study the relative frequency of transitions and transversions caused by chemical mutagens. With acetoxin, for example, a transversion occurs to produce wild-type enzyme. The investigators are now studying the effects of other mutagens.

A team at the Du Pont Company headed by Irving Sigal converted serine-70 of the active site of β-lactamase to a cysteine residue, producing a thiol-β-lactamase. Bacteria containing the mutant enzyme have a reduced resistance to penicillin, but the enzyme activity was not completely abolished as was the case with thiol-subtilisin. This is probably because the β-lactam ring is more labile than the amide bonds of peptides.

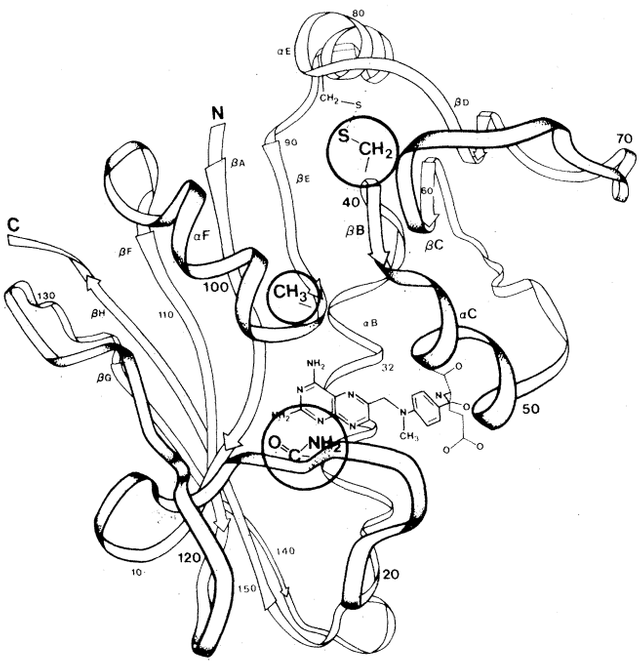
A more recent study with pure enzymes shows that the binding constant for the mutated enzyme with penicillin is about the same as that of the wild-type enzyme, but that the rate is only about 1 or 2 percent of that of the wild-type enzyme. The mutant enzyme is also as active as, or more active than, the wild type against certain activated cephalosporins, the so-called third-generation antibiotics that also contain β-lactam rings; in essence, says Sigal, "we've changed the specificity of the enzyme." And finally, Sigal has found that the mutant enzyme appears to be more resistant to proteases that destroy activity by breaking the enzyme down; this suggests that the mutant is more stable thermally.

A team headed by Joseph Kraut of the University of California, San Diego, and John Abelson of the California Institute of Technology, reported in *Science* last year (18 November, p. 782) on three separate mutations of the enzyme dihydrofolate reductase (DHFR) performed at the Agouron Institute. This enzyme catalyzes the reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate, which plays an important role as a carrier of one-carbon units in the biosynthesis of nucleotides and certain amino acids.

Aspartic acid-27 of DHFR is buried below the enzyme surface, sequestered from external solvent and poised to form a hydrogen-bonded salt linkage with the pteridine ring of the substrate. It seems to serve as a proton donor, and the negatively charged carboxylate thus formed could serve to stabilize the transition state by hydrogen bonding with the resultant positively charged pteridine ring. To test this, the group converted the residue to asparagine, which leaves the geometry at the site unchanged but eliminates hydrogen bonding and formation of a cation. The mutant enzyme has only 0.1 percent of the activity of the normal enzyme, indicating that this hypothesis is correct.

The second mutation was designed to study conformation rather than mechanism. DHFR contains no disulfide bridges, but the group speculated that one could form with cysteine-85 if proline-39 were converted to a cysteine. This change was made, and the reduced form of the enzyme (with no disulfide) showed normal activity. When the enzyme was oxidized to form a disulfide, activity was significantly reduced, presumably from "a loss of dynamic flexibility in the molecule when the alpha E helix is tethered by a disulfide to the beta B strand."

It is known that all DHFR enzymes studied to date contain a glycine-glycine dyad at positions 95 and 96. Investigators believe that this dyad plays some role in the working of the molecule, possibly as a conformational switch of some kind. For their third mutation, the group thus decided to replace glycine-95 with alanine. This mutation completely inactivated the enzyme. The mutant protein also had a lower mobility on nondenaturing gels, which suggests that a change in conformation has occurred.




***Dihydrofolate reductase***

*Ribbon representation of the* E. coli *DHFR molecule showing the locations of three mutations made at the Agouron Institute. The upper circle shows the residue 39 mutation of proline to cysteine, the middle circle the residue 95 mutation of glycine to alanine, and the lower circle the residue 27 mutation of aspartic acid to asparagine. Beta strands (arrows) and alpha helices are labeled. The approximate position of every tenth residue is indicated. A bound methotrexate molecule is depicted in the enzyme active site, but the cofactor NADPH is not.*

Glutamic acid-139 of DHFR has been replaced with lysine by Stephen Benkovic, Robert Matthews, and C.-P. D. Tu of Pennsylvania State University. X-ray structures show that glutamic acid-139 may form a salt bridge with histidine-141 which may help stabilize the beta structure in that region; the lysine would not form a salt bridge. The group will report soon that the lysine substitution decreases the stability of the enzyme-substrate complex by 3 to 4 kilocalories per mole, but that the catalytic activity of the enzyme is the same. "This is our first evidence," says Benkovic, "that we can uncouple structural stability and catalytic activity."

Many investigators are attempting to perform similar studies with these and other enzymes, but none of these studies is as far along—or, in the case of some industrial laboratories, the results have not yet been revealed. In several cases, the groups are having a hard time getting the new proteins produced in sufficient amounts for study. Despite the fact that the work has become routine, adds Benkovic, "time and material and cost add up quickly." Furthermore, notes Ulmer, no one has yet "closed the loop" by producing x-ray crystallographic structures for a mutant enzyme, so that some conclusions about structural changes must remain speculations. Nonetheless, the results to date show that the concept of altering enzymes in a rational manner is a viable one and suggest that much greater achievements shall be possible in the future—**THOMAS H. MAUGH II**