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Tinkering with Enzymes: What Are We Learning?

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It is now possible, by site-directed mutagenesis of the gene, to change any amino acid residue in a protein to any other. In enzymology, application of this technique is leading to exciting new insights both into the mechanism of catalysis by particular enzymes, and into the basis of catalysis itself. The precise and often delicate changes that are being made in and near the active sites of enzymes are illuminating the interdependent roles of catalytic groups, and are allowing the first steps to be taken toward the rational alteration of enzyme specificity and reactivity.

THE DEVELOPMENTS IN MOLECULAR BIOLOGY OF THE PAST few years have created the opportunity to change essentially any amino acid in any protein (*1*). To mechanistic enzymologists, interested in the origins of the formidable catalytic efficiency of enzymes, this opportunity is proving irresistible. Yet where is the resulting flood of new work leading us? Are we being starry-eyed to allow that "the ultimate goal is to design tailor-made enzymes for every reaction. . ." (*2*)? Are we illuminating existing problems, or merely creating a large number of new ones? There are perhaps 10^6

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functionally different enzymes extant: will it help to make this number much larger? We have long been able to synthesize unnatural substrates; will it be more useful now to synthesize unnatural enzymes? Is the act of replacing one amino acid by another using site-directed mutagenesis any more informative than that of modifying proteins by chemical reagents, which enzymologists have done for decades? In the old days, the chemical modification of enzymes was sometimes likened to an effort to understand the workings of a motor car by studying the effect of firing a shell through the engine compartment. Do today's sophisticated new approaches amount to any more than trying to solve the same problem by carefully fitting one square wheel? For, as will become apparent, most modifications to proteins are either silent (having no effect on catalytic function) or deleterious (producing a catalyst of lesser effectiveness), and one might wonder whether even site-directed damage to an enzyme can be informative.

Happily, the situation is far from being as depressing as the above questions might suggest. Exciting new insights into the nature of enzyme catalysis *are* emerging: they depend entirely on the new skills of DNA surgery, and will surely lead to the just (as distinct from the simply hopeful) use of the phrase "protein engineering" (*3*). Even "gee-whiz mutagenesis" (the change-it-and-see-what-happens approach) is producing results that are aiding our understanding. For although we have known for some time about the principles that govern enzyme catalysis, the assessment of how those principles apply to particular enzymes has remained elusive. As with any new method, the biggest danger is in overinterpretation, and the only cloud in an otherwise invitingly clear sky is that mutations are easy to make, but the mechanistic and catalytic consequences of those changes are usually laborious to evaluate.

In this article, I first describe the types of change that we can produce in an enzyme protein, then discuss the effects on the protein anatomy (that is, on structure and stability), and finally look at the consequences on protein physiology (that is, the effects on enzyme specificity and mechanism that illuminate the question of catalysis). My focus here is on catalysis at active sites: such important areas as enzyme regulation and subunit cooperativity are neglected only for want of space.

Types of Change

Although some fascinating information is coming from studies where major surgery at the DNA level is practiced and whole domains of the enzyme are deleted or switched (*4*), I will limit the discussion here to changes in just one or two amino acids in the protein. By simple site-directed mutagenesis, the range of modifications is constrained, of course, to the "other" 19 natural amino acids. How useful a repertoire is this? For most enzymological purposes, there are only two kinds of alteration that one strives for: (i) replacement of one amino acid by an isosteric residue of different function (for example, Asp by Asn, a replacement that is beautifully isosteric, maintains most of the hydrogen bonding characteristics, but removes the charge), or (ii) replacement of one amino acid by another of identical function but different structure (for example, Glu by Asp, where—if one were fortunate enough that no consequential distortions were propagated through the protein—a putatively functional carboxyl group would be "moved" by something less than 1 Å). The first type of change probes function while keeping structure constant, and the second type looks narrowly at the dependence of function on structure. Truly isosteric changes are few (for example, Glu to Gln, or Asp to Asn), though there are several alterations (for example, Cys to Ser, or His to Asn), that are reasonably conservative in space-filling terms. But the range is not large, and the importance of evaluating the structural integrity of mutant enzymes cannot be overemphasized. One can, in general, try to avoid major structural disruptions by opting always for a smaller amino acid. (This is something that chemical modification can only very rarely achieve: essentially all chemical treatments increase the bulk of the modified residue.) But even so, we cannot presume that such changes are not disruptive, and there are certainly no rules yet available to guide us.

The most thorough studies on the structural integrity of mutant enzymes have been done by Kraut and his group, who have, for example, evaluated crystallographically the D27N and D27S mutants [for the identification of this shorthand, see (*5*)] of dihydrofolate reductase (*6*). At the impressive resolution of 1.9 Å, the clearest structural changes other than those at the substitution site are in the positions of two water molecules nearby, and one may be encouraged by the fact that, in these cases at least, structural distortions are not detectably propagated to other parts of the protein. In a related study, however, the temperature factors of a mutant reductase that contains a new disulfide bridge between residues 39 and 85 suggest that the cross-link actually loosens the structure near to the new bond (*7*). Much caution is evidently needed in predicting the structural consequences of changing any amino acid in a protein.

