Relationships between Enzymatic Catalysis and Active Site Structure Revealed by Applications of Site-Directed Mutagenesis

JOHN A. GERLT

Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742, and Center for Advanced Research in Biotechnology, Rockville, Maryland 20850

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Dr. John A. Gerlt is Professor of Chemistry and Biochemistry at the University of Maryland in College Park, MD, and Senior Staff Scientist at the Center for Advanced Research in Biotechnology in Rockville, MD. Dr. Gerlt received his B.S. degree in Biochemistry from Michigan State University in 1969. His graduate research training was obtained in the laboratory of Professor F. H. Westheimer at Harvard University where he received the Ph.D. in Biochemistry and Molecular Biology in 1974. Following a year of postdoctoral study in the laboratory of Dr. Christian B. Anfinsen at the National Institutes of Health in Bethesda, MD, he joined the faculty of the Department of Chemistry at Yale University. In 1984 Dr. Gerlt moved to the University of Maryland. Dr. Gerlt's research interests are in mechanistic enzymology, with his laboratory currently being active in applying the techniques of site-directed mutagenesis to Staphylococcal nuclease as well as investigating the mechanisms of other enzymes including adenylate cyclase and endonucleases involved in DNA repair processes.

A. Introduction

The elucidation of the mechanism of an organic reaction as practiced by physical organic chemists relies upon variation of the structure of the reacting molecules and formulation of a structure–function relationship that explains the observed effect of structure on reaction rates and products.[1] Even the most casual reader of this review is familiar with the use of linear reactivity–free energy correlates in general acid–base catalysis to deduce transition-state structure by assessing the impact of relative acidity or basicity on reaction rates.[2] A major advance in understanding the large rate accelerations characteristic of enzyme-catalyzed reactions was the realization that entropic effects are important in enzymatic catalysis; this conclusion was the result of investigations of the effect of restriction in degrees of translational and rotational freedom on reaction rates.[3-5] The physical organic chemistry approach for elucidating the mechanisms of nonenzymatic reactions has also been applied to the study of mechanisms of enzyme-catalyzed reactions.[6] For example, rational variations in leaving group pK_a have been instrumental in the elucidation of the mechanisms of the serine proteases, where the manipulation of leaving group ability can cause a change in the rate-determining step.[7] Methods of stereochemical analyses developed for studies of nonenzymatic reactions have been used successfully in the study of such enzymatic reactions as alkyl[8] and phosphoryl[9] group transfer. The differing leaving group abilities of fluoride, chloride, and bromide ions are frequently exploited in the design of substrate analogues for enzymatic reactions that can probe the detailed mechanism of the processing of the "normal" substrate[10,11] as well as often alkylate, and thereby implicate in catalysis, functional groups in the active sites of enzymes.[12] And, in the extreme, medicinal chemists have developed approaches for formulation of quantitative structure–activity relationships (QSARs) that allow optimization of the properties of inhibitors of enzyme-catalyzed reactions in terms of the apparent

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steric, lipophilic, and electronic properties of enzyme binding sites for which high-resolution structural information is not available.[13] Thus, the traditional strategies of the physical organic chemist for establishing mechanisms of both nonenzymatic and enzymatic reactions are based upon the study of the reactivities of small organic molecules that can be readily synthesized.

An alternate approach for the elucidation of enzymatic mechanisms is based upon modification of the structures of amino acid functional groups that are present in the active site. Two conceptually similar but experimentally different methods have been used for the specific modification of amino acid functional groups present in active sites. The classical method is the use of chemically reactive small molecules that are designed to react with a restricted class of functional groups, with the discrimination for a specific type of group in the active site being largely determined by the unique chemical reactivities of functional groups as found in the environment of the active site. A large number of reagents are available for the modification of active site thiolate, imidazolate, hydroxylic, phenolic, and carboxylate functional groups, with the success of this approach for uniquely labeling a functional group in the active site being dependent upon the enhanced avidity of such groups to either electrophilic or nucleophilic reagents.[14] This approach has widely recognized interpretative limitations such as lack of absolute specificity for active site functional groups, the uncertain impact of the steric and electrostatic properties of the modified amino acid on the mechanism of the reaction (in terms of both catalysis and binding specificity), and the effect of the modification on the three-dimensional structure of the covalently modified enzyme.

Within the past 5 years, physical organic chemists studying the mechanisms of enzyme-catalyzed reactions have begun to utilize a complementary approach in which amino acid functional groups present in the active site of an enzyme are changed genetically by specific alteration of the codons for these amino acids in a cloned or chemically synthesized gene for the enzyme. The subject of this review is this more recent method based on recombinant DNA techniques commonly referred to as site-directed or site-specific mutagenesis. Although this approach for chemical modification of functional groups in the active site is very specific (limited only by the fidelity of ribosomal protein synthesis) and need not involve the introduction of sterically or electrostatically important additions to the active site (such as is frequently encountered in assessing whether a cysteinyl sulfhydryl group or an aspartyl or glutamyl carboxylate group is required for catalysis), it is limited by both the small number of substitutions possible for an amino acid (19, given the current genetic code) and by the problem of assessing the effect of the amino acid substitution on the three-dimensional structure of the variant enzyme. However, within these constraints, this approach for studying the mechanisms of enzyme-catalyzed reactions is being used in a large number of laboratories, with the hope being that the application of this methodology might allow a better description of the participation of active site functional groups in catalysis than could be achieved either by alteration of substrate structure of chemical modification of these functional groups.

Within the context of studying enzymatic reaction mechanisms by site-directed mutagenesis, this review will focus only on those enzymes for which high-resolution information was available about the three-dimensional structure for the wild type enzyme prior to the initiation of the mutagenesis-based studies and, accordingly, for which high-resolution structural information can be anticipated for mutant enzymes. In these systems, the rationale for the amino acid substitutions that have been created and enzymologically characterized can be clearly described in terms of active site structure. In addition, the enzymes discussed in this review are restricted to those for which sufficient kinetic and/or chemical data have been obtained for both the wild type enzyme and its site-directed mutants to allow at least rudimentary formulation of structure–function relationships. Finally, this review will consider only those enzymological studies performed on site-directed mutants of well-characterized enzymes that pertain to the formulation and examination of structure–function relationships regarding amino acid residues within the active site and will not extend to other investigations of the physical properties of these enzymes; for example, the introduction of cysteine residues by site-directed mutagenesis and the examination of whether these residues will undergo oxidation to cystine residues and thereby alter (increase?) the stabilities of the folded proteins will not be discussed.

The first section will detail the studies that have been performed on enzymes to investigate substrate binding (specificity) and the relationship of binding energy to catalysis (approximation effects) to increase the catalytic efficiency of the enzymatic reaction. The last section will detail the studies that have been performed on enzymes to evaluate the importance of general acid, general base, and/or nucleophilic catalysis to increase catalytic efficiency. The rationale for this organization is that in the former category mutations of active site functional groups may produce a broad spectrum of phenotypic changes, whereas in the latter category mutations of active site functional groups are likely to produce either no effect on catalysis (no participation of the mutated residue in catalysis) or a significant decrease in catalytic activity (*possible* direct involvement of the mutated residue in catalysis). Whereas unequivocal interpretation of the results obtained for either class of enzymatic reaction *requires* knowledge of the impact of the mutated amino acid residue on the three-dimensional structure of the enzyme, the currently available evidence suggests that studies of amino acid residues involved in substrate binding and catalysis by approximation may be more confidently interpreted than studies of amino acid residues that participate directly in catalysis by facilitating the bond-breaking and -making reaction(s); the reason for this belief that interpretation of binding processes is easier than interpretation of bond-breaking processes is that the strengths of the noncovalent interactions between two molecules are likely to be less sensitive to small changes in geometry than the actual reactions leading to formation of covalent bonds.

The recombinant DNA methods that are used to generate site-directed mutants of enzymes were de-

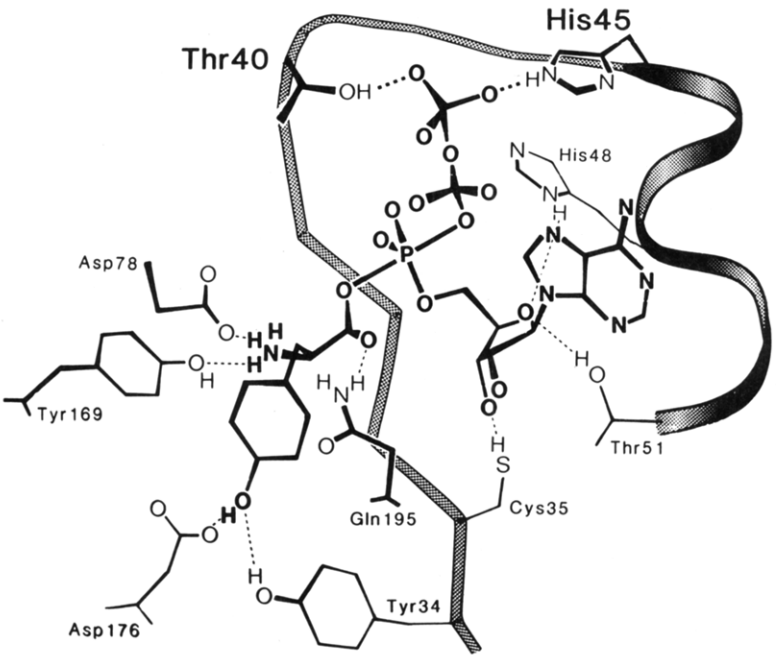

Figure 1. Active site of wild-type tyrosyl tRNA synthetase showing the position derived from model building of the trigonal-bipyramidal intermediate or transition state presumed to lie on the reaction pathway. Reproduced with permission from ref 21. Copyright 1985 Wiley.

scribed recently and will not be discussed in this review.[15–18]

The articles and manuscripts used in writing this review appeared in print or were made available to the author prior to January 1, 1987.

B. Studies of Amino Acid Residues Involved in Substrate Binding and/or in Catalysis by Approximation

1. Tyrosyl tRNA Synthetase from *Bacillus stearothermophilus*

Tyrosyl tRNA synthetase catalyzes two half-reactions that lead to the ATP-dependent aminoacylation of the tRNA for tyrosine ($tRNA^{Tyr}$) by tyrosine. The first half-reaction involves the in-line nucleophilic attack of the carboxylate group of tyrosine on the α-phosphorus of the β,γ-complex of ATP with magnesium to displace magnesium pyrophosphate and generate the enzyme-bound tyrosyl adenylate;[19] the second half-reaction involves the transfer of the tyrosyl moiety from the tyrosyl adenylate to $tRNA^{Tyr}$ to form the aminoacylated $tRNA^{Tyr}$. The reactive and thermodynamically unstable tyrosyl adenylate is not released but is sequestered and stabilized in the active site. The first half-reaction involves no general acid, general base, or nucleophilic catalysis (the carboxylate of tyrosine is fully ionized and magnesium pyrophosphate is a good leaving group) and has been studied by site-directed mutagenesis in Fersht's laboratory.

The tyrosyl tRNA synthetase from *B. stearothermophilus* is a dimeric enzyme that is composed of two identical subunits containing 419 amino acids; the total molecular weight of the dimeric enzyme is 94 600.[20] The synthetase contains two cysteine residues but no disulfide bonds. The three-dimensional structure of the unliganded enzyme has been determined to 2.1-Å resolution in Blow's laboratory.[21] In the presence of tyrosine and ATP, the tyrosyl adenylate is generated and stabilized in the active site, and a 2.7-Å structure of this complex has been determined.[22] Tyrosine binds tightly to the enzyme (K_d = 20 μM), and a 2.7-Å structure of this complex has also been determined;[23] ATP does not bind very tightly to the enzyme (K_m = 3.9 mM), and no definitive structure of ATP in its presumed binding site is available. In the crystal, only the 320-residue domain responsible for the first half-reaction, i.e., generation of the bound tyrosyl adenylate from tyrosine and ATP, is well resolved; the remaining carboxyl-terminal 99 residues are highly disordered. These carbonyl-terminal residues have been genetically deleted, and this truncated enzyme neither binds $tRNA^{Tyr}$ nor transfers tyrosine from the tyrosyl adenylate intermediate to $tRNA^{Tyr}$.[24] Thus, the available crystallographic structural information is adequate for analysis of structure–function relationships involved in the binding of the substrates and generation and stabilization of the tyrosyl adenylate intermediate.

Given the apparent absence of general acid and base catalytic residues in the active site, catalysis of the first half-reaction is the result of specific binding and approximation of the substrates within the active site and the stabilization of both stable enzyme-bound intermediates and the transition states involved in their interconversions. Fersht and his co-workers utilized site-directed mutagenesis to alter the amino acid residues present in the active site that either by direct crystallographic observation or by model building are known or presumed to interact with tyrosine, ATP, and the tyrosyl adenylate. The results of these experiments provide considerable insight into the identification and quantitation of binding interactions and energies in stabilizing both reactive intermediates and transition states.

The X-ray structure of the bound tyrosyl adenylate reveals the presence of eight hydrogen-bonding interactions involving active site functional groups (Figure

1): Cys-35 donates a hydrogen bond to the 3′-hydroxyl group of the adenylate moiety; His-48 and Thr-51 donate hydrogen bonds to the endocyclic oxygen of the adenylate moiety; the amide of Gln-195 donates a hydrogen bond to the carbonyl oxygen of the carboxylate anhydride; Asp-78 is a hydrogen-bond acceptor from and the phenolic hydroxyl of Tyr-169 and the carboxamide oxygen of Gln-173 are hydrogen-bond donors to the α-amino group of the tyrosyl moiety; and Asp-176 is a hydrogen-bond acceptor from and the phenolic hydroxyl of Tyr-34 is a hydrogen-bond donor to the phenolic hydroxyl group of the tyrosyl moiety. Model-building exercises suggest the presence of two additional hydrogen-bond donors to the γ-phosphoryl group of the polyphosphate moiety of ATP during the transition state in the nucleophilic displacement reaction, namely Thr-40 and His-45.[25] Quantitative investigations of the importance of most of the (charge-neutral) hydrogen-bond donors in stabilization of the enzyme-bound substrates and tyrosyl adenylate intermediate and in stabilization of the transition state leading from bound substrates to the tyrosyl adenylate intermediate have been made by site-directed mutagenesis and subsequent kinetic analyses. In particular, the differential effects, if any, of mutations lacking functional groups at these positions on the stabilities of the various enzyme-bound species are used to deduce whether the strength of the interaction between the functional group and the bound species changes as the reaction proceeds from bound substrates to the bound tyrosyl adenylate; in this way, the quantitative importance of the functional group in catalysis might be determined. In addition, attempts to alter (but not eliminate) hydrogen-bonding interactions by systematic variation of the functional group have been made so that the impact of these variations on the stabilities of bound species can be studied.

Cys-35 → Gly (or Ser). Cys-35 is hydrogen bonded to the 3′-hydroxyl group of the tyrosyl adenylate intermediate. Site-directed mutagenesis has been used to introduce glycine and serine substitutions at this position,[26-28] with either substitution giving quantitatively very similar results. The presence of the mutant side chain has an insignificant effect on the affinity of the enzyme for either tyrosine or ATP in contrast to the pronounced decrease in rate of formation of the tyrosyl adenylate intermediate and in the stability of the bound tyrosyl adenylate intermediate; in fact, the largest effect of these mutations is on the stability of the bound tyrosyl intermediate. These observations imply that the side chain of Cys-35 does not interact with the bound ATP substrate but that interaction develops and its magnitude increases as the reaction proceeds. This effect demonstrates that the active site functional groups can distinguish whether the 3′-hydroxyl group of the bound adenylate group is present as substrate, transition state, or product and preferentially stabilize the bound product.

His-48 → Gly. The N-1 tautomer of His-48 is observed to be within hydrogen-bonding distance of the endocyclic ribose oxygen of the bound tyrosyl adenylate intermediate. In this case, this quantitative results imply that His-48 interacts with about ATP (but not bound tyrosine) and that the interaction with the adenylate moiety again increases as the reaction proceeds to bound products.[27,28]

Thr-51 → Ala, Pro, or Cys. Multiple substitutions have been made at residue-51, and the results that have been obtained were the first to illustrate that improvements in the catalytic efficiencies of enzymes (as measured by k_{cat}/K_m) can be achieved by site-directed mutagenesis.[29,30] The crystal structure of the bound tyrosyl adenylate intermediate has been interpreted by Fersht as revealing that the potential hydrogen bond that is formed between the side-chain hydroxyl group of Thr-51 and the endocyclic ribose oxygen is suboptimal, suggesting the possibility that the strength of this hydrogen bond and/or the interaction of the enzyme with the adenylate group can be improved by altering the identity of the amino acid residue at this position. What is particularly striking about these mutants is that improvements in binding have been made both in the presumed formation of a better hydrogen bond between the adenylate group and the enzyme (Cys-51) and the certain loss of ability to form a hydrogen bond between the adenylate group and the enzyme (Ala-51 and Pro-51).

The Cys-51 substitution leads to a uniform increase in binding of all species involving the adenylate moiety of ATP, including the bound substrate in the ternary complex with tyrosine, the transition state in the formation of the tyrosyl adenylate, and the bound product both in the presence and absence of pyrophosphate. This observation suggests that the hydrogen bond from Thr-51 in the wild type enzyme is suboptimal, with the improvement in binding interactions being attributable to either the difference in hydrogen-bond strengths inherent in O–H–O and S–H–O systems and/or in the anticipated changes in hydrogen-bonding geometry. In either case, it is important to realize that the net stabilization free energy due to the occurrence of hydrogen bonds between a enzymatic binding site and its ligand is the composite of the free energies of hydrogen bonds that are present between solvent and both the unoccupied binding site and free ligand *and* the free energy consequences of breaking these interactions and forming the hydrogen bond in the binding site. These interactions involve not only enthalpic considerations but also entropic contributions associated with the release of ordered water molecules, i.e., either hydrogen bonded to enzyme and ligand heteroatoms or located in a hydrophobic pocket, to bulk solvent.[31]

The phenotype of the Ala-51 substitution is somewhat more complex in that the stability of the ternary substrate complex is not altered by the absence of the thiol group, but the transition state as well as the ternary and binary product complexes of the tyrosyl adenylate are stabilized, with the stabilization increasing modestly as the reaction proceeds. The phenotype of the Pro-51 mutant is still more complex but interesting: the stability of the ternary complex is somewhat enhanced by the substitution, but the transition state and both product complexes are significantly stabilized. Again, the phenotypes of both of these mutants reflect the net energetic consequences of hydrogen-bond formation between the enzyme and adenylate group.

Perhaps the most satisfying interpretation of the phenotypes of all of the substitutions made in tyrosyl tRNA synthetase, including those at residue-51, is based

upon the hypothesis of Albery and Knowles that enzymes can accelerate the rates of catalyzed reactions by three fundamental factors:[32] uniform binding of the various intermediates and transition states, differential binding of intermediates and transition states, and catalysis of individual steps (through preferential binding or stabilization of transition states). The Cys-51 substitution is an example of catalysis by uniform binding, since all enzyme-bound species that include the adenylate moiety, including the transition state for the formation of the tyrosyl adenylate intermediate, are stabilized by essentially the same amount of energy. The Ala-51 substitution constitutes an example of differential binding, since the ternary substrate complex is not stabilized but the transition state and product complexes are stabilized; this mutant enzyme can distinguish between the adenylate group in the bound substrate and those present further along the reaction coordinate. The Pro-51 substitution is also an example of a differential-binding mutation, although in this case the substrate complex is stabilized, albeit significantly less than the transition state and product complexes.

Since the stabilization of the various bound adenylate species by the amino acid substitutions at residue-51 includes the transition state for the formation of the tyrosyl adenylate intermediate, none of the substitutions result in impairment of catalysis; in fact, the Pro-51 enzyme accelerates the formation of the intermediate from the ternary substrate complex by a factor of approximately 20 relative to the wild type enzyme. The quantitative kinetic and energetic analyses of the reactions catalyzed by the three mutants at residue-51 reveal that each has a k_{cat}/K_{ATP} greater than that measured for the wild type enzyme, with the alanine substitution resulting in a 2-fold increase and the proline substitution resulting in a 35-fold increase in catalytic efficiency for formation of the tyrosyl adenylate intermediate from bound ATP and tyrosine. This increase in catalytic efficiency certainly demonstrates that improvements in catalysis are possible with at least some enzymatic systems. However, an increase in catalytic efficiency in the formation of the tyrosyl adenylate intermediate does not imply that such mutations confer any selective evolutionary advantage on the mutant tRNA synthetases, given the fact that the second half-reaction involves the charging of the cognate $tRNA^{Tyr}$ with the bound tyrosyl adenylate intermediate formed in the first half-reaction. In fact, the stabilization of the bound tyrosyl adenylate intermediate results in a decrease in the rate of aminoacylation by a factor of nearly 2 in the Ala-51 mutant and a factor of 4 in the Pro-51 mutant; presumably this rate diminution results from the thermodynamic accumulation of the tyrosyl adenylate intermediate and the accompanying increase in the free energy difference between this species and the rate-limiting transition state for the charging reaction.

Tyr-34 → Phe and Tyr-169 → Phe. The structure of the bound tyrosyl adenylate intermediate is consistent with the phenolic hydroxyl group of Tyr-34 participating as a hydrogen-bond donor to the phenolic hydroxyl group of the bound tyrosine moiety and with the phenolic hydroxyl group of Tyr-169 participating as a hydrogen-bond acceptor to the α-amino group of this moiety. Both of these hydrogen-bonding interactions have been probed by independent replacement of each tyrosine residue with a phenylalanine.[28] The kinetic analyses reveal that the effect of both mutations is to decrease the affinity of the synthetase for the tyrosyl moiety, irrespective of the species with which it is associated. The effect of the substitution at residue-34 is modest (approximately a factor of 3), with a small but significant decrease in affinity as the reaction proceeds to the bound tyrosyl adenylate intermediate. The substitution at residue-169 produces a much larger decrease in affinity (approximately a factor of 100), with no significant changes as the bound tyrosyl adenylate intermediate is formed. Thus, the phenotypes of the mutant proteins indicate that these residues participate in catalysis by enhancing uniform binding. As might be expected, the amino acid substrate specificity of the Phe-34 synthetase is relaxed but still favors tyrosine, with the discrimination against phenylalanine being reduced by a factor of 15 (from 1.5×10^5 to 1.0×10^4). Presumably, a further decrease in specificity for tyrosine might be achieved by elimination of the second active site hydrogen-bond participant, Asp-176, but results of such a mutagenesis experiment have not been reported.

Thr-40 → Ala and His-45 → Gly. Whereas the previously described applications of site-directed mutagenesis to probe stabilization of various species along the reaction coordinate in the first half-reaction catalyzed by the synthetase were directed toward residues that X-ray crystallography implicated as being important, Fersht and his co-workers have also used model building to implicate two additional residues in catalysis that are modestly removed from the binding site for the tyrosyl adenylate intermediate. The nucleophilic displacement reaction at the α-phosphorus atom of the ATP substrate proceeds by inversion of configuration at phosphorus, and this process, which should proceed via a trigonal-bipyramidal transition state or intermediate, must be accompanied by motion of the β- and γ-phosphate groups of the ATP. Although Mg^{2+} is required for the formation of the tyrosyl adenylate intermediate from bound substrates and certainly is coordinated to the polyphosphate structure such that Mg^{2+}-pyrophosphate is the leaving group and product of the reaction, Fersht hypothesized that additional binding interactions of the γ-phosphoryl group with enzyme functional groups might be important for catalysis; model building suggests that interactions of the γ-phosphoryl group with Thr-40 and His-45 might be important.[25]

The independent mutations of Thr-40 to alanine and of His-45 to glycine as well as the double mutation incorporating both amino acid substitutions resulted in mutant proteins that had essentially the same binding constants for tyrosine and ATP as wild type enzyme. However, the rate of formation of the tyrosyl adenylate intermediate was depressed approximately 7000-fold by the mutation at residue-40 and 200-fold by the mutation at residue-45; when both mutations are present in the same protein molecule the rate is decreased by a factor of 3×10^5 to a value very close to that predicted for the uncatalyzed reaction. (This estimate of the uncatalyzed rate provides an explanation for the fact that the decrease in reaction velocity of the double mutant was not simply the product of the rate

reductions by the independent single mutations; such a test has been advocated by Fersht as an indirect method for ascertaining whether the presence of a mutant side chain alters the conformation of the mutant enzyme molecule relative to that of the wild type enzyme.[33]) Rate reductions of this magnitude must be interpreted carefully, since structural alterations and the misincorporation of very low levels of the wild type amino acid could occur by errors in ribosomal protein synthesis. Although structural alterations have not yet been definitively eliminated as the explanation for the observed decreases in reaction velocity, the observation that each mutant enzyme was competent in the slow accumulation of a stoichiometric amount of tyrosyl adenylate in the active site argues against the contamination of completely inactive mutant protein by a very low level of wild type enzyme.

That the binding constants for the substrates were essentially unchanged in the three mutant enzymes whereas the rate of formation of the tyrosyl adenylate intermediate was significantly impaired indicate that these residues provide examples of active site groups that function only by the catalysis of an individual step. Albery and Knowles[32] argued that this is perhaps the most difficult type of catalysis for an enzyme to achieve, since it implies an interaction between the enzyme active site and the reacting substrates only in a transition state and not in a stable enzyme-bound species; such an interaction is logically possible during the transient formation of a pentacoordinate intermediate as the nucleophilic attack on the α-phosphorus atom occurs and the geometry of the polyphosphate portion of ATP changes. Whereas most of the interactions between the enzyme active site and reacting substrates have been implicated in stabilization of all bound species, including the transition state for formation of the tyrosyl adenylate intermediate, and thereby serve to cause approximation of the reacting groups of the substrates, Thr-40 and His-45 interact only to stabilize the crucial transition state in which bound substrates are converted to products.

Quantitation of Binding Energy between the Enzyme and Bound Substrates and Its Utilization in Catalysis. As noted in the Introduction to this review, physical organic chemists have traditionally used linear free energy relationships to establish mechanism, with deviations from linear relationships often providing important clues for elucidating mechanism. Fersht and his co-workers used an analogous treatment of the kinetic data obtained on a large number of mutants to estimate the importance of the binding interaction in the stabilization of the transition state for the reaction as well as the bound tyrosyl adenylate intermediate, since these are the unstable species whose formation must be favored by the enzyme.[34] In successfully demonstrating that linear free energy relationships can be applied, the persuasive argument can be made that the properties of all of the mutants can be correlated and understood on a unifying basis; a corollary is that artifacts that would lead to problems of interpretation, i.e., structural perturbations in the mutant enzymes by virtue of the amino acid substitution, must not be important in the kinetic properties of the mutants. Of course, the ability to use such linear relationships requires measurements of the rates of interconversion of enzyme-bound intermediates as well as the equilibrium constants relating their concentrations; depending upon the precise mechanism and identity of the rate-determining step, these relationships may be difficult, if not impossible, to apply. However, irrespective of whether a linear free energy relationship can be formulated and measured, the ability to demonstrate that structural changes are unimportant and that an amino acid substitution has properties in accord with other substitutions at the same position constitutes the most difficult but fundamental requirement associated with the successful application of site-directed mutagenesis to establishing structure–function relationships in enzyme-catalyzed reactions.

In formulating linear free energy relationships, Fersht assumed that a linear relationship exists between the equilibrium constant K and the rate constant k for a reaction:[35]

$$k = AK^{\beta} \tag{1}$$

where A is a proportionality constant and β measures the dependence of k on K. Since the log of the rate constant k is proportional to the activation free energy for the reaction, $\Delta G^{\ddagger}$, and the log of the equilibrium constant K is proportional to the free energy change for the reaction, ΔG, eq (1) can be recast as

$$\Delta G^{\ddagger} = \text{constant} + \beta \Delta G \tag{2}$$

Equation (2) may be interpreted as follows. If the linear free energy relationship is such that β is close to zero, the transition state resembles the substrates of the reaction; if β is close to unity, the transition state resembles the products of the reaction. For example, a plot of the logarithm of the rate of formation of the tyrosyl adenylate intermediate from bound substrates vs. the logarithm of the equilibrium constant for the formation of the intermediate from bound substrates is miraculously linear with a slope (β) equal to 0.79 when the data for the mutants in the adenylate and tyrosyl binding sites are considered; this correlation indicates that 79% of the change in binding energy that occurs by proceeding from bound substrates to bound tyrosyl adenylate and Mg^{2+}-pyrophosphate is utilized in lowering the activation energy barrier for forming the product ternary complex. The linear nature of this correlation, which includes several substitutions at some residues, certainly implies that structural changes are not induced by the presence of the mutant functional groups; since Fersht reported no crystallographic data for any of his mutants, this correlation is exceedingly important evidence that the rather interesting conclusions about the roles of several residues in catalysis are likely to be reliable. The binding energy utilization of mutants of residues that are involved only in stabilization of the transition state (Thr-40 and His-45) does not conform to this behavior, since these residues do not interact with the bound substrates or products and, therefore, the kinetic properties of these mutants cannot be expected to correlate with binding energy; a plot of the logarithm of the rate of formation of the tyrosyl adenylate intermediate vs. the logarithm of the equilibrium constant for the formation of bound tyrosyl adenylate intermediate and dissociation Mg^{2+}-pyrophosphate has a slope indistinguishable from infinity, indicating that there is no correlation between the rate of formation

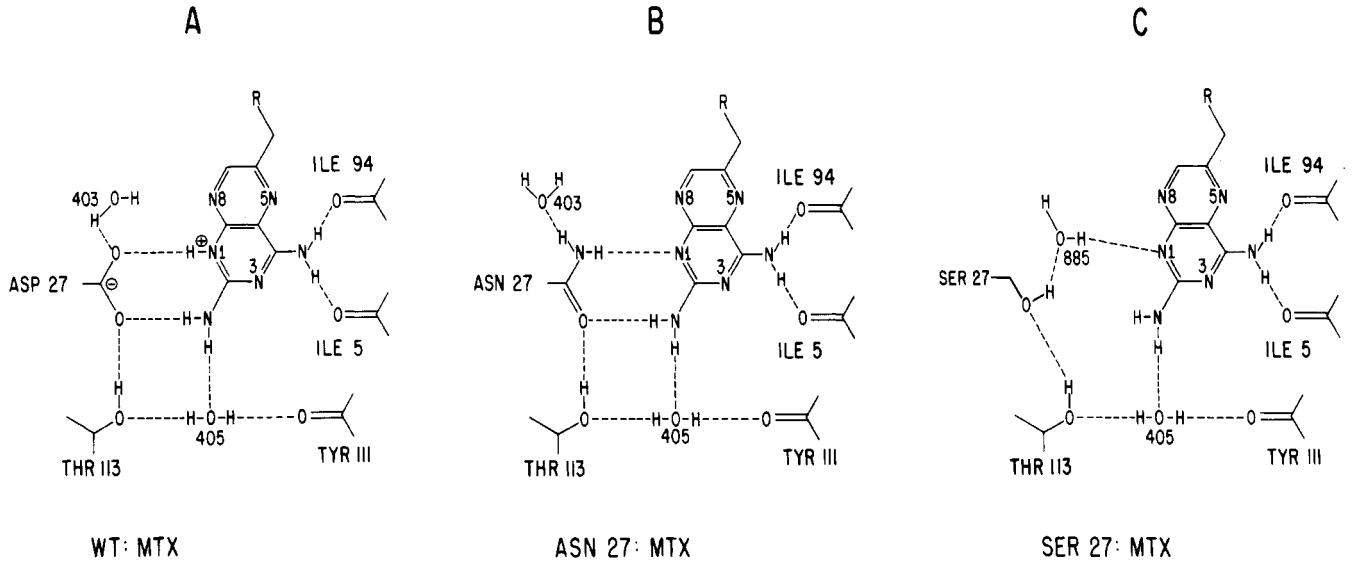

Figure 2. Probable hydrogen-bonding geometries between methotrexate and the active sites of wild-type (Asp-27) dihydrofolate reductase and the Asn-27 and Ser-27 mutants. Reproduced with permission from ref 34. Copyright 1986 Macmillan.

of the intermediate and its stability in the active site.

In summary, the structure–function studies on tyrosyl tRNA synthetase utilizing site-directed mutagenesis appear to provide reliable mechanistic information. Several lessons can be learned from these studies. First, catalysis of the reaction by approximation of bound ATP and tyrosine is accomplished by the participation of several amino acid functional groups that form hydrogen bonds with species in the active site; one residue is not exclusively responsible for catalysis. Second, the principles originally hypothesized by Albery and Knowles[32] for enhancement (or perfection) of catalysis have been demonstrated to apply to the various hydrogen-bonding interactions present in the active site of the synthetase. Third, the kinetic data obtained for several substitutions at each residue and for a wide range of mutants at a variety of residues appear to indicate that structural changes do not occur (at least to a kinetically significant extent) in the mutant proteins. (It might be noted that all of the substitutions described by Fersht and his co-workers involve the conservative replacement of uncharged amino acid residues in the wild type protein with uncharged substitutions; with such substitutions electrostatic effects that might induce conformational changes should not occur, and none are suspected on the basis of the kinetic evidence that has been presented.)

2. Dihydrofolate Reductase from *Escherichia coli*

Dihydrofolate reductase catalyzes the reduction of the 5,6 double bond (imine) of dihydrofolate to form tetrahydrofolate by the direct transfer of a hydride ion from NADPH to carbon-6. This reaction should be facilitated by protonation of the imine at nitrogen-5 in dihydrofolate, since the reduced product contains a hydrogen atom on nitrogen-5 and protonation would also increase the electrophilicity of carbon-6. Site-directed mutagenesis is being used in Kraut's laboratory and in Benkovic's laboratory to ascertain structure–function relationships in the active site.

The enzyme from *E. coli* contains 159 amino acids; the molecular weight of the monomeric enzyme is 18000. The reductase contains two cysteine residues but no disulfide bonds. The three-dimensional structure of the enzyme complexed with methotrexate, a structural analogue of folate and a potent competitive inhibitor, has been determined to 1.7-Å resolution in Kraut's laboratory.[36,37] Methotrexate and folate are very similar in structure, with methotrexate bearing a methyl group on the exocyclic nitrogen-10 and an amino group in the 4-position rather than an oxo substituent; the latter structural difference is responsible for the observed very tight binding to the reductase (K_d falls in the range of 10^{-9}–10^{-12} M, whereas the K_d for folate is approximately 10^4–10^5 larger). However, it is accepted that the geometric disposition of methotrexate in the active site differs from that of folate, with the heterocyclic rings having "flipped" relative conformations. Thus, the complex of the reductase with methotrexate shows bidentate hydrogen-bonding interactions between Asp-27 and both the protonated ring nitrogen-1 and the exocyclic 2-amino group (Figure 2); in the presumed complex of dihydrofolate with the enzyme, Asp-27 is envisaged to stabilize the formation of positive charge at nitrogen-5 of dihydrofolate, although model building suggests an indirect stabilization since the carboxylate of Asp-27 is presumed to interact with the exocyclic 2-amino group and nitrogen-3 of dihydrofolate. The high-resolution structure of the enzyme also reveals a highly conserved hydrophobic binding pocket for the folate cofactor; although the residues in this binding pocket certainly do not participate directly in catalysis, they presumably are important for cofactor binding and the potential manifestation and utilization of binding energy that may influence the hydride-transfer reaction. Site-directed mutagenesis is being used to study structure–function relationships important in hydride transfer to dihydrofolate, including the function of Asp-27, and in binding of the cofactor and the relationship of the strength of this interaction to the rate of the hydride-transfer reaction.

Asp-27 → Asn and Ser. Kraut and his co-workers examined the role of Asp-27 in the generation and stabilization of the protonated imine that is reduced by NADPH. Site-directed mutagenesis was used to replace

Asp-27 with asparagine; the serine mutant was obtained by reversion of the asparagine mutant under the selective pressure of enhanced resistance to trimethoprim, another tight-binding competitive inhibitor of the reductase.[38,39] The structures of both mutants as well as of the wild type enzyme were determined crystallographically to 1.9-Å resolution in the presence of methotrexate, thereby allowing correlation of observed kinetic differences with structural changes.

Although both mutants involve the loss of the anionic carboxylate side chain of aspartate, very few changes were observed in the high-resolution structures. In the asparagine mutant, the only structural differences evident in the electron-density difference map relative to the wild type enzyme were the positions of two water molecules. A water molecule that was hydrogen bonded to one of the carboxylate oxygens of the wild type enzyme, thereby requiring that the water molecule be a hydrogen-bond donor, was shifted by 0.9 Å, with this change in geometry presumably reflecting that in the mutant enzyme the water molecule is a hydrogen-bond acceptor to the amide nitrogen of the asparagine side chain. The second water molecule that was observed to change position is hydrogen bonded to the first water molecule and moves by 1.4 Å, with this movement reflecting the geometric changes required to maintain the interwater hydrogen-bonding interactions. The disposition of the asparagine side chain relative to the bound inhibitor is consistent with the bidentate hydrogen-bonding interactions. No experimentally significant changes in the positions of methotrexate or of the protein side chains or backbone were observed. In the serine mutant, a "hole" is created relative to the wild type enzyme (consider the structures of aspartate and serine), and this void is occupied by a new water molecule; thus, in effect, the aspartate side chain is replaced by a hydrated serine side chain. The serine hydroxyl group interacts with nitrogen-1 of the bound methotrexate via the new water molecule, and a gap indicating the absence of an interaction is present between the serine side chain and the 2-amino group of the inhibitor. Again, no change was noted in the position of the bound methotrexate or in the positions of the remainder of the protein molecule. These observations are consistent with the introduction of mutant side chains having essentially no impact on the global conformation of the mutant protein molecules.

The mutant enzymes have significantly reduced affinities for methotrexate, with the measured dissociation constants for the wild type enzyme and the aspartate and serine mutants being 0.07, 1.9, and 210 nM, respectively. UV difference spectroscopy[39] and ^{1}H NMR spectroscopy[40] have been used to ascertain the protonation state of methotrexate bound to each of the three enzymes: the wild type enzyme binds methotrexate protonated at nitrogen-1, but the mutant enzymes bind unprotonated methotrexate. Thus, in the absence of the aspartate carboxylate anion, binding of the protonated form of the inhibitor is strongly disfavored, and the binding of the neutral species to the mutants is also less favorable due to changes in the hydrogen-bonding interactions.

What is the function of the aspartate carboxylate group in the wild type enzyme? Taking careful precautions to ensure that the mutant enzymes were not contaminated with wild type enzyme (the mutants are expressed in an *E. coli* host, and the carboxamide of the asparagine side chain could be hydrolyzed to generate an aspartate functional group), kinetic analyses were performed at pH 7, the pH optimum of the wild type enzyme. The observed k_{cat} values for the wild type enzyme and the aspartate and serine mutants are 30, 0.10, and 0.44 s^{-1}, respectively; the K_m values for dihydrofolate are 1.2, 44, and 140 μM, respectively. Since the crystallographic analyses did not reveal any conformational changes in the mutant proteins, these data are interpreted to demonstrate the importance of the aspartate functional group in catalysis.