Efforts to delineate the structural integrity of mutant enzymes are helping to evaluate the usefulness of the structural methods themselves. Thus Gerlt and his collaborators have shown that changing a glutamate (E43) at the active site of staphylococcal nuclease to aspartate reduces the activity by 1300-fold, and results in changes in the ^{1}H NMR of (amongst other things) two upfield methyl groups that are close (from NOESY spectra of specifically deuterated mutant enzyme) to phenylalanine residues (*8*). The most likely candidate methyl groups are about 25 Å away from the active site E43D change (which is, indeed, about as far away as they could be in this rather small protein). There is no discernible difference in the crystal structures of mutant and wild-type enzymes at 3 Å resolution other than at the active site, and these results require that we define (or redefine) what we mean by a subtle structural change. Is this methyl group movement (of perhaps 0.5 Å relative to a Phe residue) subtle and significant, or is it functionally unimportant? Only when all structural methods are pushed to their limits shall we be able to begin to answer such questions.

Enzymologists would most like, of course, to make changes of a delicacy that is not offered by the 20 natural amino acids. We should like to insert, for example, histidine that contains pyrazole in place of imidazole, norleucine in place of methionine, or 4-fluoroglutamate to perturb the pK_a of the side-chain carboxyl group, each at a unique position in a protein sequence. The present methods do not allow this, for even if the unnatural amino acid were to fool the specificity sieves of the relevant transfer RNA (tRNA) synthetase, incorporation into protein would be ubiquitous. Perhaps the methods being developed by Hecht and his group [in which a purified and chemically mischarged suppressor tRNA is added to an in vitro translation system containing the desired gene having the cognate nonsense codon at the site of interest (*9*)] can be persuaded to produce mechanistically useful amounts of modified protein. But there is some way to go, and for the moment, we must accept the more limited range of the natural amino acids (*10*).

Protein Anatomy

The nature of enzyme catalysis cannot be divorced from the question of protein folding and structure, since the forces that maintain the three-dimensional structure of a protein are the same as those that bind substrates and transition states to enzyme active

sites. Site-directed mutagenesis is making powerful contributions in this area, and our understanding of the nature of hydrogen bonds, electrostatic interactions, and disulfide bridges is already deeper than it was 5 years ago. Fersht's group has made a careful analysis of the role of complementary hydrogen bonding in determining the specificity of tyrosyl-tRNA synthetase (*11*), and has concluded that hydrogen bonds between uncharged partners are worth 0.5 to 1.5 kcal/mol, whereas if either the donor or the acceptor is charged, the hydrogen bond is some 3 kcal/mol stronger. These rules of thumb, along with the timely reminder (*11*) that in assessing the net energetic contribution of a hydrogen bond one must consider the interaction of each noninteracting site with solvent and of "liberated" solvent with itself, will prove increasingly useful in model-building (not only of enzyme:substrate complexes, but of all kinds of protein:ligand interactions), in energy minimization (the application of which, we must hope, will ultimately allow the precise prediction of the structures of mutant proteins), and in the design of receptor sites and of ligands to fill them.

To probe the nature of electrostatic interactions, the effects of new surface charges (inserted at loci that are presumed to be relatively innocuous in terms of perturbing the protein structure) on the *p*H-dependence (*12*) and kinetics (*13*) of subtilisin have been studied. The conclusion is that the apparent dielectric constant between point charges (a parameter that includes contributions from unusual water structure) is around 40 to 50 at low ionic strength, whether the electrostatic interaction is predominantly through protein itself or through the hydrating water near the protein surface. The free energy contribution to an enzyme-substrate charge:charge interaction could then be about 1.5 to 2.5 kcal/mol. We must expect, however, that charge burial will increase the interaction energy by lowering the apparent dielectric constant, and these early data are simply the first steps in the analysis of electrostatic interactions.

We were all brought up to believe that disulfide bonds are stabilizing devices [contributing as much as 7.5 kcal/mol (*14*)] that increase the resistance of proteins to thermal denaturation in the more rugged environment outside the cell. This belief is now being tested, as disulfide bonds are being introduced into several proteins [for example, subtilisin (*15, 16*), T4 lysozyme (*17*), and dihydrofolate reductase (*7*)]. Opinions still differ both about the contribution of disulfide bridges to thermal stability and to protease and denaturant resistance, and about the need for precisely controlling disulfide dihedral angles and side-chain packing in the protein interior. On the one hand it has been suggested that only relaxed, exactly positioned disulfides will provide useful stabilization (*18*), yet on the other hand it has been observed that minor deviations from ideal dihedral angles can be accommodated by small shifts in the positions of the participating main chains (*16*). We must not be surprised that both more and less (thermally) stable proteins have been produced. The jury on the disulfide issue is still out, and is likely to remain so until we have a better view of all the contributions to the configurational energy of a folded protein molecule.

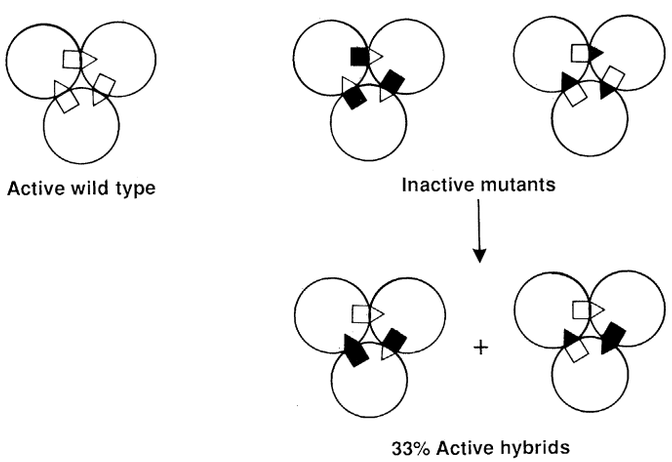

Fig. 1. Regeneration of enzyme activity from hybrid trimers of aspartate transcarbamoylase. Inactive homotrimers from site-directed damage to the active site are illustrated by filled segments. Only those active sites comprising two open segments are active.

The existence of unusually stable proteins, often isolated from thermophilic organisms, has fueled the search for the origins of thermostability. Unfortunately, proteins isolated from mesophiles differ at many places from their counterparts from thermophiles, and our ability to assess all the contributions to protein stability has been much too crude to separate out those residues that stabilize from those that are merely a consequence of genetic drift. Now, however, the phenomenon can be investigated directly. Transformation of an essential gene from a mesophile into a thermophilic host and selection for increasing stability of the target protein should allow one to follow routes to increased protein stability. In a different approach, Shortle and Lin (*19*) have found that a number of mutations producing low-activity staphylococcal nucleases where the alterations are remote from the active site can be suppressed at one or more of only three second sites. These three suppressors have the property of "global" suppression of many different alleles, and it has been conjectured that the suppressors act by increasing the conformational stability of the protein as a whole. It may therefore be that a perturbation at one site in a protein can be compensated for by a substitution quite remote from the original lesion. In general, there is no doubt that the rapidly increasing number of point mutants of proteins with known crystal structures will be recruited into studies of protein folding and stability [see, for example, (*20*)]. Finally, it should be noted that protein stability is more than a matter of commerce (*21*) or convenience (*22*). For example, triosephosphate isomerase deficiency in humans results in hemolytic anemia and neuromuscular dysfunction, and some homozygous individuals have only a small fraction of the normal isomerase activity, all of which is thermolabile. Daar *et al.* (*23*) have recently identified the allele as an E104D change, which from the known crystal structure appears to disrupt a network of charges and hydrogen bonds in an otherwise hydrophobic pocket of the native enzyme.

Apart from the physical changes that accompany thermal unfolding and denaturation, there are several irreversible chemical processes that destroy the integrity of a protein molecule. These reactions include (*24*) the hydrolysis or rearrangement of aspartyl peptide bonds, the deamidation of asparagine and glutamine, the β-elimination of cystine, and the oxidation of methionine to the sulfoxide. In an effort to eliminate the possibility of oxidative inactivation of subtilisin, which takes the susceptible methionine (M222) to the sulfoxide, all of the other 19 amino acids have been tried at this position (*21*). While not all the catalytic consequences of these changes were entirely expected, the resistance to inactivation by H_2O_2 was as predicted. The M222A mutant, for example, is completely resistant to oxidative damage and has a catalytic constant, k_{cat}, that is 80% that of the wild-type enzyme (*21*). In a second example, the groups of Klibanov and Petsko have examined the effect of altering an asparagine residue (N78) that lies at the subunit interface of triosephosphate isomerase. The monomers of this dimeric enzyme are inactive, and replacement of N78 by aspartate does indeed reduce the thermal stability of the enzyme (*25*). It is interesting that an isoleucine at this position (as N78I) stabilizes the enzyme, and the half-life at 100°C and *p*H 6 is nearly twice that of the wild type (*26*). These results suggest that the N78I mutant, which cannot deamidate to give the labile N78D enzyme,

gains in stability by avoiding this route of chemical degradation.

Site-directed mutagenesis has been nicely used to test the view that monomeric aspartate transcarbamoylase is inactive simply because the active site is made up of residues from two adjacent subunits of the catalytically active trimer. Schachman and his group (*27*) produced hybrid trimeric species from one inactive $(S52H)_3$ trimer and another inactive $(K84Q)_3$ trimer. The purified hybrid 2:1 and 1:2 trimers [that is, $(S52H)_2(K84Q)_1$ and $(S52H)_1(K84Q)_2$] were 10^5 times more active than either of the parental mutant proteins, and the activities were close to the 33% (of wild-type activity) expected for the shared active site hypothesis (see Fig. 1).