The pH dependence of k_{cat} was also examined for the wild type enzyme and both mutants. Whereas the wild type protein shows a bell-shaped dependence with a maximum at pH 7 and apparent pK_as equal to 5.0 and 8.0 (the K_s for dihydrofolate is constant at 1 μM), the mutant enzymes have a strikingly different behavior in that k_{cat} decreases with increasing pH (the K_s for unprotonated dihydrofolate is 40 μM for the asparagine mutant and 17 μM for the serine mutant; the K_s for protonated dihydrofolate is 40 μM for the asparagine mutant and 135 μM for the serine mutant). At low pH (pH 4.5) the mutant enzymes are almost as active as the wild type enzyme, and, in fact, the extrapolated V_{max} values for the mutant enzymes (60 s^{-1}) exceed that of the wild type enzyme (30 s^{-1}). These kinetic data are consistent with a kinetic scheme in which the mutant enzymes bind both the unprotonated and protonated forms of substrate, but only the bound protonated substrate is competent for reaction to product. Furthermore, the pK_a of the bound substrate is estimated to be 3.8, a value identical with that of nitrogen-5 of dihydrofolate in solution. Accordingly, the carboxylate group of Asp-27 is viewed as being required for protonation of nitrogen-5 of the bound substrate. Consistent with this hypothesis is the observation that completely oxidized folate is not a substrate for either mutant enzyme, whereas the wild type enzyme catalyzes its reduction; the pK_a of nitrogen-5 of oxidized folate is −1.5, with this implying that at pH 5, the lowest pH for which enzyme-catalyzed reactions can be studied, essentially no protonated folate is present in solution and, therefore, could be bound to the mutant enzymes.

Deuterium isotope effects utilizing NADPD as substrate have been performed on the wild type and mutant enzymes at pH 5.1; whereas the wild type enzyme shows deuterium isotope effects that do not deviate significantly from unity (on both V_{max} and V_{max}/K_m), the values for the asparagine and serine mutants were 2.2 and 3.5, respectively, for V_{max} and 3.4 and 1.8, respectively, for V_{max}/K_m. These measurements show that hydride transfer is at least partially rate determining for the mutant enzymes, but in the absence of a more detailed study of presteady-state kinetics a distinction could not be made between a decrease in the rate of hydride transfer or an increase in the rate of product dissociation, thereby potentially causing a change in the rate-determining step. (In recent detailed kinetic studies, Benkovic and his co-workers concluded that dissociation of the tetrahydrofolate product is rate determining for the wild type enzyme, with $NADP^+$ dissociation being rapid; in fact, the binding of NADPH to the product binary complex facilitates the dissocia-

tion of tetrahydrofolate.[41])

These structural and kinetic studies on mutants of Asp-27 demonstrate that although the velocity of the enzymatic reaction is decreased significantly at neutral pH, the enzyme is not impaired with respect to its ability to catalyze hydride transfer, since at low pH when protonated substrate can be bound from solution the V_{max}s for the mutants are actually greater than that measured for the wild type enzyme at its pH optimum. Thus, the carboxylate side chain of Asp-27 helps to generate and/or stabilize the protonated form of the substrate in the active site either by significantly increasing the pK_a or by participating in the transfer of a proton from solution to the bound substrate. In either case, the model accepted for the geometry of bound substrate requires that other active site residues and/or bound water molecules participate in the formation of the bound protonated substrate. Furthermore, the structural studies suggest that substitutions of (charged) active side residues are not accompanied by conformational changes that would prevent detailed mechanistic explanations for the observed phenotypes of the mutant enzymes.

Thr-113 → Val. The X-ray structure of the wild type enzyme reveals that Asp-27 not only participates in a bidentate hydrogen-bonding interaction with bound methotrexate but also is a hydrogen-bond acceptor to the hydroxyl group of Thr-113; given the fact that the precise mechanism for generation and/or stabilization of bound protonated substrate is unknown (although Asp-27 is acknowledged as being important), Thr-113 is potentially involved. Benkovic and his co-workers mutated Thr-113 to the sterically similar valine and assessed the impact of this substitution on catalysis.[42] Although the catalytic phenotype of this mutant has not been as thoroughly characterized as other mutant dihydrofolate reductases, the conclusion is that the threonine hydroxyl group is not essential for the hydride-transfer reaction. The V_{max} for this mutant is essentially the same as that of the wild type enzyme, although the pK_as noted in the V_{max} and V_{max}/K_m profiles are decreased by about 0.5 pH units; however, the K_m for dihydrofolate is increased about 25-fold, with this increase being associated with an enhanced rate of dissociation of substrate from the enzyme. This kinetic behavior suggests that the hydroxyl group stabilizes the protonated form of the enzyme (although the precise identity of this acid is unclear) but is not essential for conducting a proton into the active site. No information regarding the conformational integrity of this mutant is available.

Phe-31 → Tyr and Val. As mentioned previously, Benkovic's group reinvestigated the kinetic scheme for the wild type dihydrofolate reductase.[41] The essence of this analysis is that at neutral and acidic pH the rate-determining step is the dissociation of product tetrahydrofolate from the enzyme. The measured values of k_{off} for $NADP^+$ and tetrahydrofolate from the ternary product complex, of k_{off} for tetrahydrofolate from its binary product complex, and of k_{on} for NADPH to the binary product complex with tetrahydrofolate reveal that the dissociation of $NADP^+$ is much faster than that of tetrahydrofolate from the ternary complex and that the rate of association of NADPH to the binary tetrahydrofolate complex is faster than the dissociation of tetrahydrofolate. Thus, the preferred kinetic pathway involves the dissociation of the tetrahydrofolate product from a ternary complex that contains the reductant for the next catalytic cycle. Furthermore, the measured rate of dissociation of tetrahydrofolate from this ternary complex (12 s^{-1}) is equal to the observed V_{max}. Consistent with this conclusion about the identity of the rate-determining step is that the rate of hydride transfer within the ternary substrate complex was found to be very fast (950 s^{-1}). Knowledge of the precise details of the kinetic mechanism is necessary for understanding the effect of the Tyr-31 and additional substitutions on the enzymatic reaction.

Phe-31 is an invariant residue that is located in the hydrophobic binding pocket for the pteroyl moiety of dihydrofolate. Site-directed mutagenesis has been used to replace this residue with the hydrophobic valine and the more hydrophilic (but aromatic) tyrosine side chains.[43] The measurements of the pH dependence of the kinetic parameters for both substitutions reveal an approximate 2-fold increase in V_{max} (30 and 26 s^{-1} for tyrosine and valine, respectively) and significant increases in the K_m for dihydrofolate, with the net result being that V_{max}/K_m is reduced 2-fold for the tyrosine mutant and 10-fold for the valine mutant. The k_{off} for product tetrahydrofolate from the ternary complex with NADPH was again measured to be equal to V_{max} for the tyrosine substitution (and presumably also for valine). The rates of hydride transfer within the substrate ternary complexes were also measured for both mutants; the rate constants for the tyrosine and valine mutants, 320 and 130 s^{-1}, respectively, were decreased relative to that for the wild type enzyme, 950 s^{-1}, but greater than the observed V_{max}. Thus, the effect of either substitution for Phe-31 was to increase the rate of dissociation of product from the enzyme but also to decrease the rate at which product was formed in the active site. Neither of these substitutions give the mutant enzymes any selective benefit, even though the V_{max} values are greater than that of the wild type, since the catalytic efficiencies are lower due to the increased K_ms for dihydrofolate. In terms of binding energies, the phenotypes of the mutant enzymes may be understood as arising from the weakened interaction of substrate with the enzyme increasing the activation energy barrier for the hydride-transfer reaction. The conformational properties of these mutants have not been investigated in any detail. One measure of potential conformational differences may lie in the observed amplitudes of the biphasic rates of binding of NADPH to the unliganded enzymes. The mutants are observed to have a significantly smaller fast-phase component (24% and 14% for the tyrosine and valine substitutions) than does the wild type enzyme (72%); the significance of this difference may be explained by the relative energies of two conformational states for the unliganded enzyme but is not easily translated into useful information about the presence or importance of conformational changes induced by the amino acid substitutions. However, the valine mutation has been combined with the mutation described in the next section, and the observed differences in kinetic properties have been observed to be additive, suggesting that the two mutations have independent and, therefore, localized effects on conformation.

Leu-54 → Gly. Benkovic's laboratory also reported the preliminary kinetic characterization of a mutant dihydrofolate reductase in which the conserved Leu-54 is replaced with a glycine residue.[44] This residue interacts with the benzoyl glutamate side chain of the bound dihydrofolate substrate. This interesting mutant has a V_{max} essentially unchanged from that measured for the wild type enzyme, but both the K_m for dihydrofolate and the rate of dissociation of this substrate from the enzyme are increased about 300-fold. In contrast to the wild type enzyme, the deuterium isotope effect for the Gly-54 mutant is 3.3 rather than a value near unity; thus, hydride transfer appears to be at least partially rate determining in the mutant. Since the rate of hydride transfer in the wild type enzyme is 950 s^{-1} and V_{max} for the Gly-54 mutant is 14 s^{-1}, the effect of the amino acid substitution is to decrease the rate of hydride transfer approximately 70-fold and to increase the rate of dissociation of the product such that the identity of the rate-determining step is changed from product dissociation to hydride transfer. Again, the phenotype of this mutant emphasizes the importance of the strengths of binding interactions in the magnitude of the energy barrier for the chemical step. As mentioned previously, the structural integrity of this mutant was investigated by combining it with the Val-31 substitution; also, the biphasic binding of NADPH was observed to be the same as that of wild type enzyme.

His-45 → Gln. The high-resolution structure of the reductase reveals that His-45 is appropriately situated to interact electrostatically with the phosphate associated with the nicotinamide portion of the NADPH cofactor. This residue was replaced with a glutamine in Benkovic's laboratory with the expectation that the carboxamide functional group could participate as a hydrogen-bond donor.[41] The rate constants describing the association and dissociation of NADPH were quantitated; for the mutant enzyme, k_{on} was observed to decrease and k_{off} to increase by factors ranging from 3 to 10, with the net result that the K_m for the cofactor is higher in the mutant enzyme. Essentially no change was observed on V_{max}. The conclusion is that the presence of an ionic interaction rather than a hydrogen-bonding interaction between the enzyme and the cofactor does not have a dramatic impact on the catalytic properties of the protein. In this case, the aromatic portion of the ^1H NMR spectrum of the mutant enzyme was compared with that of the wild type, and the only described difference is the absence of the resonance associated with carbon-2 of the imidazole side chain that was removed. In addition, the biphasic kinetics for NADPH binding to the unliganded enzyme were identical with that for binding to the wild type enzyme.

3. Subtilisin BPN′ from *Bacillus amyloliquefaciens*

Subtilisins (the enzymes are produced and secreted by a variety of *Bacillus* species) are typical serine endopeptidases that utilize the classic catalytic triad of Asp-His-Ser. The enzyme from *B. amyloliquefaciens* is relatively nonspecific with respect to the amino acid residue contributing the carboxyl of the scissile peptide bond (designated the P_1 residue; the amino acid contributing the amino group is designated the P_1' residue); high-resolution X-ray crystallographic studies reveal that the oligopeptide binding cleft has residues (or subsites) that can potentially interact with a hexapeptide having the structure P_4-P_3-P_2-P_1-P_1'-P_2' and that the P_1 binding site is a large open cleft, thereby explaining the observed lack of specificity at this subsite.

The subtilisin from *B. amyloliquefaciens* (subtilisin BPN′, but herein referred to simply as subtilisin) contains 274 amino acids; the molecular weight of the monomeric enzyme is 27 500. Subtilisin contains no cysteine or cystine residues. The three-dimensional structure of the enzyme was solved recently at 1.8-Å resolution both by groups at Genentech and at Genex; earlier lower resolution crystallographic studies performed in Kraut's laboratory examined the modes of interaction of peptide inhibitors,[45] product complexes,[46] and transition-state analogues[47,48] with the enzyme. The group at Genentech has been particularly active in using site-directed mutagenesis to study structure–function relationships in the active site, with the majority of the studies being directed toward understanding the factors that are involved in the specificity (or lack thereof) expressed for the P_1 residue or, more generally, that are involved in the recognition of small molecules by enzymes. In addition, substantial effort is being directed both at Genentech and at Genex toward understanding the factors involved in enhancing the stability of the enzyme,[49,50] given its use in laundry detergents. Finally, both groups reported studies concerning the participation of an active site residue (Asn-155) in stabilizing the tetrahedral intermediate presumed to lie on the reaction coordinate.[51,52] This residue will be discussed in the last section of this review, which examines those residues intimately involved in catalysis.

The binding site for the P_1 residue is a large, solvent-accessible cleft. The bottom of the cleft is occupied by Gly-166, thereby explaining the relative lack of specificity for uncharged residues at this subsite. The X-ray structure of a covalent complex of subtilisin derived from reaction with a chloromethyl ketone affinity label that has a lysine residue in the P_1 subsite revealed that this charged side chain forms an ion pair with Glu-156 at the mouth of the cleft rather than extending into the cleft toward Gly-166.[45] Both Gly-166 and Glu-156 have been the target of studies using site-directed mutagenesis, with the mutations of the former residue allowing studies to examine the effect of mutant side-chain volume and hydrophobicity on P_1 specificity[53] and mutations of the latter residue allowing studies to examine the importance of electrostatic interactions on P_1 specificity.[54]

Gly-166 → Ala, Ser, Thr, Val, Leu, Ile, Phe, Tyr, Trp, Cys, Met, and Pro. Cassette mutagenesis was employed to construct the extensive range of mutants at this position so that the effect of hydrophobicity and steric constraints on catalytic efficiency and specificity could be examined.[49]

Using a series of ten structurally homologous substrates (succinyl-L-Ala-L-Ala-L-Pro-L-[X]-*p*-nitroanilide, where X is an uncharged P_1 amino acid), Wells and his co-workers at Genetech and Genencor examined the dependence of k_{cat}/K_m for the wild type enzyme on P_1 residue hydrophobicity (where hydrophobicity is defined as the free energy of transfer of each amino acid

from water to ethanol or dioxane relative to glycine). The correlation was remarkably linear with a slope of -0.89 ± 0.07, with the only deviant data point being that for glycine, which binds nonproductively to the enzyme. When the effect of hydrophobicity on this ratio is partitioned into the separate effects on k_{cat} and on $1/K_m$, these correlations are also remarkably linear. Since the rate-determining step in the hydrolysis of amide substrates is the acylation of the enzyme, k_{cat} can be assumed to be a measure of the acylation rate constant and K_m the true dissociation constant for bound substrate. The correlations of these kinetic parameters on hydrophobicity show that both in substrate binding and in stabilization of the transition state for acylation the interaction of the P_1 residue with its subsite is influenced by the hydrophobicity (or steric volume) of the side chain. Given this correlation, the more ambitious experiment of assessing the influence of P_1 subsite hydrophobicity in various mutant enzymes on substrate binding and acylation with the previous series of homologous substrates is worthwhile. These analyses also involve the correlation of k_{cat}/K_m, k_{cat}, and $1/K_m$ on subsite residue properties, since these comparisons allow the free energy changes associated with total catalytic efficiency, rate of acylation, and substrate binding to be assessed.

The effects of the substitutions at residue-166 are complicated but, in general, can be explained by enhanced binding between the P_1 subsite and the substrate P_1 side chain by virtue of increased hydrophobic interactions until the total steric bulk of the side chain of residue-166 in the P_1 subsite and the P_1 amino acid side chain exceed the volume of the cleft; when this limit is exceeded, catalytic efficiency drops precipitously. The best example that illustrates this conclusion is the observed substrate specificity of the isoleucine substitution. With the alanine-containing oligopeptide as substrate, the isoleucine enzyme was 16-fold more active (in terms of k_{cat}/K_m) than the wild type enzyme. The activity of a valine-containing substrate was slightly enhanced relative to the alanine substrate, but further modest increases in size (methionine and leucine) reduced the catalytic efficiency to the level observed with the wild type enzyme. Finally, phenylalanine- and tyrosine-containing substrates caused reductions in catalytic efficiency of approximately three orders of magnitude. Whereas the modest increase with the valyl substrate can be best explained in terms of a hydrophobic effect, the marked decreases associated with the phenylalanyl and tyrosyl substrates are clearly examples of steric contributions. Additional evidence for the importance of steric factors is obtained from the observation that the catalytic efficiencies of the enzymes having P_1 site substituents with β- and γ-branched side chains are significantly less than substituents with linear side chains having the same volume. These data also illustrate that increases in catalytic efficiency over that found for the wild type enzyme can be achieved, at least for some substrates. However, these increases in catalytic efficiency may be made at the expense of changing specificity; *B. amyloliquefaciens* requires a relatively nonspecific protease since it produces only two extracellular proteases, including subtilisin, and changes in specificity may not provide a selective advantage and, therefore, evolutionary pressure for selective enhancements in catalytic efficiency. Although the structural characterizations of the various mutants at residue-166 have not been explicitly described in publications, at least some of the mutant proteins have been examined crystallographically, and the structures of these mutants were not found to have changes with the exception of the expected side-chain substitution.

Glu-156 → Gln or Ser and/or Gly-166 → Asp, Glu, Asn, Gln, Met, Arg, and Lys. As described previously, the side chains of positively charged amino acids are thought to bind to subtilisin via interactions with the carboxylate group of Glu-156 rather than with the cleft partially defined by Gly-166. This observation suggests that changes in specificity for charged substrates might be accomplished by variation of the charge associated with residue-156 and also by the introduction of a charged side chain at residue-166. Cassette mutagenesis was used to introduce mutations at residue-156, and these were ligated to appropriate restriction fragments obtained from the previously described mutants at residue-166.[54] Various combinations of side chains were constructed, with these leading to variation in the total charge associated with the P_1 site. These mutants were then used to examine substrate specificity, with pairs of sterically similar but charge dissimilar substrates being used to detect changes in specificity; in particular, a glutamate/glutamine pair was used to ascertain the effect of binding site charge on specificity for an anionic substrate, and a lysine/methionine pair was used to ascertain the effect of charge on specificity for a cationic substrate. By the use of k_{cat}/K_m as a measure of the effect of electrostatic interactions between enzyme and substrate on specificity, the glutamyl and glutaminyl substrates were both found to have direct dependencies of different magnitude on the total charge in the P_1 site, with k_{cat}/K_m for the glutamyl substrate increasing more rapidly with an increase in positive charge in the binding site; however, k_{cat}/K_m for the glutaminyl substrate might have been expected to be independent of binding site charge but was not. Quantitative differences in behavior were also observed for the lysyl and methionyl substrates, with k_{cat}/K_m for the methionyl substrate increasing with an increase in positive charge in the binding site but that for the lysyl substrate having a slight inverse dependence on binding site charge; in this case, k_{cat}/K_m for the methionyl substrate might have been expected to be charge independent and that for the lysyl substrate might have been expected to be significantly inversely dependent on increasing positive charge. Although the observed trends are qualitatively in the right direction, the fact that behavior of neither substrate pair was that expected if only electrostatic effects are important demonstrates the complexity of the problems associated with understanding the specificity of enzyme–substrate interactions. In one extreme, the explanation for the failure of naive predictions may be explained by insufficient data to clearly discern trends; in the other, a complete quantitative dissection of the multitude of factors involved in binding may be impossible to attain.

4. Trypsin from Rat

Trypsins are also serine endopeptidases that utilize the catalytic triad of Asp-His-Ser. The mechanism and structure of the bovine enzyme have been intensely

studied; along with the mechanistically analogous enzyme chymotrypsin, trypsin represents perhaps the best understood protease. Trypsin is specific for cationic amino acid residues at the carboxyl side of the scissile peptide bond.