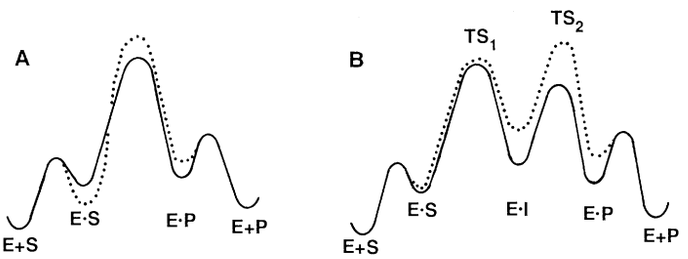

Fig. 2. Free energy profiles for one-step and two-step reactions. (**A**) The interconversion of S and P is catalyzed by the enzyme, E, in a single step the transition state of which is rate limiting. The profile for a slower mutant enzyme is shown by the dotted line. (**B**) The interconversion of S and P proceeds via the formation of an enzyme-bound intermediate, I. The profile for a slower mutant enzyme is shown by the dotted line, and the change in the rate-limiting transition state from TS_1 (solid line) to TS_2 (dotted line), is apparent.

Protein Physiology

The heart of enzymology lies, of course, in catalysis, and an understanding of how enzymes achieve their extraordinary rate accelerations while maintaining exquisite substrate specificity is enzymology's challenge for the physical organic chemist. Here, site-directed mutagenesis is providing splendidly unambiguous information about substrate specificity, the role of particular amino acid residues in the catalyzed reaction, the nature of intermediates, and the energetic basis of catalysis itself. Yet it is misleading to discuss each of these areas in isolation, as has been past practice, without the new wisdom that site-directed changes have already brought to the field. Historically, enzymologists have tended to use two languages, switching from one to the other depending on what was being discussed. In one language, the mechanism of the catalyzed reaction was described in terms of the sequence of steps leading from substrate to product. High-energy reaction intermediates such as enolates, carbanions, and radicals, as well as lower energy covalent intermediate states such as imines and acyl enzymes, were defined and characterized, and roles for amino acids at the active site (as general bases, general acids, and nucleophiles) were suggested. Frequently, it was presumed that the protein recognition elements that determine substrate specificity are different from the catalytic elements of the active site that are directly involved in the making and breaking of covalent bonds. For example, the serine proteases all possess an identical catalytic apparatus: a triad of residues—Asp-His-Ser—the terminal serine of which attacks the carbonyl group of the scissile peptide, plus a site that stabilizes the developing charge on oxygen as the acyl carbon becomes tetrahedral. Yet these enzymes show individual preferences for the substrate amino acids whose peptide bonds are cleaved. Crystallographic work illuminated the molecular basis for this specificity, and the preference of α-chymotrypsin for hydrophobic side chains, of trypsin for the cationic residues of Arg and Lys, and of elastase for Ala, were all happily accounted for by the nature of the so-called "specificity pocket" of these enzymes. These were tidy (if naïve) days for enzymology, when recognition was one thing and the chemistry of catalysis was another, the latter presumably being made efficient by some unspecified cleverness of Nature in creating super acids, super bases, and super nucleophiles from the modestly reactive building blocks provided by the 20 amino acids. At the other linguistic extreme, enzymologists described the acts of recognition and catalysis together, simply in terms of the total binding energy of substrates, of transition states, and of products. According to such heroes as Haldane (*28*) and Pauling (*28*), catalysis *is* recognition. If the enzyme recognizes and binds the transition state of the reaction more tightly than it binds the substrate or product ground states, the reaction is accelerated and we have a catalyst.

The dichotomy described above has been put into much sharper focus by the first flush of results from site-directed mutagenesis. Neither language is wrong, of course, but we must now be clearer when to speak which. If an active-site amino acid is changed, we may alter the chemical mechanism (that is, the path taken, and the sequence of reaction intermediates involved), and we shall probably affect the energetics of that path. To make any sensible statement about the effect of the substitution on the rate, we must be sure that neither the reaction mechanism nor the rate-limiting transition state has changed. [If the substitution has a large effect on the reaction rate, the transition state of the rate-limiting step will not, of course, have exactly the same structure as before, but the point here is that the rate-limiting transition state for each of the enzymes being compared must be that of the same elementary step.] For some enzyme reactions [for example, reactions involving displacements at phosphate esters (*29*) that proceed by associative S_N2-like transition states], there is only one transition state in which chemical changes occur, and provided that the chemistry is not so fast that substrate or product "off" steps are rate limiting, all mutations that affect the rate will relate to that single chemical event (see, for example, Fig. 2A). The encompassing contributions of Fersht and his group (*2, 30*) on tyrosyl-tRNA synthetase fall into this class, and this system seems to be uncomplicated by enzymatic acids, bases, and nucleophiles. For most enzyme-catalyzed reactions, however, there are real dangers that amino acid substitutions at the active site will slow the rate of a step that in the wild type is not, or is only partially, rate limiting (see, for example, Fig. 2B). Indeed, there are persuasive arguments that efficient multistep enzyme reactions have several transition states of roughly comparable free energy (*31*), which makes it especially important that the kinetic consequences of any mutation be properly evaluated.

With this background, let us select examples where site-directed changes have illuminated questions of enzyme specificity, of the chemical role of individual amino acids, of the nature of reaction intermediates, and, finally, of the energetics of catalysis.

Enzyme Specificity

The gratifying correlation between the observed kinetic substrate preferences of the serine proteases and the structures of their specificity pockets from crystallographic studies made these enzymes obvious candidates for early attempts to manipulate enzyme specificity. Thus the specificity pocket in trypsin has glycines (G216 and G226) in the middle, and aspartate (D189) at the bottom, and accommodates the side chains of substrate lysyl or arginyl residues. Craik *et al.* (*32*) made G216A and G226A mutants in the hope of producing a trypsin that could better discriminate between lysyl and

arginyl substrates. Surprisingly both mutants were much less active than the wild type, and although some shift in the specificity was observed the dominant effects were on k_{cat} and not on the Michaelis constant, K_m. These results emphasize the problems raised above, that even if it seems that recognition and catalysis are determined by different parts of the active site, the functional separation may be poor, and the structural consequences of rather minor changes in the pocket may be unexpected. Recent efforts to reverse the charge preference of trypsin by exchanging the pocket's aspartate for lysine (D189K), in the hope of producing a trypsin now selective for aspartyl and glutamyl peptides, has again led to a surprising result: of a protein that prefers neutral substrates (*33*). This finding can be rationalized (albeit post hoc) by the suggestion that the new lysine side chain may extend through the wall of the pocket and form hydrogen bonds to various acceptors outside it.

Subtilisin has been subjected to extensive modification in its specificity pocket by the Genentech-Genencor group (*13, 34*) and the mutant enzymes have been tested against a range of substrates. The results are encouraging, in the sense that the specificity trends are more or less as expected. The effects on overall catalytic activity are not, however, intuitive, and the k_{cat} values vary independently of and as dramatically as the K_m values. More detailed structural and kinetic work will be needed before we properly understand these data at the molecular level.

In an analogous study, Kirsch and his group (*35*) have successfully reversed the substrate charge preference of aspartate transaminase, by an R292D mutation of the arginine residue that appears to bind the side-chain carboxylate group of substrate aspartate. Once again, although the specificity has been reversed (the substrate preference, Asp/Arg, for the wild-type enzyme is 6×10^5, and for the R292D mutant is 0.2), there is a precipitous fall in k_{cat}/K_m (wild-type enzyme with substrate Asp: $18{,}500 M^{-1}$ sec^{-1}; mutant enzyme with substrate Arg $0.4 M^{-1}$ sec^{-1}). The message seems clear: it may often be impossible to change one part of an enzyme's machinery without risking catastrophic consequences elsewhere. Yet the continuing quest for an understanding of the interdependence of specificity and activity, given such dramatic examples as those quoted, will undoubtedly improve our ability to tinker more effectively. For example, might it be possible to improve the ratio of carboxylase to oxygenase activity in ribulose 1,5-bisphosphate carboxylase/oxygenase, and reduce, in a plant, wasteful photorespiration (*36*)?

The Role of Particular Residues

Before the advent of site-directed mutations, the role of particular amino acid residues was deduced either from chemical modification experiments (since modification of X destroys the activity, X must be functionally essential) or from crystallographic results (since X is at the active site, X must be important). The looseness of these deductive statements was clear, of course, but aside from the use of mechanism-based enzyme inhibitors, there was little that could be done to tighten the logic. Now, however, the validity of an increasing number of such conclusions is being tested. The list of active-site amino acids that have been changed is already long, and in what follows, I shall simply mention some of the more startling or more gratifying results, as well as some of those that illustrate general principles.

Carboxypeptidase A is a zinc-dependent exopeptidase, chemical modification of which had strongly suggested that a tyrosine (Y248) is essential for the enzyme-catalyzed cleavage of peptide substrates. In other modification work, a second tyrosine (Y198) had also been implicated, and the proximity of both of these residues (especially Y248) to the active site, shown crystallographically, served strongly to buttress the presumption that each of the phenolic hydroxyl groups had some role in the peptidase action of the enzyme. The substantial literature on this subject has now been clarified: when the hydroxyl group of tyrosine 248 is removed (in Y248F), the k_{cat} values for both ester and peptide substrates are not seriously affected over a wide *p*H range (*37*). Replacement of the other implicated tyrosine by phenylalanine (in Y198F) is also without dramatic effect, and even the double mutant Y198F,Y248F has a k_{cat} that is 25% that of the wild type (*37*). Here one sees a real advantage of site-directed mutagenesis over chemical modification: the modified residue can be both smaller and nonfunctional, thus minimizing irrelevant and misleading effects on the enzyme's activity (*38*).