Trypsin is a monomeric enzyme that is derived proteolytically from the precursor trypsinogen. The enzyme has a molecular weight of 26 000; it contains 12 cysteine residues in the form of six disulfide bonds, which maintain the basic tertiary structure of the proteolyzed trypsinogen precursor. High-resolution X-ray structures are available for bovine trypsin both in the absence and presence of inhibitors, including bovine pancreatic trypsin inhibitor, which has a lysyl bond in the to be described specificity pocket,[55] and benzamidine, which mimics the binding of an arginyl side chain.[56,57] The structural analyses reveal that the origin of the binding specificity for cationic amino acids is most certainly the presence of an aspartyl side chain at the bottom of a hydrophobic pocket in which arginyl side chains are envisaged to ion pair directly and lysyl side chains are envisaged to interact indirectly via an intervening water molecule (Figure 3). The structures of trypsin and the homologous elastase molecule reveal similar active site geometries, except that side chains are sterically excluded from the binding pocket of elastase by the presence of valine and threonine residues at the mouth of the pocket; in trypsin the analogous amino acids are both glycine. Site-directed mutagenesis of the gene for rat trypsin has been used in the laboratories of Rutter and of Craik to examine the influence of binding pocket structure on substrate specificity[58,59] as well as the function of the aspartate residue in the catalytic triad.[60,61] By DNA sequence analysis of the gene for the rat enzyme, this enzyme has 74% identity with the well-characterized bovine trypsin,[62] with most of the 57 differences in amino acid primary structure being located on the surface and four of the nine remaining interior changes being structurally conservative. For example, the catalytic triad, the aspartate group in the binding cleft and the two glycine residues at the mouth of the cleft, are conserved residues in the two enzymes. Thus, the assumption was initially made that the structural data and mechanistic information available for the bovine enzyme would, by and large, be applicable to the rat enzyme; subsequent high-resolution X-ray analysis of the rat enzyme has fulfilled the structural expectations. The specificity studies will be described in this subsection, and the importance of the aspartate residue in the catalytic triad will be discussed in the following section.

Gly-216 → Ala and/or Gly-226 → Ala. Model building based upon the structure of the bovine enzyme and the knowledge that in the homologous elastase amino acid substitutions at the mouth of the binding pocket appear to place steric limitations on substrate specificity suggested that the specificity of trypsin might be altered by amino acid substitutions in the binding pocket.[58] In particular, the initial studies that were performed attempted quite subtle changes in binding specificity: modulation of the observed preference for arginyl side chains relative to lysyl side chains (approximately 12:1 in the rat enzyme). This reversal was to be achieved by the following changes. The X-ray structures of the complexes with bovine pancreatic

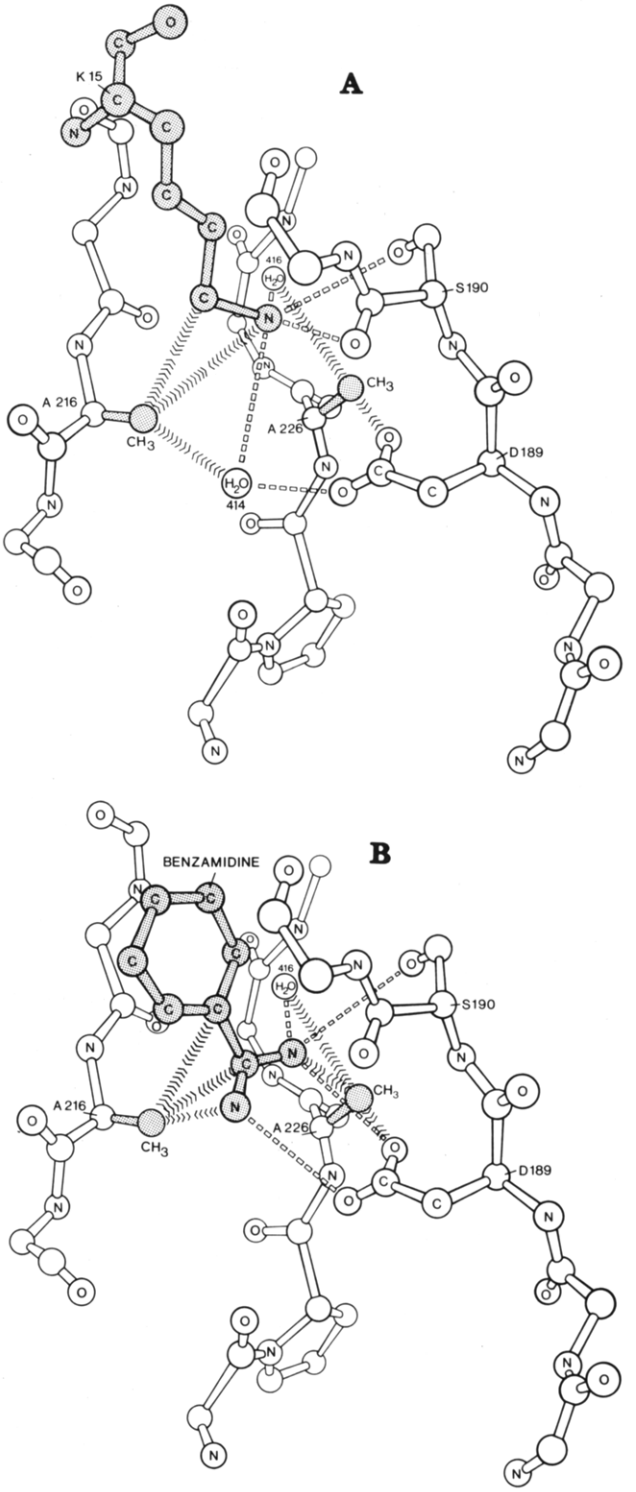

Figure 3. Model building derived predictions of the effects of Ala-216 and Ala-226 substitutions on the binding pocket of trypsin. Panel A shows the geometry of binding of Lys-15 of bovine pancreatic inhibitor, and Panel B shows the geometry of binding of benzamidine. Reproduced with permission from ref 55. Copyright 1976 Academic.

trypsin inhibitor and with benzamidine suggested that since the ϵ-ammonium group of the lysyl side chain interacts via a water molecule with the carboxylate of Asp-189, displacement of this water molecule by the substitution of alanine for Gly-216 should prevent the binding of lysine; since the presence of the additional methyl group was not expected to interact with the arginyl side chain, the Ala-216 enzyme should have enhanced specificity for arginine. In contrast, model

building suggested that the substitution of alanine for Gly-226 would interfere sterically with Asp-189 and that this presumed geometric alteration in the binding pocket would significantly impair the binding of arginyl side chains; however, since lysyl side chains interact indirectly with the aspartyl carboxylate group via a water molecule, movement of this water molecule might allow the unperturbed (or minimally perturbed) binding of lysyl side chains.

Site-directed mutagenesis of the codons for residues-216 and -226, subsequent isolation of the mutant trypsinogens, and activation with enterokinase permitted the isolation of the desired mutants of rat trypsin. Fluorescence-based kinetic analyses utilizing D-Val-Leu-Lys-aminofluorocoumarin and the analogous D-Val-Leu-Arg-aminofluorocoumarin and measuring k_{cat}, K_m, and k_{cat}/K_m revealed that the glycine to alanine mutation at residue-216 had the desired enhanced specificity (in terms of k_{cat}/K_m) for arginine (approximately 30:1 in favor of arginine) and the glycine to alanine mutation at residue-226 had the desired enhanced specificity for lysine (2:1 in favor of lysine). However, these specificity changes were achieved primarily by changes in k_{cat} and to a lesser extent by changes in K_m. Implicit in the predictions based upon model building was the expectation that specificity changes would be achieved by changes in binding, i.e., K_m, and not by changes in catalytic rate. The double mutant with alanine substitutions at residues-216 and -226 was also constructed, and this protein was observed to be virtually catalytically inactive rather than fully catalytically active with significantly reduced affinities for substrates. Although crystallographic analyses have not been reported for any of these site-directed mutants, plausible explanations for the unexpected kinetic results include alteration of the conformation of the active site of the enzyme to produce catalytically impaired active sites and nonproductive binding of substrates, preventing "normal" hydrolysis of peptide bonds by the catalytic triad. Interestingly, Warshel and Sussman proposed on the basis of calculations based upon free energy perturbation methods that the effect of these glycine to alanine mutations is to alter the activation energy barrier to catalysis by altering the electrostatic potentials within the "oxyanion hole" that stabilizes the presumed tetrahedral intermediate on the reaction pathway[63] (mutations within this hole will be discussed in the next section). In any event, these studies also show that the specificity of an enzyme can be altered by site-directed mutagenesis, although the origin of the specificity change was not that predicted a priori.

Asp-189 → Lys. The specificity of trypsin for cationic amino acid side chains is presumed to be dependent on Asp-189. An interesting hypothesis was that the specificity of trypsin might be changed to anionic amino acid side chains if Asp-189 were substituted with a cationic amino acid residue.[59] Site-directed mutagenesis was used to mutate the codon for residue-189 to one specifying lysine, and the desired Lys-189 mutant was obtained by enterokinase digestion of the mutant trypsinogen precursor. As hoped, the mutant protein was completely inactive against the previously described lysyl and arginyl substrates; however, it was also inactive on aspartyl and glutamyl substrates! A successful search for proteolytic activity was performed by extensive exposure of several natural proteins and oligopeptides to the mutant trypsin and analyzing the N-terminal sequences of fragments that were produced. Sperm whale myoglobin was observed to undergo cleavage preferentially at a Leu-Ala bond. Porcine β-lipotropin and human adrenocorticotropic hormone were more extensively cleaved, with N-terminal analyses of the fragment products revealing cleavage at Leu, Phe, and Tyr residues. Luteinizing hormone-releasing hormone was also cleaved at Tyr and Leu bonds. The hydrolytic behavior observed on these substrates is characteristic of chymotrypsin. In retrospect, the low level of chymotryptic activity was not surprising, given earlier reports that highly purified trypsin had some chymotryptic activity, i.e., peptide bonds involving Phe and Tyr were slowly cleaved; however, whereas the earlier reports could have been erroneous due to actual contamination by chymotrypsin,[64,65] contamination of the mutant trypsin by chymotrypsin is not possible given the fact that the mutant trypsin was expressed in *E. coli*. However, the ability of the Lys-189 mutant trypsin to cleave leucyl peptide bonds was unexpected. Retrospective model building suggests that the explanation for this behavior is likely to reside in an unanticipated conformation for the lysyl side chain such that it would be inaccessible to anionic substrates and, in fact, make the binding pocket hydrophobic: hence the chymotryptic activity. No high-resolution X-ray studies are yet available to support this suggestion. However, these studies serve as yet another example of altered substrate specificity achieved by site-directed mutagenesis, although the phenotype to be achieved via hypotheses developed by model building was not realized.

5. Aspartate Aminotransferase from *E. coli*

Aspartate aminotransferase catalyzes the pyridoxal phosphate (PLP) dependent interconversion of L-aspartate and 2-oxoglutarate to form oxaloacetate and L-glutamate; the overall reaction occurs via two half-reactions, the first in which L-aspartate is converted to oxalacetate with the intermediate formation of enzyme-bound pyridoxamine phosphate, and the second in which 2-oxoglutarate is converted to L-glutamate with the regeneration of the pyridoxal phosphate cofactor.

A 3.0-Å X-ray structure was recently determined for the enzyme from *E. coli*,[66,67] and this structure shows that the active site of the enzyme is very similar to those of the previously characterized mammalian enzymes.[68] In addition, the residues at the active site that interact with bound substrate and pyridoxal phosphate have both sequence and structural homology among the eukaryotic and prokaryotic enzymes: Lys-258 participates in Schiff base formation with the bound cofactor, Arg-386 ion pairs with the α-carboxylate groups of bound substrates, and Arg-292 ion pairs with the side-chain carboxylate groups. Site-directed mutagenesis is being used in Kirsch's laboratory to probe the functions of active site functional groups in catalysis.

Arg-292 → Asp. In analogy to the previously described efforts to alter the substrate specificity of trypsin and subtilisin by mutation of amino acids present in their binding pockets, Arg-292 has been mutated to an aspartate residue with the intent being

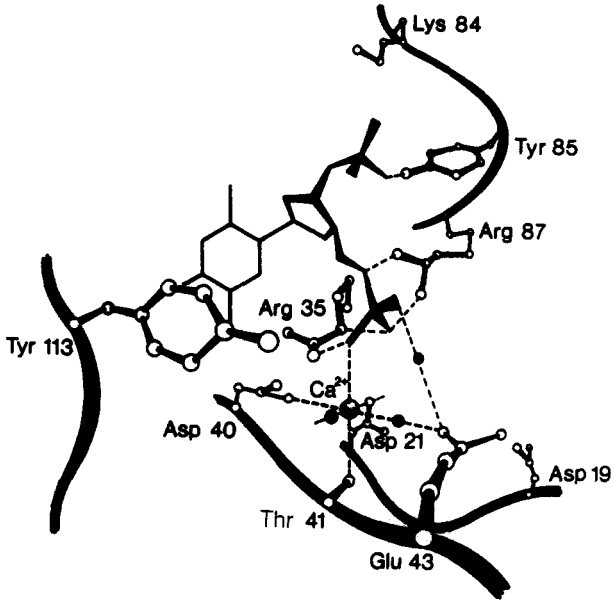

Figure 4. Active site of wild type Staphylococcal nuclease with bound active site ligands Ca^{2+} and thymidine 3′,5′-diphosphate. *Reproduced with permission from ref 67.*

to make the enzyme specific for substrates with cationic rather than anionic side chains.[69] This goal was at least partially accomplished in that the Asp-292 enzyme does prefer either arginine or lysine over aspartate, but this change in specificity occurred at the price of a disappointingly inefficient value for k_{cat}/K_m. The wild type enzyme prefers aspartate relative to arginine by a factor of 6.5×10^5 and aspartate to lysine by a factor of 1.0×10^6; the Asp-292 mutant prefers arginine to aspartate by a factor of 5.7 and lysine to aspartate by a factor of 2.4. This reversal in specificity has its origins in the effect of the active site substitution on k_{cat}/K_m for the various pertinent amino acid substrates: whereas the catalytic efficiency for aspartate in the first half-reaction is decreased by a factor of 2.6×10^5 and the catalytic efficiency for 2-oxoglutarate is diminished by essentially the same factor in the second half-reaction when the specificity substitution is made, the catalytic efficiency for arginine increases only 14-fold and that for lysine only 9-fold. Thus, the cationic amino acid specific aminotransferase is a rather inefficient catalyst when compared with the parent wild type enzyme.

C. Studies of Amino Acid Residues Directly Involved (or Thought To Be Directly Involved by Virtue of X-Ray Crystallographic Studies) in Covalent Bond Formation

1. Staphylococcal Nuclease from *Staphylococcus aureus*

Staphylococcal nuclease catalyzes the Ca^{2+}-dependent hydrolysis of single-stranded DNA and RNA to yield 3′-mononucleotides as products. The enzyme is monomeric, contains 149 amino acids, and has a molecular weight of 17 000; no cysteine or cystine residues are present.

A nominal 1.5-Å resolution X-ray structure of the nuclease crystallized in the presence of Ca^{2+} and the competitive inhibitor thymidine 3′,5′-diphosphate (pdTp) has been reported[70] (Figure 4), and this crystallographic information has been used to formulate a mechanism for the reaction. The metal ion is octahedrally coordinated, with the ligands being the carboxylate groups of Asp-21 and of Asp-40, the carbonyl of a backbone peptide bond involving Thr-41, two water molecules, and one of the anionic phosphoryl oxygens of the 5′-phosphate of the bound inhibitor. The 5′-phosphate group of the bound inhibitor is also within hydrogen-bonding distance of the guanidino side chains of Arg-35 and Arg-87. Assuming that the structure of this nonproductive complex mimics that of a productive complex with a substrate, the extensive neutralization of the anionic charge on the phosphate group appears to enhance the electrophilicity of the phosphorus to facilitate attack by water (or hydroxide ion). The attack of water on the bound substrate has been proposed to be general base catalyzed by the carboxylate group of Glu-43, which is hydrogen bonded to one of the water molecules in the inner coordination sphere of the metal ion, and a water molecule, which is also hydrogen bonded to one of the oxygen atoms of the 5′-phosphate of the bound inhibitor. This mechanism predicts that the cleavage of the phosphate ester bond to the 5′-oxygen of a substrate should proceed with inversion of configuration at phosphorus, and my laboratory has confirmed this expectation.[71] The identity of the general acid catalyst, if any, that facilitates the departure of the 5′-oxygen of the substrate is unknown, although the unlikely candidates of Arg-35 and Arg-87 are proximal to this oxygen atom in the X-ray structure. In addition, in the X-ray structure the ϵ-ammonium group of Lys-84 and the hydroxyl group of Tyr-85 are sufficiently close to hydrogen bond with the 3′-phosphate group of the bound inhibitor; since the presence of a 3′-phosphate group is essential for tight binding of substrates and inhibitors, these interactions are assumed to be the origin of this binding interaction. Various aspects of this proposed mechanism have been investigated in Mildvan's laboratory and in my laboratory with random[72,73] and site-directed[74,75] mutants, respectively, of the nuclease. Although the results of these studies have been proposed by Mildvan as being sufficient for the complete dissection of the importance of each active site residue in catalysis, such an interpretation is in doubt in the opinion of this author given the evidence that at least some substitutions of active site residues are accompanied by conformational changes of currently unknown importance in catalysis.

Glu-43 → Asp, Gln, Asn, Ser, and Ala. The kinetic properties of the multiple substitutions made for Glu-43 could allow the importance of this residue in catalysis to be evaluated, or at least its impact on the free energy profile of the reaction, if the substitutions are not accompanied by conformational changes within the mutant proteins.[74,75] The homologous, charged conserved aspartate substitution results in a 1400-fold decrease in k_{cat}/K_m relative to wild type, with k_{cat} decreasing 200-fold and K_m increasing 7-fold; the charge-neutral substitutions ranging from the isosteric glutamine to the noninteractive alanine substitution had approximately equal values for k_{cat}/K_m that were decreased about 5000-fold relative to wild type enzyme. In the absence of conformational differences between the wild type and mutant proteins, these differences in catalytic efficiency appear to define the importance of Glu-43

and, presumably, general base catalysis in the estimated 10^{15}-fold rate acceleration accomplished by the wild type enzyme.

However, in the current absence of high-resolution crystallographic information regarding the conformations of the mutant enzymes, data are available that demonstrate that the conformations of complexes of the mutant enzymes with Ca^{2+} and pdTp differ from that of the wild type enzyme in solution. All five substitutions for Glu-43 were observed to increase the melting temperature of the unliganded mutant enzymes relative to the wild type enzyme; a modest 1 °C increase in T_m was measured for Asp-43 protein and a more impressive 5 °C increase was measured for the Ser-43 protein. In the absence of differential scanning calorimetry studies that yield the thermodynamic parameters necessary to compare ΔG for unfolding at any temperature, these differences in T_m tentatively *may* be indicative of an enhancement in thermal stability. If this supposition is correct, enhancement in stability may arise from increased stability of the folded form or decreased stability of the unfolded form or a combination of both effects. (The thermal unfolding of wild type nuclease is truly reversible.[76])

^1H NMR spectroscopy can be used to detect, localize, and describe conformational changes in the various substitutions for Glu-43 in the nuclease. Although the nuclease is considerably larger than proteins for which complete or extensive chemical shift assignments have been made, both the aromatic and upfield-shifted methyl regions of the one-dimensional spectrum as well as isotopic labeling with deuteriated amino acids allow sufficient spectral simplification to extract at least some meaningful conclusions. Obvious changes in the chemical shifts of some aromatic and upfield-shifted methyl resonances are evident when the spectra of the mutant and wild type proteins are compared. Although the relationship between changes in chemical shifts of the aromatic resonances and conformation is uncertain, at least some of the changes in the chemical shifts of the upfield-shifted methyl resonances are likely to occur by changes in ring current effects, i.e., the changes in chemical shifts arise from altered geometric relationships between the aromatic rings and proximal methyl groups. More convincing evidence for conformational changes is obtained by the observed changes in nuclear Overhauser effect correlations between the aromatic and (upfield-shifted) methyl resonances; nuclear Overhauser effects describe dipolar coupling between spatially proximal protons that is sensitive to both the interproton distance and the motional correlation times of the proximal protons and occur when protons are separated by 3–5 Å. Changes in NOEs of factors of two are found when the wild type and mutant two-dimensional NOESY spectra are compared, and these imply either changes in interproton distances and/or local dynamics of the side chains; if the changes in NOEs are entirely associated with changes in interproton distance, conformational changes on the order of 0.3–0.5 Å are detected. Comparisons of NOESY spectra of samples of the nuclease labeled with deuteriated aromatic amino acids allow the conclusion that the most obvious spectral changes occur in the regions of the three phenylalanine residues present in the nuclease, and these are present at distances of 15, 25, and 30 Å from the position of the substitutions. The presently incomplete crystallographic studies of these mutants have revealed that the mutants are isomorphous with the wild type enzyme. However, even though the X-ray studies have not progressed to high-resolution structures, the NMR data clearly demonstrate that extensive conformational changes of perhaps modest magnitude accompany the amino acid substitution; the impact of these changes on the kinetic properties of the mutant proteins is unknown. As a result of these conformational changes and the uncertainty regarding their impact on the catalytic properties of the mutant proteins, the conservative assessment of the relationship of the wild type and mutant proteins is that the kinetic phenotypes should not be used to deduce the quantitative or even qualitative importance of the carboxylate group of Glu-43 in catalysis.