The alteration of amino acid side chains that seem to have a clear mechanistic role is being used to provide estimates of the contribution of a particular feature to the overall catalytic power of the enzyme. Thus the removal of one of the two groups in subtilisin (N155) that are believed to stabilize the developing substrate oxyanion in the transition state as the tetrahedral intermediate forms (or collapses), reduces k_{cat} by 200- to 4000-fold, depending on the substitution. The value of K_m is relatively unaffected, and the results are entirely in accord with the proposed stabilization of the nascent oxyanion (*39*). In other experiments, replacement of the aspartate in trypsin's catalytic triad by asparagine (as D102N) cuts k_{cat} by about 10^4-fold (*40*). Such data are satisfying, and confirm particular contributions to the catalyzed reaction, and it is tempting to imagine that one is dissecting the overall rate enhancement into its components. This is usually false: the elements of catalysis (for example, general base, or electrophilic) are unlikely to be independent of one another, even in the absence of a change in rate-limiting step. Such presumptions are as unhelpful as the suggestion that a person with one leg can run half as fast as someone with two.

In an attempt to delineate the role of histidine residues, it has been

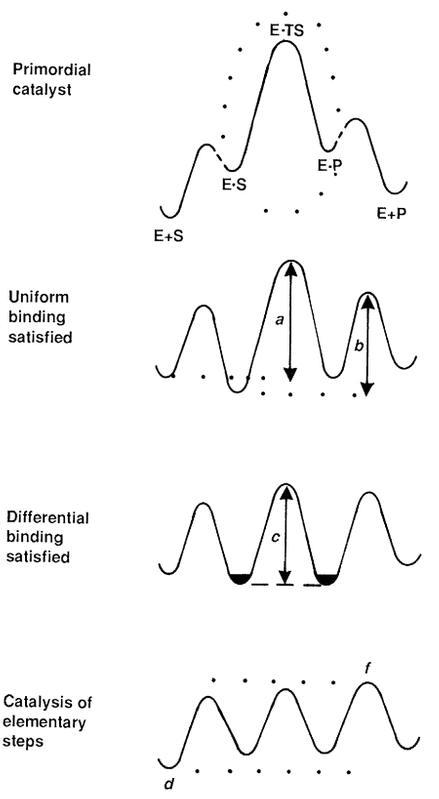

Fig. 3. Free energy profiles illustrating the three types of binding energy. Uniform binding does not discriminate amongst S, TS, and P, and all the internal states (enclosed in the dotted egg) are bound more or less tightly to the enzyme. At the optimum, $a = b$. Differential binding discriminates between S and P, and equalizes the internal thermodynamics so that the free energies of E·S and E·P are equal, resulting in a lower energy barrier (c). Finally, the enzyme discriminates between S and TS, and reduces the barrier c. The lowest free energy profile is that for a "perfect" enzyme, where in the downhill direction (right to left) the diffusive transition state f is the highest barrier, and in the uphill direction (left to right) no intermediate accumulates because at the ambient concentration of S, d has the lowest free energy.

suggested that asparagine and glutamine can mimic the hydrogen-bonding capability of the δ and ε nitrogens of histidine, respectively. In tyrosyl-tRNA synthetase, H48N is as active as the wild-type enzyme yet H48Q is much less active, suggesting both that H48 is not involved in any electrostatic interactions and that its δ nitrogen is responsible for hydrogen bonding to substrate (*41*).

It has been known for many years that the pyridoxal phosphate–dependent enzymes of amino acid metabolism bind the cofactor as an aldimine by using a unique lysine residue. In the case of the aspartate transaminase from *Escherichia coli*, this residue (K258) has been replaced by alanine, and the mutant enzyme in the pyridoxal form is found not to react with either of the amino acid substrates, alanine or aspartate (*42*). Yet the pyridoxamine form of the mutant reacts with oxaloacetate to yield stoichiometric levels of the tightly bound aldimine of alanine with pyridoxal phosphate! The protein appears to have become a (slow) decarboxylase. Evidently the critical lysine K258 acts both to form the aldimine with pyridoxal phosphate and to mediate the necessary azallylic proton shifts in the aldimine-ketimine interconversion (if this were not so, we should expect the normal reaction, the enzyme having successfully formed the ketimine with oxaloacetate). The accumulation of the alanine aldimine from the oxaloacetate direction but not from the alanine direction is an unexpected bonus, and supports the view that transaldimination is (despite not infrequent doubts) a critical kinetic feature of pyridoxal phosphate–dependent enzymes.