Asp-21 → Glu and Tyr, Thr-41 → Pro, and Asp-40 → Glu and Gly. Mildvan and Shortle and co-workers examined the kinetic properties of amino acid substitutions at additional positions within the active site that were generated by in vitro random mutagenesis of the gene for the nuclease;[72,73] as such, the replacements were not chosen with the intent of systematically probing structure–function relationships but as substitutions that lead to a decrease in the amount of enzyme activity in *E. coli* cells transformed with the mutant plasmids.[77] In any event, Mildvan studied several mutants and used their kinetic phenotypes to quantitate the contribution of each functional group to the catalytic efficiency of the reaction catalyzed by the wild type enzyme. The amino acid substitutions discussed in this section involve ligands to the bound Ca^{2+} ion.

Some of the amino acid substitutions involving the metal ion ligands have surprising little impact on catalysis. For example, the substitutions for Asp-40 (Glu and Gly) decrease the affinity for the metal ion less than 10-fold and decrease k_{cat} by factors of 12 and 30, respectively. The presumed alteration in backbone geometry at residue-41 and, therefore, position of the peptide carbonyl thought to be a metal ion ligand decreases metal ion binding only 3-fold and reduces k_{cat} by a factor of 37. However, the amino acid substitutions for Asp-21 (Glu and Tyr) also have little influence on metal ion binding but decrease k_{cat} by factors of 1500 and $\geq$ 29 000, respectively. In the absence of any structural characterization of the mutants, the properties of these mutant enzymes are difficult to understand. However, Mildvan used his data to deduce that the metal ion ligands and the resulting positioning of the metal ion and bound water molecule can account for approximately half of the total decrease in activation energy for hydrolysis (a factor of approximately 10^{15} in rate or 21 kcal/mol in activation free energy) that is provided by the wild type nuclease relative to the uncatalyzed reaction.

Arg-35 → Gly and Arg-87 → Gly. Glycine substitutions for both arginines that are in close proximity to the 5′-phosphate of pdTp were also identified in Shortle's library of random mutations.[77] The kinetic properties of the mutant proteins were evaluated, and both were observed to severely decrease k_{cat} (by factors $\geq$ 35 000, i.e., enzyme activity could not be reliably detected). Interestingly, while neither mutation seriously effects the affinity for Ca^{2+}, the affinity of Gly-35

for pdTp is decreased 100-fold while that for Gly-87 is indistinguishable from the value characteristic of the wild type enzyme. This data may suggest that Arg-35 is involved in both binding of substrate and the presumed trigonal-bipyramidal transition state, while Arg-87 is important only for binding of the transition state, i.e., according to the concepts developed by Albery and Knowles,[32] Arg-35 contributes to uniform binding of the internal states but Arg-87 functions by the very exacting stabilization of a transition state and, therefore, catalysis of an elementary step. Even though no catalytic activity could be detected for either of these mutants, Mildvan proposed that the large decrease in k_{cat} observed for the Gly-87 mutant indicates that it is very important in catalysis by binding the transition state and that this interaction is responsible for one-fourth of the rate acceleration; curiously the equally large decrease in k_{cat} observed for the Gly-35 mutant was considered to be unimportant in accounting for the magnitude of the total catalytic efficiency of the enzyme(?). No characterization of the impact, if any, of the amino acid substitutions on the conformational integrity of these mutants has been reported, and given the lack of this information the validity of the quantitative analysis of the factors that contribute to catalysis is difficult to assess.

The quantitative analysis described by Mildvan also used the previously discussed data from my laboratory regarding the importance of the effect of amino acid substitutions for Glu-43 on catalysis. The decreases in k_{cat}/K_m were interpreted as evidence that general base catalysis by the carboxylase at residue-43 contributes the remaining one-fourth of the observed rate acceleration. In light of the accumulating evidence that amino acid substitutions for Glu-43 are accompanied by far-reaching conformational changes, the dissection of catalysis reported by Mildvan should be viewed with caution.

Additional Mutations. We also examined whether the side chains of Lys-84 and Tyr-85 are responsible for the tight binding of substrates and inhibitors with 3′-phosphate groups as suggested by the high-resolution X-ray structure.[78] These residues have been independently mutated by site-directed mutagenesis to Ala and Phe, respectively; in addition, the Lys-84–Phe-85 double mutant has also been constructed. The affinities of the Ala-84 and Phe-85 single mutant for pdTp are decreased approximately 20- and 15-fold, respectively, relative to the wild type value; the affinity of the double mutant is decreased 150-fold to a value similar to that observed for the binding of thymidine 5′-phosphate, which lacks the 3′-phosphate required for tight binding.

Finally, Shortle and Linn reported the isolation and characterization of four "global suppressor" mutations that at least partially revert the phenotypes of a number of first site mutations that decrease the stability of the enzyme with respect to either thermal- or denaturant-induced unfolding.[77,79] These mutations, Val-66 → Leu, Gly-88 → Val, His-124 → Leu, and His-124 → Arg, can complement a variety of remote mutations that decrease the stability of the folded state of the nuclease, but the mechanism by which this suppression is accomplished is unknown as is the effect of these mutations on the catalytic properties of any of the active site mutations.

2. Trypsin from Rat

The serine proteases constitute a large family of mechanistically homologous enzymes, with chymotrypsin and trypsin being the most intensely studied members of this family. Prior to X-ray crystallographic studies of these enzymes, chemical modification studies had implicated histidine[80] and serine[81] residues as being present in the active sites of these enzymes. This expectation was verified by the high-resolution crystallographic studies that revealed the presence of these residues as well as aspartic acid in a mechanistically tantalizing geometric relationship in the active site.[82-84] Ser-195 serves as the nucleophile involved in formation of the acyl enzyme intermediate. The relationship of the hydroxyl group of this serine to the imidazole functional group of His-57 suggests that the unprotonated N-1 of the side chain is hydrogen bonded to the serine hydroxyl group; this arrangement is assumed to enhance the nucleophilicity of the hydroxyl group. However, the mechanistic surprise was that the carboxylate group of Asp-102 was found also associated with the imidazole side chain of His-57. The precise function of this interaction has remained uncertain: in one extreme a mechanism can be formulated in which the proton from N-3 of the diprotonated (imidazolium ion) His-57 is actually transferred to the carboxylate anion of Asp-102 to generate the carboxylic acid and the neutral monoprotonated His-57; in the other extreme, no transfer of a proton occurs, but the proximity of the carboxylate anion of Asp-102 to His-57 increases the basicity of the neutral imidazole, since it can stabilize the imidazolium ion and also properly position the imidazole functional group for maximum interaction with the hydroxyl group of Ser-195. The results of neutron diffraction[85] and NMR studies[86] are consistent with the resting form of the enzyme having an anionic carboxylate group at Asp-102. The role of the carboxylate group of Asp-102 has been probed by site-directed mutagenesis of the gene for the previously discussed trypsin from rat.[60]

Asp-102 → Asn. The pH dependence of the kinetic parameters for hydrolysis of the benzyl thioester of *N*-carbobenzoxy-L-lysine (Z-Lys-S-Bz) were compared for the wild type and Asn-102 mutant proteins. At pH 7.15, the ratios of k_{cat} and k_{cat}/K_m for the wild type enzyme relative to the asparagine substitution are 4 400 and 11 300, respectively; at pH 10.2, these ratios decrease dramatically to 18 and 152, respectively. That these decreases in catalytic properties result from a dimunition in the nucleophilicity of the hydroxyl group of Ser-195 is supported by the observation that the Asn-102 enzyme is approximately 10 000-fold less reactive to inactivation by diisopropyl fluorophosphate than the wild type enzyme. However, the rate of reaction of tosyl-L-lysine chloromethyl ketone, an active site titrant that reacts irreversibly with His-57, with Asn-102 was reduced only 5-fold at neutral pH and was little effected at alkaline pH when compared with the kinetics of inactivation of the wild type Asp-102 enzyme. Taken together, these data suggest that the greatest effect of replacing the carboxylate functional group with a carboxamide functional group is a decrease in the nucleophilicity of the serine hydroxyl group, with the imidazole functional group of His-57 remaining in the active site.

Measurements of the dependence of k_{cat} and k_{cat}/K_m on pH reveal that the wild type enzyme has a sigmoidal dependence on pH, with activity increasing to a constant value and a pK_a of 6.9 quantitatively describing the behavior; this pK_a may reflect the ionization of His-57. In contrast, the activity of the Asn-102 enzyme continues to increase with pH, with the rate of catalysis still increasing at the highest pH value examined (10.2). Careful quantitative analysis of the dependence of k_{cat}/K_m on pH for the mutant enzyme suggests contribution by two components: a low-pH component in which the activity increases sigmoidally, with a pK_a of 5.4 describing the behavior; and a high-pH component that continues to increase in magnitude. One interpretation of the low-pH component pK_a is that the pK_a of His-57 has been decreased by 1.5 pH units by the removal of the proximal carboxylate anion; the high-pH component is interpreted to describe the specific base-catalyzed hydrolysis of the acyl enzyme intermediate. If it is assumed that acylation of the hydroxyl group of Ser-195 is rate limiting for both enzymes at neutral pH, comparison of the rate constants for acylation suggests that acylation is 24 000-fold slower for the mutant enzyme than for the wild type but is estimated to be 400-fold greater than the uncatalyzed rate of hydrolysis of the thioester substrate. It is interesting to note that the characterization of the kinetic properties of the Asn-102 mutant has not assumed any profound change in mechanism; for example, if the nucleophilicity of the hydroxyl group of Ser-195 is greatly diminished, an interesting possibility is that acylation of the enzyme occurs on the imidazole functional group of His-57 rather than the hydroxyl group of Ser-195.

The Asn-102 is one of the few mutant enzymes for which high-resolution structural data have been reported.[61] Structures have been determined for mutant enzyme at two pH values, pH 6 (above the kinetically estimated pK_a of 5.4 for His-57 in the Asn-102 enzyme) and pH 8. The active site of the Asn-102 mutant of the rat enzyme is very similar to that of the bovine enzyme at pH 8. However at pH 6, the mutant enzyme shows disorder in the placement of the side chain of His-57, with model building suggesting an occupancy ratio of 2:1 for the native conformation relative to a conformation in which the imidazole side chain is rotated from the active site to interact with solvent. This altered conformation is presumed to reflect steric interactions in the active site between Asn-102 and the protonated imidazolium ion, which would not be present in the wild type enzyme. In particular, the carboxylate of Asp-102 interacts with both the imidazole of His-57 and with a backbone amide NH; the NH hydrogens of the carboxamide of Asn-102 must interact with the imidazole side chain of His-57, and the carbonyl oxygen must be a hydrogen-bond acceptor from the backbone NH. Thus, the carboxamide of Asn-102 can only interact with His-57 as a hydrogen-bond donor, the opposite role of the carboxylate of Asp-102 in the wild type enzyme. This implies the existence of a steric interaction with the protonated imidazolium ion that would force the occupancy of a different conformation by the side chain of His-57. This reasoning also has significant mechanistic implications. In the wild type enzyme, the nucleophilicity of the hydroxyl group of Ser-195 is assumed to be enhanced by hydrogen-bond donation to His-57 which, in turn, donates a hydrogen bond to the carboxylate of Asp-102; in the mutant enzyme, the side chain of His-57 can only accept a hydrogen bond from the carboxamide of Asn-102 and, as a result, can only donate a hydrogen bond to Ser-195. Thus, the decreased nucleophilicity of the hydroxyl group of Ser-195 in the Asn-102 mutant can be easily rationalized on the basis of the crystallographic data. It should be realized, however, that the crystallographic data are not based on the established positions of the extraordinarily crucial hydrogen atoms in the hydrogen bonds, which can only be obtained from neutron diffraction data.

3. Subtilisin BPN′ from *B. amyloliquefaciens*

The high-resolution crystallographic studies of subtilisin reveal the presence of a catalytic triad of active site residues (Asp-32, His-64, and Ser-221) reminiscent of that previously described for trypsin;[46] the importance of these residues in catalysis has not been examined for subtilisin. However, the X-ray studies of the serine proteases reveal other interactions involving active site functional groups that are presumed to be important in the formation and breakdown of the acylated enzyme intermediate. The mechanism of any serine protease catalyzed reaction is envisaged to involve the transient formation of tetrahedral intermediates both in the formation and hydrolysis of the acylated enzyme intermediate. The formation of these intermediates involves developing negative charge on the carbonyl oxygen of either the substrate or acylated enzyme intermediate, and the structural studies on a number of these proteases reveal the presence of a putative oxyanion hole in which the negative charge of the tetrahedral intermediate is stabilized by hydrogen bonding to hydrogen-bond donors provided by the protein. In the case of trypsin and chymotrypsin, backbone peptide NH bonds appear to serve as the hydrogen-bond donors in the oxyanion hole; obviously, the importance of these interactions cannot be assessed by site-directed mutagenesis. However, in subtilisin, one of the two hydrogen-bond donors in the oxyanion hole is provided by a carboxamide NH of Asn-155; the second is provided by the peptide NH of the catalytic triad Ser-221. The groups at Genentech and at Genex have used site-directed mutagenesis to probe the energetic importance of the interactions between Asn-221 and the tetrahedral intermediates occurring on the reaction pathway; analogous conclusions have been reported by both groups.

Asn-155 → Leu, Thr, Gln, Asp, and His. The Genex group reported a single amino acid substitution at residue-155, namely the isosteric leucine mutation.[51] The Genentech group reported four substitutions at residue-155, threonine, glutamine, aspartate, and histidine.[52] The leucine and threonine substitutions were observed to have similar phenotypes; the K_m for substrate (and, therefore, K_s, since acylation is rate determining) was unchanged, but k_{cat}s were decreased 200-fold and 2000-fold for the leucine and threonine enzymes, respectively. The lack of effect on K_m can be interpreted as an indication that active site geometry is effectively unchanged by the presence of the mutant side chains; however, the significant effect on k_{cat} certainly suggests that stabilization of the tetrahedral in-

termediate(s) does lower the activation energy for the reaction. In the case of the leucine substitution, no hydrogen-bonding interaction with the intermediate(s) is possible; hydrogen-bond donation from the threonine hydroxyl group is conceivable but considered to be unlikely given the presumed increase in distance between the oxyanion and the OH relative to the position of the carboxamide NH in asparagine. Similar decreases in k_{cat} were observed for the remaining three substitutions, which have either different steric requirements (glutamine and histidine) or different charge (aspartate and, potentially, histidine) than the wild type asparagine, but the K_m values for substrate were observed to decrease relative to that characteristic of the wild type enzyme. In the absence of crystallographic determinations of the position of these side chains in the mutant enzymes, a definitive explanation for the alterations of affinity for substrate cannot be made. However, if the decreases in k_{cat} are directly used as measures of the stabilization of the anionic tetrahedral intermediate by hydrogen-bond interactions with the asparagine carboxamide, all four mutations suggest that this interaction is worth approximately 4 kcal/mol in stabilization. This value is similar to that observed by Fersht for hydrogen-bonding interactions involving one charged and one uncharged hydrogen-bond participant.[31] However, it should be realized that no structural data are available for any of these mutant enzymes.

4. Carboxypeptidase A from Rat

Carboxypeptidase A is a zinc-dependent enzyme that can catalyze the hydrolysis of either amide or ester bonds of amino acids at the carboxy termini of oligopeptides. The mechanism of this monomeric enzyme (molecular weight 27 600) has been studied intensely and must be regarded as one of the most controversial subjects in modern mechanistic enzymology.[87-89] The high-resolution three-dimensional structures of the enzyme both in the absence and presence of a wide variety of inhibitors and substrate analogues are available as a result of studies in Lipscomb's laboratory.[90] These structures suggest that the side chains of Glu-270 and Tyr-248 can assume positions sufficiently close to the presumed position of bound substrate and the essential Zn^{2+} ion to be potentially involved in catalysis. Despite this rather limited selection of functional groups, the results of direct mechanistic studies as well as those of chemical modification have not allowed an unequivocal choice between the various proposed mechanisms. For example, in the context of the results obtained from reported mechanistic studies of site-directed mutants of carboxypeptidase, the role of Tyr-248 in catalysis is particularly interesting. Although in the unliganded enzyme Tyr-248 is removed from the immediate vicinity of the zinc ion, as judged by the crystallographic studies, the presence of bound ligand causes a conformational change that approximates the phenolic hydroxyl adjacent to the scissile bond. This tyrosine residue has been the subject of intense study by diverse types of chemical modification, with the resulting modified proteins usually having significantly decreased peptidase activity and increased esterase activity. These observations have suggested different mechanistic details for peptidase and esterase activities, with the phenolic group of Tyr-248 being *essential* for peptidase activity. For example, one explanation for this behavior is that hydrolysis of esters involves the departure of an alkoxide ion whereas that of amides involves the departure of an amide ion; since alkoxide ions are more stable than amide ions, their departure from the presumed tetrahedral intermediate need not involve protonation by a general acid, i.e., the phenolic group of Tyr-248.

Phenylalanine substitutions for Tyr-248[91,92] and Tyr-198,[93] a second active site tyrosine, have been made by Rutter and Craik in the cloned gene for carboxypeptidase A from rat. Just as the rat trypsin is homologous to bovine trypsin, the DNA sequence of carboxypeptidase A from rat[94] reveals that it is highly homologous to the bovine enzyme, which was the subject of the previously summarized mechanistic and structural scrutiny. Given the fact that the sequences of the two mammalian enzymes are 76% homologous and that the pertinent active site residues are perfectly conserved, the assumption has been made that characterization of the mechanistic properties of the wild type enzyme and its active site phenylalanine mutants will be directly applicable to the controversy surrounding the bovine enzyme.

Tyr-248 → Phe. The pH dependence of the kinetic constants for the phenylalanine substitution for tyrosine-248 was studied in detail for several amide and ester substrates.[91,92] For example, the peptide Cbz-Gly-Gly-Phe was found to be a reasonably good substrate for the mutant enzyme. The limiting (maximal) values for k_{cat} (dissected from analyses of their bell-shaped dependencies on pH) were found to be 51.9 and 20.0 s^{-1} for the wild type and phenylalanine mutant, respectively; for each enzyme the pH optimum for k_{cat} was approximately 8. A larger difference between the wild type and mutant enzyme was found for k_{cat}/K_m; the limiting value for the wild type enzyme was 150 000 M^{-1} s^{-1} while that for the mutant was 84 000 M^{-1} s^{-1}. As noted, the kinetic behavior of both enzymes revealed a descending basic limb for both k_{cat} and k_{cat}/K_m, and the detailed kinetic analyses revealed very little change in the apparent pK_a associated with the decrease in k_{cat} at high pH; earlier studies suggested that this ionization should be attributed to the phenolic hydroxyl group of Tyr-248. Clearly, the results obtained for the mutant enzyme invalidate this hypothesis and demonstrate that the phenolic hydroxyl group of Tyr-248 is not essential for the hydrolysis of peptide substrates. In addition, the wild type enzyme was observed to be subject to substrate inhibition at high concentrations of the tripeptide substrate. No such inhibition was observed for either the phenylalanine mutant or enzyme that had been acetylated with acetylimidazole; this reagent has been shown to acetylate both tyrosines-248 and -198. This observation implicates the phenolic hydroxyl group as being involved in but not totally responsible for binding interactions with the peptide substrate.

The pH dependence of the kinetics of hydrolysis of the ester substrate (*trans*-*p*-chlorocinnamoyl)-L-phenyllactate (ClCPL) by the mutant was also assessed. Again, this was a substrate for the mutant enzyme, and the limiting values for k_{cat} were 14.0 and 3.9 s^{-1} for the wild type and mutant enzymes, respectively; the limiting values for k_{cat}/K_m were 530 000 and 15 000 M^{-1} s^{-1}, respectively. In contrast to the essentially identical pH

dependencies for hydrolysis of the peptide substrate by the wild type and mutant enzymes, the pH dependencies for the hydrolysis of ClCPL differed for the two enzymes; whereas k_{cat} for the wild type enzyme had a basic *ascending* limb, no such increase in k_{cat} at high pH was observed for the mutant enzyme. The data demonstrate that the phenolic hydroxyl group of Tyr-248 is also not essential for the hydrolysis of ester substrates, although the differing kinetic behaviors observed at high pH are not understood. Although substrate inhibition does not occur for ClCPL, such data were reported for the more labile ester substrate benzoylglycyl-D,L-phenyllactic acid. The wild type enzyme is substrate inhibited at high concentrations of this ester substrate but acetylated wild type enzyme is not; however, the phenylalanine mutant is also substrate inhibited, but acetylation does not eliminate this kinetic anomaly. Thus, the phenolic hydroxyl group of Tyr-248 does not appear to be important in the binding of ester substrates (in contrast to the conclusion reached for peptide substrates).

The necessary conclusion that the phenolic hydroxyl group of Tyr-248 is *not essential* for hydrolysis of either peptide or ester substrates can be made confidently in the absence of structural information about the mutant enzyme. Unlike the uncertainty in interpretation of large decreases in catalytic activity that may be associated with amino acid substitutions of residues presumed to be important for catalysis, i.e., is the phenotype caused by an important conformational change of unknown but perhaps small magnitude in the mutant enzyme or is the amino acid residue really intimately involved in catalysis, the results that have been obtained on the Phe-248 mutant of carboxypeptidase are easy to interpret: the phenolic hydroxyl group of Tyr-248 is not essential for catalysis.

Tyr-198 → Phe and Tyr-198 and Tyr-248 → Phe-198 and Phe-248. Chemical modification studies also suggested that the phenolic hydroxyl group of Tyr-198 may participate in catalysis even though the crystallographic studies revealed that it appears to be too far away from the position of the scissile bond to directly participate in the hydrolysis reaction. Nonetheless, site-directed mutagenesis has been used to substitute this tyrosine with a phenylalanine as well as to construct the double mutant in which both Tyr-248 and -198 are replaced by phenylalanine residues.[93]

The kinetic parameters that were measured for the Phe-198 were mechanistically analogous to those described previously for the Phe-248 mutant. The limiting value for k_{cat} was found to be 32.4 s^{-1} for the tripeptide substrate, a value approximately two-thirds that observed for the wild type enzyme; the pH dependence of k_{cat} was bell shaped, and the apparent ionization constants derived from the kinetic analysis were similar to those measured for the wild type enzyme. The limiting value for k_{cat}/K_m was found to be 100 000 M^{-1} s^{-1}, a value two-thirds that observed for the wild type enzyme; again, the pH dependence of k_{cat}/K_m was bell shaped, with the apparent ionization constants again being similar to those found for the wild type enzyme. The ester ClCPL is also a good substrate for the Phe-198 mutant, the observed k_{cat} and k_{cat}/K_m values are decreased by approximately one-sixth and one-third, respectively, relative to the wild type enzyme. Again, the pH dependence of these kinetic constants were qualitatively similar to the behavior noted for the Phe-248 mutant, i.e., whereas k_{cat} for the wild type enzyme has an ascending basic limb, that for the Phe-198 mutant is pH independent at alkaline pH. For both the tripeptide and ester substrates, substrate inhibition was observed at high concentrations, and the observed values for K_m were seen to increase nearly 10-fold. The increase in K_m is consistent with the crystallographic results that place this residue in an amino acid subsite (S_2) and suggests the importance of hydrogen-bonding interactions between the enzyme and substrates in this subsite. Thus, the conclusion was reached that the phenolic hydroxyl group of Tyr-198 is not *essential* for catalysis. Taken together, the phenotypes of the Phe-248 and Phe-198 mutants provide an extraordinarily valuable demonstration that mechanistic conclusions based upon the results of chemical modification studies need not be correct. Fortunately, the amino acid substitutions at these positions did not perturb the active site geometry sufficiently that the question of the relevance of their phenotypes to the mechanism of the reaction catalyzed by the wild type enzyme was obscured.

The double mutant combining both phenylalanine substitutions in the active site was also constructed; interestingly, the observed effect on the kinetic parameters was found to be the additive composite of the two single mutants. This observation provides persuasive evidence that neither substitution alters the conformational properties of the active site region and that catalysis can proceed in the absence of both phenolic hydroxyl groups.

5. Triose Phosphate Isomerase from Chicken Muscle and Yeast

Triose phosphate isomerase catalyzes the *cis*-enediolate-mediated interconversion of dihydroxyacetone 3-phosphate (DHAP) and (*R*)-glyceraldehyde 3-phosphate (G3P). The reaction has received intense mechanistic scrutiny in the laboratories of Rose[95] and Knowles.[96] An active site base, identified initially by affinity labeling[97-99] and later by X-ray crystallography[100] as Glu-165 in the enzyme from chicken muscle, abstracts the *pro-R* proton from carbon-1 of DHAP or the 2-proton of G3P to generate the enediolate intermediate. The proton abstracted by the carboxylate of Glu-165 exchanges significantly with solvent prior to reprotonation to generate either DHAP or G3P. The complete free energy profile for the reaction catalyzed by the chicken muscle enzyme has been quantitated in Knowles' laboratory.

Triose phosphate isomerases from chicken muscle and from yeast are currently being scrutinized with the assistance of site-directed mutagenesis; Knowles' laboratory has reported work on the chicken muscle enzyme, and Petsko's laboratory is examining the yeast enzyme. Both enzymes are dimeric with monomer molecular weights of 26 000. The structures of both enzymes have been studied crystallographically. The structure of the chicken muscle enzyme has been determined to 2.5-Å resolution in Phillip's laboratory,[101] the structure of the unliganded enzyme from yeast has been solved to 1.9-Å resolution,[102] a complex of the yeast enzyme with substrates has been solved to 3.5 Å,[101] and a complex of the

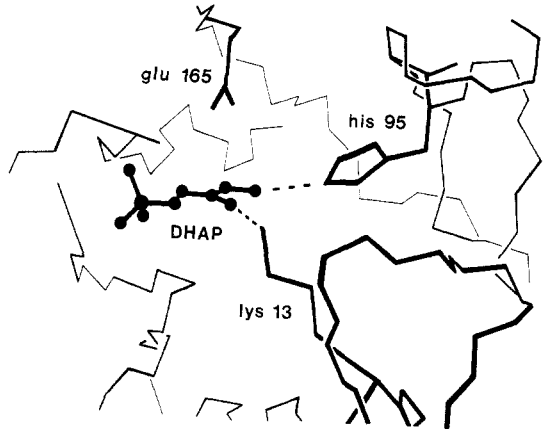

Figure 5. Active site of wild type chicken muscle triose phosphate isomerase showing the presumed position of the *cis*-enediolate of DHAP derived from model building. Reproduced with permission from ref 104. Copyright 1975 American Chemical Society.

yeast enzyme with the competitive inhibitor phosphoglycolohydroxamate has been solved to 1.6 Å[103] in Petsko's laboratory. These structures confirmed the presence of an active site glutamate (residue-165 in the chicken muscle enzyme) appropriately placed to shuttle a proton between carbons-1 and -2 in the substrates as well as two electrophilic residues that are presumed to interact with and stabilize the enediolate intermediate; the imidazole functional group of His-95 is appropriately located to interact with oxygen-1 of the intermediate, and the ϵ-ammonium group of Lys-13 is proximal to oxygen-2 (Figure 5). Earlier chemical[104] and spectroscopic[105] work in Knowles' laboratory suggested the presence of electrophilic catalysts in the active site. Site-directed mutagenesis based investigations are underway in Knowles' and Petsko's laboratories; the role of Glu-165 in catalysis has been scrutinized in Knowles's laboratory, and those of Lys-13 and His-95 are under study in Petsko's laboratory.

Glu-165 → Asp. The codon for Glu-165 in the chicken muscle enzyme was replaced with one for aspartate in Gilbert's laboratory; both the wild type and mutant enzymes are expressed in *E. coli*, although only the former can complement a chromosomal defect in the host triose phosphate isomerase.[106] The effect of the amino acid substitution on enzymatic activity was to decrease k_{cat} 240-fold in the direction of DHAP isomerization and 1500-fold in the direction of G3P isomerization; since the equilibrium constants for the wild type and mutant enzymes cannot differ, the K_m for DHAP was increased two-fold and the K_m for G3P was decreased 3.6-fold. No effect was noted on the binding constant for phosphoglycolate, a potent competitive inhibitor that is assumed to mimic the binding of the enediolate intermediate.

Subsequent more detailed investigation has allowed quantitation of the complete free energy profile for the reaction catalyzed by the Asp-165 mutant so that the effect of the amino acid substitution on catalysis can be more precisely described.[107] The predominant effect was on the rates of proton abstraction from enzyme-bound DHAP and G3P and the rates of protonation of the enediolate intermediate to form enzyme-bound DHAP and G3P. The rate-determining step in the reaction catalyzed by the wild type is dissociation of G3P from the enzyme in the direction of DHAP isomerization and binding of G3P to the enzyme in the direction of G3P isomerization;[108] in the reaction catalyzed by the Asp-165 mutant, the rate determining step in the direction of DHAP isomerization is abstraction of a proton from enzyme-bound DHAP, and in the reverse reaction the protonation of the enediolate intermediate to form enzyme-bound DHAP. Whereas the rate of the reaction catalyzed by the wild type enzyme is limited by the physical step of dissociation and binding of G3P, the reaction catalyzed by the Asp-165 mutant is limited by the rate of a chemical step. According to the criteria suggested by Albery and Knowles,[32] the wild type enzyme is perfect but the mutant enzyme is not. Careful controls have been performed to establish that such interpretations are correct. For example, the mutant enzyme is as rapidly inactivated by bromohydroxyacetone phosphate as the wild type enzyme, and no hydrolysis of the affinity label can be detected; in addition, no difference in solvent isotope effect could be detected in the reactions catalyzed by the wild type and mutant enzymes. Presumably, the catalytic activity of this mutant is decreased by the greater distance between the carboxylate group at residue-165 (considering the tetrahedral geometry of the methylene group that is, in effect, excised by this mutation, the distance between the carboxylate group and the bound substrates and intermediate should increase by about 1 Å); however, no crystallographic data are yet available for the mutant protein.

Experiments in progress in Knowles' laboratory at the time of the preparation of this review are attempting to improve the catalysis by the Asp-165 mutant so that it too might be perfect.[109] Random in vitro mutagenesis of the gene for the Asp-165 mutant has been performed, and the expectation that a second site mutation would significantly improve catalytic activity has been used as the necessary metabolic screen. As previously noted, the Asp mutant is unable to complement a triose phosphate isomerase deficiency in the host, and a selection based upon this metabolic block was used to detect the desired second site mutants (as well as a few true revertants). The mutation of Ser-96 to a proline residue was found to increase the activity of the Asp-165-containing protein about 25-fold such that this protein containing two mutations relative to the wild type is about 20-fold less active than wild type. As previously noted, His-95 is thought to act as an electrophilic catalyst, and model building suggests that proline at the adjacent residue could reposition the imidazole functional group of His-95 to bring the bound substrates and intermediates closer to the carboxylate group of Asp-165; this hypothesis is simply speculation given the absence of crystallographic characterization of this double mutant. Efforts are in progress to further improve the catalytic efficiency of the Ser-96–Asp-165 double mutant by the random introduction of additional mutations, thereby allowing assessment of whether perfection in catalysis can be achieved with either a glutamate or an aspartate side chain at residue-165.

His-95 → Gln. As noted previously, one of the electrophilic catalysts is His-95. The role of this residue in catalysis is being studied in Petsko's laboratory. The one substitution at this position that has been characterized in any detail, i.e., for which crystallographic

information is available, is the glutamine substitution.[103] Whereas His-95 could participate in catalysis by donation of a proton to the enediolate intermediate to form an enediol intermediate, Gln-95 cannot donate a proton and could only stabilize the enediolate intermediate by hydrogen-bonding interactions. The preliminary characterization of the kinetic properties of this mutant revealed that the rate of the reaction was decreased 100-fold. A 3.0-Å X-ray structure of the mutant protein complexed with the inhibitor phosphoglycolohydroxamate revealed that the side chain of Gln-95 was present in a conformation that differs from that characterizing the side chain of His-95 in the wild type, i.e., the phenotype of the mutant protein could not be used to definitively quantitate the role of His-95 in catalysis. This study serves to illustrate the problems associated with understanding the kinetic properties of mutant proteins that have significantly less catalytic activity than the wild type parent. Meaningful conclusions can only be reached if the active site structure and interactions between the enzyme functional groups and bound substrates (or inhibitors that mimic the binding of the substrate) of the mutant protein are structurally analogous to that of the wild type protein.

6. Aspartate Transcarbamoylase from *E. coli*

Aspartate transcarbamoylase (ATCase) catalyzes the first reaction of the pyrimidine biosynthetic pathway, the carbamoylation of aspartate by carbamoyl phosphate to form carbamoyl aspartate and inorganic phosphate. The enzyme from *E. coli* is end-product regulated (inhibited by CTP) and activated by ATP, a product of the purine biosynthetic pathway. Given these regulatory mechanisms, it is not surprising that ATCase should have an oligomeric subunit structure; the enzyme from *E. coli* is composed of two catalytic units (each composed of three identical polypeptide chains having a molecular weight of 33 000) and three regulatory units (each composed of two identical polypeptide chains having a molecular weight of 17 000). The complex allosteric properties of ATCase and its well-characterized but also complex subunit structure have been the subject of extensive literature on the allosteric structure–function relationships of the enzyme.[110–112] However, little attention has been directed toward understanding the chemical mechanism of and accompanying structure–function relationships important in the conversion of substrates to products. In the context of the focus of this review, the use of site-directed mutagenesis to further probe the structure–function relationships involved in the allosteric properties and regulation of ATCase will not be discussed in detail, although this use of mutagenesis is being actively pursued in Kantrowitz's laboratory.[113–115] Instead, the discussion will center on a novel use of site-directed mutagenesis that permits important insight into the structure of the active site.

High-resolution X-ray structures of ATCase have been determined under a variety of states of ligand binding. The structures of the unliganded enzyme[116] and that with the feedback inhibitor CTP[117] have been compared; in addition, the structure of the complex of the enzyme with the potent bisubstrate analogue inhibitor *N*-phosphonoacetyl L-aspartate (PALA) is also available.[118] The latter structure (reported at 2.9-Å resolution) reveals a striking conformational reorganization of the oligomeric enzyme, with the catalytic subunits moving apart by 12 Å and changing their relative orientation by 10° about their 3-fold axes (the two trimeric units are parallel to one another and at opposite ends of the molecule) and the regulatory subunits changing their relative orientation by 15° about their 2-fold axes. This structure also forms the basis for the hypothesis that the active site of ATCase shares residues from two catalytic subunits, since the binding site for PALA is found at the interface of two catalytic subunits. For example, the phosphonic acid moiety of PALA is proximal to Ser-52, and His-134 of the same subunit is adjacent to the carbonyl of the peptide bond. The carboxylate group of the aspartate moiety is proximal to Lys-84 of the adjacent catalytic chain. This structure may then provide an explanation for the observation that whereas the catalytic trimers possess catalytic activity, the fully dissociated monomeric polypeptides that comprise the catalytic trimer possess no detectable catalytic activity. Schachman's laboratory has investigated this question by site-directed mutagenesis and provided a potentially general solution for ascertaining whether active sites are present at subunit interfaces and require the participation of the amino acid functional groups present in each subunit. Although the presently available resolution of the structure of PALA with ATCase is likely to be insufficient for formulation of detailed structure–function relationships such as those that have been discussed for the remaining enzymes in this review, these studies are included to demonstrate another type of information about an active site that can be derived by the use of site-directed mutagenesis.

Ser-52 → His, Lys-84 → Gln, and His-134 → Ala. Schachman's strategy for demonstrating that the active site of ATCase is located at the interface between two identical polypeptides in the catalytic trimer is based upon the strategy of using site-directed mutagenesis to alter a functional group known to be at the interface and interacting with PALA; if this functional group has an important interaction with PALA, the catalytic activity of the mutant catalytic trimer should be significantly decreased. However, if the catalytic trimer is dissociated into inactive monomeric polypeptide chains and then allowed to reassemble in the presence of an equal amount of dissociated wild type enzyme, a statistical distribution of trimeric catalytic species should be formed (Figure 6): one-eighth of the resulting trimers should contain three wild type polypeptide chains; three-eighths of the trimers should contain two wild type polypeptides and one mutant polypeptide, three-eighths of the trimers should contain one wild type polypeptide and two mutant polypeptides, and one-eighth of the trimers should contain three mutant polypeptides. If the active site is located at the interface and is formed by residues contributed by each of two polypeptides, the trimer containing three wild type polypeptides will contain three catalytically competent active sites and, obviously, have a specific activity equal to that of undissociated wild type trimer; the trimer containing one mutant polypeptide will contain two catalytically competent active sites (one is catalytically incompetent due to the presence of a single mutant functional group) and have a specific activity equal to

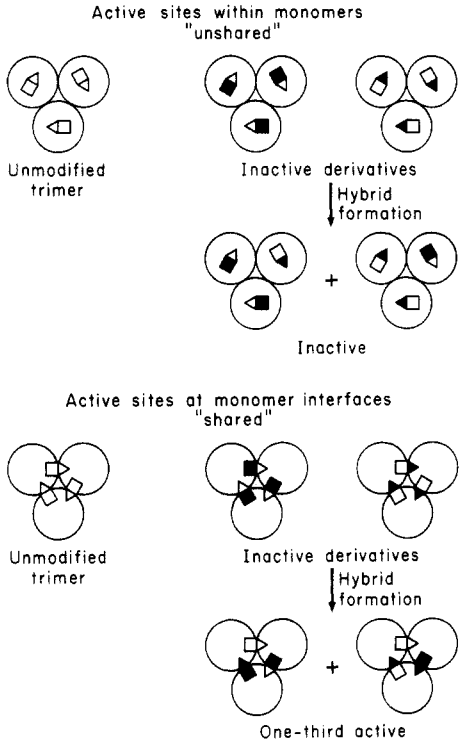

Figure 6. Schematic representation of the rationale for the subunit complementation test used to deduce whether the active sites of the catalytic trimers of aspartate transcarbamoylase require functional groups from adjacent subunits. Reproduced with permission from ref 117. Copyright 1984 National Academy of Sciences.

two-thirds of that of undissociated wild type trimer; the trimer containing two mutant polypeptides will contain one catalytically competent active site (two are catalytically incompetent due to the presence of two mutant functional groups, one at each of two interfaces) and have a specific activity equal to one-third of that of undissociated wild type trimer; and the trimer containing three mutant polypeptides will contain no catalytically competent active sites and be catalytically inactive.[119,120] By use of this reasoning, the specific activity of hybrid trimers prepared from two single mutant trimers can be predicted; for example, if two catalytically inactive catalytic trimers each containing a different single mutant are dissociated and allowed to reassociate, the two homotrimers would be catalytically inactive but each of the two hybrid trimers would be predicted to have a single interface containing only wild type functional groups and, therefore, a specific activity equal to one-third of that of wild type catalytic trimer. This study would be rendered essentially uninterpretable if it were impossible to separate the various hybrid trimers; this is possible if before dissociation one of the catalytic trimers is reversibly acylated with tetrahydrophthalic anhydride, which introduces additional negative charge and allows separation of species containing differing numbers of acylated polypeptide chains by ion-exchange chromatography.[121] Acylation, separation, and subsequent deacylation of the such separated trimers can be accomplished without an impact on enzymatic activity.