Whenever a putatively functional amino acid at the active site of an enzyme is changed, there is the fear that the enzyme may not have retained its mechanistic integrity. When we "moved" the active-site carboxyl group in triosephosphate isomerase (which is the base responsible for abstracting the enolic proton from the substrates) by substituting aspartate for glutamate (in E165D), was it possible that the space created could be filled by a water molecule and the proton abstraction was now mediated by the intervening water (*43*)? When Kaiser's group changed the critical serine that forms the phospho-enzyme intermediate in alkaline phosphatase to a cysteine (in S102C), was it possible that the mechanism changed to one in which a zinc-bound water molecule was now the nucleophile (*44*)? Clearly such questions must be answered before any mechanistic comparisons can be made between mutant enzymes and their parental wild types. In these and other cases [for example, see (*45*)], in fact, the chemical mechanisms remained unaltered, though the rate-limiting transition state was changed. In the case of triosephosphate isomerase, the reaction of wild-type enzyme is limited by the rate of substrate diffusion to the active site, whereas in the E165D mutant the transition state of highest free energy becomes that of a chemical enolization step.

A final example of how amino acid substitution can aid the understanding of mechanism is the game of "hunt the radical" in cytochrome c peroxidase. This enzyme reacts with H_2O_2 to yield a ferryl-heme and a radical species that is believed to be located on an amino acid residue. Amongst several possibilities for the guilty amino acid, a tryptophan (W51) and a methionine (M172) seemed likely candidates. Each of these has, however, been ruled out as the primary center by the study of the mutants W51F (*46*) and M172S (*47*). Although the search is still on, it is hard to imagine a more unambiguous way to solve the problem.

The Energetics of Catalysis

The rate enhancement mediated by an enzyme derives from, and can economically be described by, three types of binding interaction between the enzyme and its substrates. We have earlier (*31*) termed these three classes: *uniform binding* (where, for the simple reaction

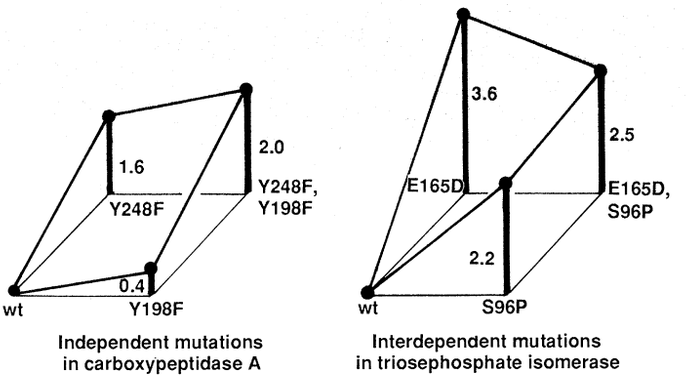

Fig. 4. Examples of independent and interdependent mutations. The kinetic effects of the indicated substitutions on k_{cat}/K_m are plotted vertically as values of $\delta\Delta G^{\ddagger}$. The substitutions in carboxypeptidase A are independent and additive, and the four points lie on a plane. This is not true for the interdependent substitutions in triosephosphate isomerase.

illustrated in Fig. 3, the enzyme does not discriminate amongst substrate (S), transition state (TS), and product (P), but merely binds all of them more or less strongly); *differential binding* (where the enzyme now recognizes the difference between S and P, and binds one more tightly than the other); and *catalysis of elementary steps* (where the enzyme is at its most discriminating, and binds TS more strongly than S or P).

The first type of binding interaction, uniform binding, optimizes the free energy of the bound states (E·S, E·TS, and E·P) en bloc with respect to the external states (E + S and E + P). An optimum exists, because if all substrate species (S, TS, and P) bind only weakly there is a large free energy barrier for passage over TS (*a* is large: Fig. 3), whereas if all substrate species bind very strongly the enzyme becomes tied up in a liganded free-energy well from which escape is slow (*b* is large: Fig. 3). The optimum situation is when the uniform binding condition is satisfied ($a = b$). The profiles in Fig. 3 must be drawn using the actual in vivo substrate level as the standard state, and it is clear that enzymes are optimized with respect to the ambient substrate levels that they experience. There are now several enzymes in which amino acid substitutions have resulted in uniform binding changes (*48, 49*), both demonstrating the feasibility of adjusting all the bound states stimultaneously, and, of more pragmatic interest, producing mutant enzymes that are better fitted for nonphysiological substrate concentrations (*50*).