The Ser-52 → His mutant trimer has a specific activity 0.003% of that of the wild type trimer,[120] the Lys-84 → Gln mutant has a specific activity 0.008% of that of the wild type trimer, and the His-134 → Ala mutant has a specific activity 6% of that of the wild type trimer.[122] In accord with the previously described reasoning and predictions, two different types of hybrid catalytic trimers were prepared from catalytically deficient mutant trimers: the Ser-52 → His trimer mixed with the Lys-84 → Gln trimer and the Lys-84 → Gln trimer mixed with the His-134 → Ala trimer. In both cases, the hybrid trimers were found to have specific activities extremely close to the predicted one-third of the wild type trimer. As a control, an inactive homotrimer containing two mutants (Lys-84 → Gln and His-134 → Ala) on the same polypeptide was constructed and allowed to hybridize with wild type trimer; as predicted, the hybrid trimer containing a single doubly mutated polypeptide was one-third as active as the wild type catalytic trimer, and the hybrid trimer containing two doubly mutated polypeptides was inactive. This control persuasively eliminates an alternate explanation that the low catalytic activities of the mutant homotrimers result from conformational changes that are propagated to other regions of the structure rather than directly participating in catalysis by interacting with the bound substrates at the interfacial active site.

7. Alkaline Phosphatase from *E. coli*

Alkaline phosphatase is present in the periplasmic space of *E. coli* that is starved for inorganic phosphate and catalyzes the nonspecific hydrolyses of a wide range of phosphate monoesters. The mechnaism of the reaction catalyzed by this zinc-dependent enzyme has been studied in detail by a variety of chemical and physical approaches; the formation of a phosphorylated enzyme intermediate is accepted as an essential step on the reaction coordinate.[123] At low pH, the rate-limiting step of the phosphatase-catalyzed reaction is the dephosphorylation of this covalent intermediate, and the accumulation of the intermediate under these conditions[124,125] has allowed its identification as the phosphate monoester of Ser-102; at high pH, the rate-limiting step of the reaction is the release of noncovalently bound phosphate from the active site of the enzyme. The stereochemical course of the reaction catalyzed by the enzyme is known to be retention of configuration,[126] and this is consistent with the necessary formation of this intermediate.

The enzyme is a dimer of two identical polypeptides having molecular weights of 43 000. A moderate resolution (3.4-Å) X-ray structure of the enzyme has been reported,[127] and this reveals that the hydroxyl group of Ser-102 is in close proximity to one of the essential zinc ions and may be a ligand of this metal ion. Kaiser's laboratory has reported the use of site-directed mutagenesis to investigate whether other nucleophilic functional groups at residue-102 are catalytically competent.

Ser-102 → Cys. The active site hydroxyl group was replaced with a thiol group by mutating the active site serine to cysteine.[128] The mutant enzyme retained catalytic activity on a variety of substrates, but the V_{max} for the mutant was observed to be dependent upon the pK_a of the ester, with alcohols of lower pK_a having higher V_{max} values; as noted previously, the rate of the reaction catalyzed by the wild type enzyme is independent of the identity of the leaving group. This ob-

servation suggests that, in contrast to the wild type enzyme, the rate-determining step of the cysteine mutant is the phosphorylation of the active site thiol rather than the release of the phosphate product. Further evidence for this change in rate-determining step is the demonstration that whereas increases in the concentration of Tris buffer increases the rate of the wild type enzyme, the rate of the reaction catalyzed by the Cys-102 enzyme is independent of the concentration of Tris even though the enzyme is capable of using Tris rather than water as the phosphoryl group acceptor. Thus, just as in the case of triose phosphate isomerase,[107] the active site substitution sufficiently alters the free energy profile of the reaction to change the rate-determining step.

Unfortunately, little additional physical and structural characterization of this mutant has been performed. The active site thiol was found to be inaccessible to thiol-modifying reagents, raising the possibility that the thiol group is directly coordinated to the active site zinc, even though it is clearly accessible to substrates. Both NMR and X-ray studies would be useful to test this hypothesis, but the results of neither of these types of investigation have been reported.

8. Ribonuclease T_1 from *Aspergillus oryzae*

Ribonuclease T_1 catalyzes the hydrolysis of RNA via a mechanism involving the formation of a 2′,3′-cyclic nucleotide intermediate. This monomeric enzyme is composed of 104 amino acids, and the structure has been determined crystallographically in the presence of active site inhibitors.[129-131] On the basis of these structures, the carboxylate of Glu-58 is envisaged to act as a general base to abstract a proton from the 2′-hydroxyl group of a substrate, leading to the formation of the five-membered ring cyclic phosphate intermediate; the attack of water on this intermediate is base catalyzed by either His-40 or His-92, with the protonated Glu-58 providing a proton to the departing 2′-oxygen. This mechanism is being examined in Ikehara's laboratory by site-directed mutagenesis, except with this system mutations are being introduced into a chemically synthesized gene (RNase T_1 is a small protein) by incorporation of an appropriately mutated double-stranded DNA fragment.[132,133]

Glu-58 → Asp and Gln. Two amino acid substitutions have been made for the putative general base, the homologous aspartate and the isosteric glutamine.[133] Whereas the K_ms of the mutant enzymes for hydrolysis of pGpC are essentially identical with that measured for the wild type enzyme, the k_{cat} for the Asp mutant was decreased to about 10% of that observed for the wild type enzyme and that for the Gln mutant was decreased to about 1%. In contrast to the results previously described for Staphylococcal nuclease,[74,75] the Asp mutant retained easily measurable catalytic activity, and the Gln mutant was further depressed in catalytic activity. No assessment of the structural integrities of these mutants have been reported, so the direct involvement of these residues in catalysis remains equivocal; however, the simplest interpretation of the kinetic data is consistent with a perhaps surprising insensitivity of catalysis to an increased distance between the substrate and the putative general base. As noted previously, that these enzymes remain catalytically active even though a general base has been altered is consistent with current notions about the origins of the large rate accelerations observed in enzymic catalysis.

Additional Mutations. Other mutations of RNase T_1 have been generated in the base binding site (the enzyme is specific for guanine residues at the scissile bond); since these have been assayed only on a single substrate (pGpC), the impact of these on substrate recognition is unknown.[132] However, these mutations retained significant catalytic activity.

It is also of interest to note that the originally synthesized gene for RNase T_1 did not yield catalytically active protein. Although the gene was correctly synthesized on the basis of the reported protein sequence, the initially determined primary sequence for the protein was in error; when the protein sequence was corrected and the gene for this sequence was synthesized, catalytically active enzyme was obtained.

9. Cytochrome *c* from Yeast

Cytochrome *c* transfers electrons derived from NADH generated in aerobic metabolism to cytochrome *c* oxidase, the enzyme that catalyzes the reduction of diatomic oxygen. The redox active element in cytochrome *c* is an iron-containing heme, with the oxidized and reduced cytochrome containing Fe^{3+} and Fe^{2+}, respectively. The evolution of this protein is perhaps the most thoroughly characterized of any protein and, as a result, considerable primary sequence information is available for assessment of functionally conserved amino acid residues.[134]

Cytochrome *c* is a small protein, with eukaryotic versions commonly containing 104 amino acids. High-resolution X-ray structures are available for cytochrome *c* from a number of sources,[135-137] so considerable information is also available about the geometric relationships between the heme, amino acids proximal to the heme, and the surface of the molecule across which electrons must be transferred to proteinaceous electron acceptors. This information revealed that Phe-87, a phylogenetically conserved residue, is located both near the surface of the protein and also is 5 Å removed from the methionine-liganded side of the heme. This juxtapositioning of the phenyl ring of Phe-87 has lead to the hypothesis that its evolutionary conservation reflects an essential role for this residue in the transfer of electrons from cytochrome *c*. A collaborative effort involving the laboratories of Smith and of Mauk have employed site-directed mutagenesis to investigate the function of Phe-87 in yeast iso-1-cytochrome *c*.

Phe-87 → Tyr, Ser, and Gly. Three amino acid substitutions have been generated in yeast cytochrome *c*, and the biochemical properties of the mutant proteins have been scrutinized.[134-136] The initial studies demonstrated that all three mutant proteins functioned in vivo, since their presence in a cytochrome-deficient yeast host complemented the chromosomal deficiency when lactate or glycerol was used as carbon source; these growth conditions require the ability to reduce cytochrome oxidase by a functional cytochrome *c*.[138]

Recent investigations of these mutant proteins have quantitatively compared the rates at which electrons can be transferred from their reduced forms to an acceptor, cytochrome *c* peroxidase reconstituted with zinc protoporphyrin IX.[140] In this system, electron transfer

from the reduced cytochrome to the peroxidase is initiated by flash photolysis, which generates the triplet state of the zinc protoporphyrin and then reduces the oxidized form of the complexed cytochrome *c* and forms the π-cation radical of the protoporphyrin; subsequently, in a thermal process, the reduced cytochrome *c* transfers an electron back to the π-cation radical of the zinc proporphyrin to regenerate the complex of starting reactants. The thermal reduction of the π-cation radical by the reduced cytochrome *c* serves as a convenient way to measure the rate of electron transfer from the wild type and mutant cytochromes *c*.

The rates of photochemically induced reduction of the various cytochromes *c* were used as control reactions to assess whether any of the substitutions at residue-87 altered the structures of the complexes with cytochrome *c* peroxidase. The wild type protein and tyrosine and serine mutants were found to have identical rates of photochemically induced electron transfer whereas that of the glycine mutant was significantly less; this suggested that the geometries of the former complexes were similar but that the geometry of the complex with the glycine variant was perturbed. Accordingly, only the rates of electron transfer from the wild type and tyrosine and serine mutants to the π-cation radical were studied to assess the role of the invariant phenylalanine in the wild type protein.

The rates of transfer of the electron from the reduced cytochromes *c* to the π-cation radical were found to be equivalent for the wild type and Tyr-87 mutant; the rate of transfer from the Ser-87 mutant was reduced from these by a factor of 10000. A precise explanation for this observation is not possible: an aromatic residue may provide a conducting hole through which electron transfer is facilitated or a conformational change may occur, the magnitude and details of which would depend upon the volume of the side chain at residue-87 of cytochrome *c*. However, additional site-directed variants at this position should allow clarification of the function of the aromatic side chain in the transfer of electrons from reduced cytochrome *c*.

10. Cytochrome *c* Peroxidase from Yeast

Cytochrome *c* peroxidase catalyzes the two-electron reduction of hydroperoxides, using cytochrome *c* as the reductant to yield water and an alcohol. The enzyme from yeast is a monomeric enzyme containing 294 amino acids and ferric protoporphyrin IX as the redox cofactor in the resting state of the enzyme. This prosthetic group reacts with the peroxide to form an intermediate that is two electrons more oxidized than the resting state. In contrast to the analogous oxidation state of other peroxidases, which contain a ferryl center (Fe^{4+}=O) coupled to a porphyrin radical cation, the two-electron oxidized form of cytochrome *c* peroxidase contains no porphyrin radical cation; instead, an EPR signal ($g = 2$) that is weakly coupled to the ferryl center is observed, and this has been proposed to be associated with a reversibly oxidizable amino acid side chain.[141-143]

A high-resolution structure has been determined for cytochrome *c* peroxidase from yeast,[144] and this information has revealed the presence of both Trp-51 and Met-172 in close proximity to the protoporphyrin. The Trp-51 is located in a plane parallel to and 3.3 Å above the distal face of the heme; Met-172 is located 3.7 Å below the opposite face of the heme. The available physical data regarding the identity of the previously described EPR signal lead to the proposal that either one of these residues may be the site of the unpaired electron. Thus, cytochrome *c* peroxidase has been a logical system for the application of site-directed mutagenesis to probe the origin of the EPR signal. Simultaneous studies are being carried out in both Kraut's laboratory and those of Mauk and Smith.

Met-172 → Ser and Cys. Mauk and Smith reported two substitutions for Met-172, serine and cysteine.[145] Both mutants are catalytically active, and the optical spectra of the mutant proteins are not significantly different than that of the wild type enzyme. Furthermore, the EPR signal of the two-electron oxidized form Ser-172 mutant enzyme has the sharp $g = 2$ signal observed for the wild type enzyme, although careful examination of the spectrum reveals that the EPR spectrum of the wild type enzyme appears to be a composition of the sharp signal and a much broader signal; the EPR spectrum of the Ser-172 mutant does not appear to contain the broad component.

These data clearly demonstrate that the catalytic activity of cytochrome *c* peroxidase is not critically dependent upon Met-172 but suggest the presence of two distinct radical species, one of which may be associated with the presence of the methionine side chain. One explanation for this behavior is that the unpaired electron associated with the protein portion of the peroxidase is delocalized on more than one amino acid residue, with the broad component being associated with electron spin delocalized on the thioether sulfur. Alternatively, the serine substitution necessarily represents a significant change in side chain volume, and the consequences of this potential source of a conformational change on the properties of the radical center may represent an indirect effect on the spectrum such that the broad component should not be associated with the thioether sulfur. In either case, it is unlikely that the sharp signal should be associated with electron delocalization on Met-172.

Trp-51 → Phe. Kraut's laboratory has used site-directed mutagenesis to introduce a phenylalanine substitution for Trp-51, the other putative candidate for delocalized electron density;[146] phenylalanine should be less readily oxidized than tryptophan. Although many of the spectral properties of the Phe-51 mutant are similar to those of the wild type protein, the Soret absorption of the mutant occurs at a lower wavelength and with a lower extinction than the wild type enzyme. In addition, the half-life of the two-electron oxidized form of the mutant peroxidase at 23 °C is approximately 70-fold less than that of the oxidized form of the wild type peroxidase. The EPR spectrum of the two-electron oxidized form of the Phe-51 mutant protein is similar to that of the Ser-172 mutant protein, with the sharp $g = 2$ signal remaining but the broader component of the spectrum missing; this EPR spectrum was also observed to decay with a half-life similar to that observed in the visible spectrum. In addition, the EPR spectrum of the resting form of the Phe-51 mutant was observed to show predominantly high-spin iron whereas that of the wild type enzyme showed a mixture of low- and high-spin iron, with the former predominating.

Clearly the data for this mutant show that Trp-51 is not essential for catalytic activity, nor is it the exclusive site of the unpaired electron, although the change in shape of the broad component again suggests that Trp-51 does influence the spin properties of whatever the location of the unpaired electron may be. Again, this substitution is accompanied by a change in volume of the side chain of substituted amino acid side chain, and the effect of this likely source of a conformational change on the stability of the radical and its detailed electronic properties cannot be evaluated.

Certainly, the hunt for the sharp $g = 2$ EPR signal is continuing and provides an attractive and (hopefully) powerful use of site-directed mutagenesis to ascertain structure–function relationships in this peroxidase.

D. Summary

This review is contained in an issue of *Chemical Reviews* having the theme "Frontiers in Biological Chemistry". The inclusion of a review such as this is noteworthy for several reasons, and I hope that those who have taken the time to read this review will understand these summary comments.

First, the term "site-directed mutagenesis" may have biological connotations that presumably stem from the common view that any field or area of investigation that relies on the properties of DNA is too complex to be viewed as chemistry and, therefore, must be biology. However, this review purposely did not discuss the experimental procedures and strategies used in site-directed mutagenesis for the fear that the chemical reader might fail to properly recognize that the purpose of applying these procedures is to allow the basic principles of experimental physical organic chemistry and reaction mechanisms to be applied to enzymatic catalysis by variation of the structure of the enzyme molecule. The mechanistic enzymologists who are using site-directed mutagenesis as one experimental technique among the many in their repertoire for understanding the mechanisms of enzymatic reactions are, by and large, physical organic chemists and not biologists by training.

Second, the new field in biological chemistry described in this review is truly at a frontier. The author considers a frontier in science to be an area that is being explored by only a few but will soon be populated by many. The examples discussed in this review are unfortunately but necessarily not an exhaustive account of all those under exploration but represent the studies being performed on the structurally and chemically best understood systems. In effect, the frontier exploration of the utility of site-directed mutagenesis in understanding problems in mechanistic enzymology must focus on those systems that are already well characterized so that the properties of mutant enzymes might be more easily understood. With at least some convincing results from such systems, the more daring may venture into less characterized systems with at least a modicum of confidence that some new insights can be achieved.

However, the primary lesson that is being repeatedly learned by those already using site-directed mutagenesis is that the weakest but most important aspect to success in its utilization is not the ability to generate and produce mutant proteins but to perform the structural characterizations of the mutant proteins so that their chemical and kinetic properties can be definitively understood. Much of the data discussed in this review cannot be definitively explained in the absence of crystal structures for essentially each and every mutant protein. Presumably, only those amino acid substitutions that create no change in phenotype (as defined by identity of the free energy profiles for the mutant and wild type enzymes) can be safely interpreted without a plethora of structural data. In fact, many of the hypotheses on which the studies were based have not yielded the unambiguous results that might have been desired. This situation is true both in those cases when rational alteration in substrate specificity was sought and when residues known to be essential in catalysis had been replaced with even functionally conserved substitutions; certainly many of the uncertainties in interpretation will be understood only when the structures of the mutant proteins are known. However, the question of what information is necessary to adequately describe the structure of a protein so that its catalytic properties can be explained looms as the major barrier to interpretation of the properties of mutant proteins. Will X-ray crystallography yield adequate descriptions of structure, or does this technique yield structural information for only one conformer of the population of conformers likely to be present in solution? Will NMR techniques be developed for proteins as large as most enzyme molecules of interest, and does the possibility exist that this technique will yield structural information for both the average conformation of a population of molecules as well as a subset of this population that may represent the catalytically competent molecules? Depending upon the point of view of the reader, these concerns may appear unrealistic, but the reality of the situation is that the ability to easily generate enzymes with known amino acid substitutions at specific sites is generating concern that both our techniques for assessing structure and our ability for predicting and understanding the structural consequences of introducing amino substitutions may be seriously inadequate. Given the recognition that inadequacies in our understanding of the principles of protein structure exist and, therefore, may explain our inability to predict and explain the catalytic properties of mutant enzymes, the determination of the detailed relationships between catalysis and enzyme structure is necessarily at a frontier. Further exploration of this frontier is certain to be both challenging and exciting.

E. Acknowledgments

I express my thanks to the many laboratories using site-directed mutagenesis to probe the relationships between enzyme structure and function for supplying a deluge of unpublished information. I also express my apologies for the stringent criteria used in the selection of topics and, therefore, for not discussing all of the applications of site-directed mutagenesis to mechanistic and structural enzymology. The research discussed in this review that was performed in my laboratory was generously supported by the National Institues of Health (Grant GM-34573).

Registry No. Tyrosyl tRNA synthetase, 9023-45-4; dihydrofolate reductase, 9002-03-3; trypsin, 9002-07-7; aspartate aminotransferase, 9000-97-9; staphylococcal nuclease, 9013-53-0;

carboxypeptidase A, 11075-17-5; triose phosphate isomerase, 9023-78-3; aspartate transcarbamylase, 9012-49-1; alkaline phosphatase, 9001-78-9; cytochrome *c*, 9007-43-6; cytochrome *c* peroxidase, 9029-53-2; subtilisin, 9014-01-1; ribonuclease T_1, 9026-12-4.