The second type of binding interaction, differential binding, discriminates between S and P, and, for enzymes that maintain their substrates at equilibrium in vivo (*51*), we have argued (*31*) that catalytic efficiency is maximized when the free energy levels of the E·S and E·P complexes are equal (that is, when the equilibrium constant for the bound species, K_{int}, is near unity: see Fig. 3). An increasing number of such systems appears to follow this prediction [for example, see (*29*)]. Tyrosyl-tRNA synthetase has recently joined these ranks (*52*), and the overall equilibrium constant for the amino acid activation reaction (tyrosine + ATP $\rightleftharpoons$ tyrosyl-adenylate + PP_i) moves from a very unfavorable 3×10^{-7} for the free substrates to a value of 2.3 for the enzyme-bound species. Our original argument, that optimized enzymes would have values of K_{int} near unity, depended on the presumption that there would be a free energy relationship (*53*) linking thermodynamic changes in E·S and E·P with concomitant kinetic changes in the activation barrier between them. Recent work has shown that such linear free energy relationships are followed when amino acid substitutions are made that do not lead to propagated structural disruptions, and that are not, for instance, uniquely involved in the binding of TS as distinct

from S or P (*54*). The existence of these Brønsted-type correlations is persuasive evidence that the mutations considered are indeed nondisruptive, and that, provided the earlier caveats are heeded (so that only changes in the same elementary step are correlated, see Fig. 2A), enzymes do follow the expectations of classical physical-organic chemistry. It has correctly been noted, however (*54*), that such correlations should only be made in the knowledge of both structural and detailed kinetic information, and with extensive sets of data for mutants. Thus even if the complexities of the situation illustrated in Fig. 2B are avoided, the question of whether the effect of a given amino acid substitution is localized and independent of other changes must be faced. For limited data sets, the independence of two different amino acid substitutions can be established by making the double mutant. When the kinetic effects of the two mutations (separately and together) are plotted as in Fig. 4, one can see that for independent mutants the four points lie in a plane, whereas for interdependent changes no plane can be drawn.

The third type of binding interaction is the most discriminating and involves the preferential binding of TS rather than S or P. This is equivalent to the Pauling view (*28*), and it leads directly to the acceleration of an elementary step in the catalyzed reaction. Examples where single amino acid substitutions have this consequence have been found in several systems (*43, 55, 56*). These examples emphasize how enzymes can recognize the geometric and electronic differences between ground states and transition states, and can successfully recruit amino acid side chains for the selective stabilization of the latter. In staphylococcal nuclease, for example, an active-site arginine appears not to affect the binding of a substrate analogue (in R87G), yet the k_{cat} for DNA hydrolysis by this mutant is reduced by 10^5-fold (*56*). The methods of site-directed mutagenesis have thus gratifyingly provided examples of each of the three kinds of binding interaction discussed above, and the study of these mutants has sharply focused our views about how catalytic efficiency and substrate specificity are achieved.

Conclusion

It is clear that, for some enzymes at least, the evidently additive and independent contributions of different amino acid residues to the overall catalysis can be delineated. In other systems, however, independence of substitutions and nondisruptive mutations may prove difficult or impossible to achieve, even if the issues of changes in mechanistic pathway or in the rate-limiting transition state are properly resolved. But the field is very young, and undoubtedly there will be massive contributions from directed mutagenesis to both of the central problems of protein chemistry, folding and catalysis, by the continued tinkering in and around the active sites of enzymes.

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3. I have argued in vain against the premature use of the word engineering, on the grounds that an engineer knows what he is doing when he designs a bridge, whereas the protein chemist has yet to define the equivalents of Hooke's law, Young's modulus, and the rest.
4. Two pretty examples of such experiments will suffice. Mas and her collaborators [M. T. Mas, C. Y. Chen, R. A. Hitzeman, A. D. Riggs, *Science* **233**, 788 (1986)] have produced chimeric phosphoglycerate kinases that contain one domain from the human enzyme and the other from the yeast enzyme. All four species (the two native enzymes and the two chimeras) have roughly the same catalytic activity, despite a 35% difference in amino acid sequence between species. This brave experiment has important ramifications for the nature of the active site cleft and for the mechanism of the domain closure event. In another study, the relation between the penicillin-binding proteins (PBP) that are involved in bacterial cell wall synthesis and the β-lactamases has been probed by J. H. Richard's group [Y-H. Chang, thesis, California Institute of Technology (1987)]. A stretch of 29 amino acid residues of the β-lactamase has been replaced by a weakly homologous sequence of 30 residues from PBP-5. The hybrid protein has less than one thousandth of the β-lactam-cleaving activity of the β-lactamase, but shows a remarkable 2% of the D,D-carboxypeptidase activity of PBP-5, lending support to the view that the D,D-carboxypeptidases and the β-lactamases are evolutionarily related.
5. We follow here the emerging protocol that, with the one-letter amino acid codes, specifies a mutant as D27S if Asp-27 has been changed to Ser. The one-letter amino acid codes are: alanine, A; arginine, R; asparagine, N; aspartate, D; cysteine, C; glutamate, E; glutamine, Q; glycine, G; histidine, H; isoleucine, I; leucine, L; lysine, K; methionine, M; phenylalanine, F; proline, P; serine, S; threonine, T; tryptophan, W; tyrosine, Y; and valine, V.
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57. I gratefully acknowledge the kindness of many discriminating practitioners of site-directed mutagenesis for their permission to cite unpublished work. I also hope for the indulgence of those authors whose work, because of space limitations, has been inadequately adumbrated. Support was provided by the National Institutes of Health and the National Science Foundation.