F. References

(1) Jencks, W. P. *Catalysis in Chemistry and Enzymology*; McGraw-Hill: New York, 1969.
(2) Brønsted, J. N.; Pedersen, K. *Z. Phys. Chem. Stoechiom. Verwandtschaftsl.*, **1923**, *A108*, 185.
(3) Page, M. I.; Jencks, W. P. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 1678.
(4) Bruice, T. C.; Brown, A.; Harris, D. C. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 658.
(5) Page, M. I. *Chem. Soc. Rev.* **1973**, *2*, 295.
(6) Fersht, A. L. *Enzyme Structure and Mechanism*, 2nd ed.; Freeman: New York, 1985.
(7) Zerner, B.; Bond, R. P.; Bender, M. L. *J. Am. Chem. Soc.* **1964**, *86*, 3674.
(8) Floss, H. G.; Tsai, M. D. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1979**, *50*, 243.
(9) Gerlt, J. A.; Coderre, J. A.; Mehdi, S. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1983**, *55*, 291.
(10) Reynolds, L. J.; Garcia, G. A.; Kozarich, J. W.; Kenyon, G. L. *Biochemistry*, **1987**, in press.
(11) Harris, G.; Ashley, G. W.; Robins, M. J.; Tolman, R.; Stubbe, J. *Biochemistry*, **1987**, *26*, 1895.
(12) Walsh, C. T. *Tetrahedron* **1982**, *38*, 871.
(13) Hansch, C. In *Drug Design*; Ariëns, E. J., Ed.; Academic: New York, 1971; Vol. 1, p 271.
(14) Means, G. E.; Feeney, R. E. *Chemical Modification of Proteins*; Holden-Day: San Francisco, 1971.
(15) Itakura, K.; Rossi, J. J.; Wallace, R. B. *Annu. Rev. Biochem.* **1984**, *53*, 323.
(16) Smith, M. *Annu. Rev. Genet.* **1985**, *19*, 325.
(17) Botstein, D.; Shortle, D. *Science (Washington, D.C.)* **1985**, *229*, 1193.
(18) Carter, P. *Biochem. J.* **1986**, *237*, 1.
(19) Lowe, G.; Tansley, G. *Tetrahedron* **1984**, *40*, 113.
(20) Winter, G.; Koch, G. L. E.; Hartley, B. S.; Barker, D. G. *Eur. J. Biochem.* **1983**, *132*, 383.
(21) Blow, D. M.; Brick, P. (1985) In *Biological Macromolecules and Assemblies: Nucleic Acids and Interactive Proteins*; Jurnak, F., McPherson, A., Ed.; Wiley: New York, 1985; Vol. 2, p 442.
(22) Rubin, J.; Blow, D. M. *J. Mol. Biol.* **1981**, *145*, 489.
(23) Monteilhet, C.; Blow, D. M. *J. Mol. Biol.* **1978**, *122*, 407.
(24) Waye, M. Y. M.; Winter, G.; Wilkinson, A. J.; Fersht, A. R. *EMBO J.* **1983**, *2*, 1827.
(25) Leatherbarrow, R. J.; Fersht, A. R.; Winter, G. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 7840.
(26) Winter, G.; Fersht, A. R.; Wilkinson, A. J.; Zoller, M.; Smith, M. *Nature (London)* **1982**, *299*, 756.
(27) Wells, T. N. C.; Fersht, A. R. *Nature (London)* **1985**, *316*, 656.
(28) Wells, T. N. C.; Fersht, A. R. *Biochemistry* **1986**, *25*, 1881.
(29) Wilkinson, A. J.; Fersht, A. R.; Blow, D. M.; Carter, P.; Winter, G. *Nature (London)* **1984**, *307*, 187.
(30) Ho, C. K.; Fersht, A. R. *Biochemistry* **1986**, *25*, 1891.
(31) Fersht, A. R.; Shi, J.-P.; Knill-Jones, J.; Lowe, D. M.; Wilkinson, A. J.; Blow, D. M.; Brick, P.; Carter, P.; Waye, M. M. Y.; Winter, G. *Nature (London)* **1985**, *314*, 235.
(32) Albery, W. J.; Knowles, J. R. *Biochemistry* **1976**, *15*, 5631.
(33) Carter, P. J.; Winter, G.; Wilkinson, A. J.; Fersht, A. R. *Cell* **1984**, *38*, 835.
(34) Fersht, A. R.; Leatherbarrow, R. J.; Wells, T. N. C. *Nature (London)* **1986**, *322*, 284.
(35) Jencks, W. P. *Chem. Rev.* **1985**, *85*, 511.
(36) Bolin, J. T.; Filman, D. J.; Matthews, D. A.; Hamlin, R. C.; Kraut, J. *J. Biol. Chem.* **1982**, *257*, 13650.
(37) Filman, D. J.; Bolin, J. T.; Matthews, D. A.; Kraut, J. *J. Biol. Chem.* **1982**, *257*, 13663.
(38) Villafranca, J. E.; Howell, E. E.; Voet, D. H.; Strobel, M. S.; Ogden, R. C.; Abelson, J. N.; Kraut, J. *Science (Washington, D.C.)* **1983**, *222*, 782.
(39) Howell, E. E.; Villafranca, J. E.; Warren, M. S.; Oatley, S. J.; Kraut, J. *Science (Washington, D.C.)* **1986**, *231*, 1123.
(40) London, R. E.; Howell, E. E.; Warren, M. S.; Kraut, J.; Blakley, R. L. *Biochemistry* **1986**, *25*, 7229.
(41) Fierke, C. A.; Johnson, K. A.; Benkovic, S. J. *Biochemistry* **1987**, *26*, 4085.
(42) Chen, J.-T.; Mayer, R. J.; Fierke, C. A.; Benkovic, S. J. *J. Cell. Biochem.* **1985**, *29*, 73.
(43) Chen, J.-T.; Taira, K.; Tu, C.-P. D.; Benkovic, S. J. *Biochemistry* **1987**, *26*, 4093.
(44) Mayer, R. J.; Chen, J.-T.; Taira, K.; Fierke, C. A.; Benkovic, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 7718.
(45) Robertus, J. D.; Alden, R. A.; Birktoft, J. J.; Kraut, J.; Powers, J. C.; Wilcox, P. E. *Biochemistry* **1972**, *11*, 2439.
(46) Robertus, J. D.; Kraut, J.; Alden, R. A.; Birktoft, J. J. *Biochemistry* **1972**, *11*, 4293.
(47) Matthews, D. A.; Alden, R. A.; Birktoft, J. J.; Freer, S. T.; Kraut, J. *J. Biol. Chem.* **1975**, *250*, 7120.
(48) Poulos, T. L.; Alden, R. A.; Freer, S. T.; Birktoft, J. J.; Kraut, J. *J. Biol. Chem.* **1976**, *251*, 1097.
(49) Estell, D. A.; Graycar, T. P.; Wells, J. A. *J. Biol. Chem.* **1985**, *260*, 6518.
(50) Bryan, P. N.; Rollence, M. L.; Pantoliano, M. W.; Wood, J.; Finzel, B. C.; Gilliland, G. L.; Howard, A. J.; Poulos, T. L. *Proteins: Struct. Funct. Genet.* **1986**, *1*, 326.
(51) Bryan, P.; Pantoliano, M. W.; Quill, S. G.; Hsiao, H.-Y.; Poulos, T. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 3743.
(52) Wells, J. A.; Cunningham, B. C.; Graycar, T. P.; Estell, D. A. *Philos. Trans. R. Soc. London A* **1986**, *317*, 415.
(53) Estell, D. A.; Graycar, T. P.; Miller, J. V.; Powers, D. B.; Burnier, J. P.; Ng, P. G.; Wells, J. A. *Science (Washington, D.C.)* **1986**, *233*, 659.
(54) Wells, J. A.; Powers, D. B.; Bott, R. R.; Graycar, T. P.; Estell, D. A. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 1219.
(55) Bode, W.; Schwager, P.; Huber, R. *Miami Winter Symp.* **1976**, *11*, 43.
(56) Bode, W.; Schwager, P. *J. Mol. Biol.* **1975**, *98*, 593.
(57) Bode, W.; Walter, J.; Huber, R.; Wenzel, H. R.; Tschesche, H. *Eur. J. Biochem.* **1984**, *144*, 185.
(58) Craik, C. S.; Largman, C.; Fletcher, T.; Roczniak, S.; Barr, P. J.; Fletterick, R.; Rutter, W. J. *Science (Washington, D.C.)* **1985**, *228*, 291.
(59) Graf, L.; Craik, C. S.; Patthy, A.; Roczniak, S.; Fletterick, R.; Rutter, W. J. *Biochemistry* **1987**, *26*, 2616.
(60) Craik, C. S.; Roczniak, S.; Largman, C.; Rutter, W. J. *Science (Washington, D.C.)* **1987**, in press.
(61) Sprang, S.; Standing, T.; Fletterick, R.; Finer-Moore, J.; Stroud, R.; Xhoung, N.-H.; Hamlin, R.; Rutter, W. J.; Craik, C. S. *Science (Washington, D.C.)*, **1987**, in press.
(62) Craik, C. S.; Choo, Q.-L.; Swift, G. H.; Quinto, C.; MacDonald, R. J.; Rutter, W. J. *J.Biol. Chem.* **1984**, *259*, 14255.
(63) Warshel, A.; Sussman, F. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 3806.
(64) Inagami, T.; Sturtevant, J. *J. Biol. Chem.* **1960**, *235*, 1019.
(65) Vajda, T.; Szabo, T. *Acta Biochim. Biophys. Acad. Sci. Hung.* **1976**, *11*, 287.
(66) Smith, D. L.; Ringe, D.; Finlayson, W. L.; Kirsch, J. F. *J. Mol. Biol.* **1986**, *191*, 301.
(67) Kirsch, J. F.; Ringe, D., personal communication.
(68) Kirsch, J. F.; Eichele, G.; Ford, G. C.; Vincent, M. G.; Jansonius, J. N.; Gehring, H.; Christen, P. *J. Mol. Biol.* **1984**, *174*, 497.
(69) Cronin, C. N.; Malcolm, B. A.; Kirsch, J. F. *J. Am. Chem. Soc.* **1987**, *109*, 2222.
(70) Cotton, F. A.; Hazen, E. E.; Legg, M. J. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 2551.
(71) Mehdi, S.; Gerlt, J. A. *J. Am. Chem. Soc.* **1982**, *103*, 3223.
(72) Serpersu, E. H.; Shortle, D.; Mildvan, A. S. *Biochemistry* **1986**, *25*, 68.
(73) Serpersu, E. H.; Shortle, D.; Mildvan, A. S. *Biochemistry* **1987**, *26*, 1289.
(74) Hibler, D. W.; Stolowich, N. J.; Gerlt, J. A. In *Mechanisms of Enzymatic Reactions: Stereochemistry*; Frey, P. A., Ed.; Elsevier: New York, 1985; pp 101–114.
(75) Hibler, D. W.; Stolowich, N. J.; Reynolds, M. A.; Gerlt, J. A.; Wilde, J. A.; Bolton, P. H. *Biochemistry*, **1987**, in press.
(76) Calderon, R. O.; Stolowich, N. J.; Gerlt, J. A.; Sturtevant, J. M. *Biochemistry* **1985**, *24*, 6044.
(77) Shortle, D.; Lin, B. *Genetics* **1985**, *110*, 539.
(78) Reynolds, M. A.; Gerlt, J. A., unpublished observations.
(79) Shortle, D., personal communication.
(80) Shaw, E.; Mares-Guia, M.; Cohen, W. *Biochemistry* **1965**, *4*, 2219.
(81) Dixon, G. H.; Neurath, H. *Biochim. Biophys. Acta* **1956**, *19*, 193.
(82) Birktoft, J. J.; Blow, D. M. *J. Mol. Biol.* **1972**, *68*, 187.
(83) Tulinsky, A.; Vandlen, R. L.; Morimoto, C. N.; Mani, N. V.; Wright, L. H. *Biochemistry* **1973**, *12*, 4185.
(84) Krieger, M.; Kay, L. M.; Stroud, R. M. *J. Mol. Biol.* **1974**, *83*, 185.
(85) Kosiakoff, A. A.; Spencer, S. A. *Nature (London)* **1980**, *288*, 414.
(86) Markley, J. L.; Ibanez, I. B. *Biochemistry* **1978**, *22*, 4627.
(87) Lipscomb, W. N. *Ann. Rev. Biochem.* **1983**, *52*, 17–34.
(88) Vallee, B. L.; Galdes, A. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1984**, *56*, 283.
(89) Makinen, M. W.; Wells, G. B.; Kang, S.-O. *Adv. Inorg. Biochem.* **1983**, *6*, 1.
(90) Rees, D. C.; Lewis, M.; Lipscomb, W. N. *J. Mol. Biol.* **1983**, *168*, 367.
(91) Gardell, S. J.; Craik, C. S.; Hilvert, D.; Urdea, M. S.; Rutter, W. J. *Nature (London)* **1985**, *317*, 551.

(92) Hilvert, D.; Gardell, S. J.; Rutter, W. J.; Kaiser, E. T. *J. Am. Chem. Soc.* **1986**, *108*, 5298.
(93) Gardell, S. J.; Hilvert, D.; Barnett, J.; Kaiser, E. T.; Rutter, W. J. *J. Biol. Chem.* **1987**, *262*, 576.
(94) Quinto, C.; Quiroga, M.; Swain, W. F.; Nikovits, W. C.; Standring, D. N.; Picket, R. L.; Valenzuela, P.; Rutter, W. J. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 31.
(95) Rose, I. A. *Philos. Trans. R. Soc. London B* **1981**, *293*, 131.
(96) Albery, W. J.; Knowles, J. R. *Biochemistry* **1976**, *15*, 5631.
(97) Hartman, F. C. *J. Am. Chem. Soc.* **1970**, *92*, 2170.
(98) Coulson, A. F. W.; Knowles, J. R.; Priddle, J. D.; Offord, R. E. *Nature (London)* **1970**, *227*, 180.
(99) Waley, S. G.; Miller, J. C.; Rose, I. A.; O'Connell, E. L. *Nature (London)* **1970**, *227*, 181.
(100) Alber, T.; Banner, D. W.; Bloomer, A. C.; Petsko, G. A.; Phillips, D. C.; Rivers, S. P.; Wilson, I. A. *Philos. Trans. R. Soc. London B* **1981**, *293*, 159.
(101) Banner, D. W.; Bloomer, A. C.; Petsko, G. A.; Phillips, D. C.; Pogson, C. I.; Corran, P. H.; Furth, A. J.; Milman, J. D.; Offord, R. E.; Priddle, J. D.; Waley, S. G. *Nature (London)* **1975**, *255*, 609.
(102) Alber, T.; Hartman, F. C.; Petsko, G. A., personal communication.
(103) Davenport, R. C.; Petsko, G. A., personal communication.
(104) Webb, M. R.; Knowles, J. R. *Biochemistry* **1975**, *14*, 4692.
(105) Belasco, J. G.; Knowles, J. R. *Biochemistry* **1980**, *19*, 472.
(106) Straus, D.; Raines, R.; Kawashima, E.; Knowles, J. R.; Gilbert, W. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 2272.
(107) Raines, R. T.; Sutton, E. L.; Straus, D. R.; Gilbert, W.; Knowles, J. R. *Biochemistry* **1986**, *25*, 7142.
(108) Albery, W. J.; Knowles, J. R. *Biochemistry* **1976**, *15*, 5627.
(109) Hermes, J. D.; Knowles, J. R., personal communication.
(110) Gerhart, J. C. *Curr. Top. Cell. Regul.* **1971**, *2*, 275.
(111) Jacobson, G. R.; Stark, G. R. *Enzymes* **1973**, *9*, 225.
(112) Schachman, H. K. *Harvey Lect.* **1974**, *68*, 67.
(113) Smith, K. A.; Nowlan, S. F.; Middleton, S. A.; O'Donovan, C.; Kantrowitz, E. R. *J. Mol. Biol.* **1986**, *189*, 227.
(114) Middleton, S. A.; Kantrowitz, E. R. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 5866.
(115) Ladjimi, M. M.; Kantrowitz, E. R. *J. Biol. Chem.* **1987**, *262*, 312.
(116) Honzatko, R. B.; Crawford, J. L.; Monaco, H. L.; Ladner, J. E.; Edwards, B. F. P.; Evan, D. R.; Warren, S. G.; Wiley, D. C.; Ladner, R. C.; Lipscomb, W. N. *J. Mol. Biol.* **1982**, *160*, 219.
(117) Ke, H.-M.; Honzatko, R. B.; Lipscomb, W. N. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 4037.
(118) Krause, D. L.; Volz, K. W.; Lipscomb, W. N. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 1643.
(119) Robey, E. A.; Schachman, H. K. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 361.
(120) Wente, S. R.; Schachman, H. K. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 31.
(121) Gibbons, I.; Schachman, H. K. *Biochemistry* **1976**, *15*, 52.
(122) Robey, E. A.; Wente, S. R.; Markby, D. W.; Flint, A.; Yang, Y. R.; Schachman, H. K. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 5934.
(123) Coleman, J. E.; Gettins, P. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1983**, *55*, 381.
(124) Schwartz, J.; Lipmann, F. *Proc. Natl. Acad. Sci. U.S.A.* **1961**, *47*, 1996.
(125) Engstrom, L. *Biochim. Biophys. Acta* **1962**, *56*, 606.
(126) Jones, S. R.; Kindman, L. A.; Knowles, J. R. *Nature (London)* **1978**, *275*, 564.
(127) Wyckoff, H. A.; Handschumacher, M.; Murthy, K.; Sowadski, J. M. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1983**, *55*, 453.
(128) Ghosh, S. S.; Bock, S. C.; Rokita, S. E.; Kaiser, E. T. *Science (Washington, D.C.)* **1986**, *231*, 145.
(129) Heinemann, U.; Saenger, W. *Nature (London)* **1982**, *299*, 27.
(130) Sugio, S.; Amisaki, T.; Ohishi, H.; Tomita, K.; Heinemann, U.; Saenger, W. *FEBS Lett.* **1985**, *181*, 129.
(131) Sugio, S.; Oka, K.; Ohishi, T.; Tomita, K.; Heinemann, U.; Saenger, W. *FEBS Lett.* **1985**, *183*, 115.
(132) Ikehara, M.; Ohtsuka, E.; Tokunaga, T.; Nishikawa, S.; Uesugi, S.; Tanaka, T.; Aoyama, Y.; Kikyodani, S.; Fujimoto, K.; Yanase, K.; Fuchimura, K.; Morioka, H. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 4695.
(133) Nishikawa, S.; Morioka, H.; Fuchimura, K.; Tanaka, T.; Uesugi, S.; Ohtsuka, E.; Ikehara, M. *Biochem. Biophys. Res. Commun.* **1986**, *138*, 789.
(134) Dickerson, R. E.; Timkovich, R. *Enzymes* **1975**, *11*, 397.
(135) Dickerson, R. E.; Takano, T.; Eisenberg, D.; Kalai, O. B.; Samson, L.; Cooper, A.; Margoliash, E. *J. Iol. Chem.* **1971**, *246*, 1511.
(136) Takano, T.; Dickerson, R. E. *J. Mol. Biol.* **1981**, *153*, 95.
(137) Ochi, H.; Hata, Y.; Tanaka, N.; Kakudo, M.; Sakurai, T.; Aihara, S.; Morita, Y. *J. Mol. Biol.* **1983**, *166*, 407.
(138) Pielak, G. J.; Mauk, A. G.; Smith, M. *Nature (London)* **1985**, *313*, 152.
(139) Pielak, G. J.; Oikawa, K.; Mauk, A. G.; Smith, M.; Kay, C. M. *J. Am. Chem. Soc.* **1986**, *108*, 2724.
(140) Liang, N.; Pielak, G. J.; Mauk, A. G.; Smith, M.; Hoffman, B. M. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 1249.
(141) Yonetani, T.; Schleyer, H.; Ehrenberg, A. *J. Biol. Chem.* **1966**, *241*, 3240.
(142) Wittenberg, B. A.; Kampa, L.; Wittenberg, J. B.; Blumberg, W. E.; Peisach, J. *J. Biol. Chem.* **1968**, *243*, 1863.
(143) Hoffman, B. M.; Roberts, J. E.; Brown, T. G.; Kang, C. H.; Margoliash, E. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 6132.
(144) Finzel, B. C.; Poulos, T. L.; Kraut, J. *J. Biol. Chem.* **1984**, *259*, 13027.
(145) Goodin, D. B.; Mauk, A. G.; Smith, M. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 1295.
(146) Fishel, L. A.; Villafranca, J. E.; Maura, J. M.; Kraut, J. *Biochemistry* **1987**, *26*, 351.