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Dissection of the Structure and Activity of the Tyrosyl-tRNA Synthetase by Site-Directed Mutagenesis†

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ABSTRACT: To understand an enzyme reaction, one has to characterize the bound substrates, intermediates, products, and transition states on the reaction pathway and determine the interaction energies between them and the enzyme as the reaction proceeds. Site-directed mutagenesis is invaluable in this task, enabling the systematic dissection of the active site. Residues involved in catalysis may be detected and the energetics probed. The contributions of each hydrogen-bonding site chain in the active site of the tyrosyl-tRNA synthetase to binding and catalysis are being determined by making sensible mutations, which remove defined interactions with the substrates. The difference in free energy between complexes of wild-type and mutant enzymes gives the apparent binding energy of the relevant side chain in each complex. By this means, the following have been determined: the contributions of different types of hydrogen bonds to specificity; their roles in catalysis; the importance of enzyme–substrate versus enzyme–transition-state versus enzyme–intermediate complementarity; the fine tuning of enzyme catalysis during "evolution"; and the existence of linear free energy relationships between rate and binding constants. Prior to these studies, the mechanism of activation by the aminoacyl-tRNA synthetases was totally unknown. It is now seen that catalysis results solely from the use of binding energy. There are residues that do not bind the substrates in the ground state but stabilize just the transition state, consistent with the classical ideas of Haldane and Pauling of enzyme–transition-state complementarity. There are, however, regions of the protein that bind the ribose ring more tightly in the intermediate tyrosyl adenylate than in the transition state. This is to stabilize the intermediate against dissociation and to increase its concentration relative to bound ATP and tyrosine. Portions of the enzyme apparently far removed from the substrates have been detected by mutagenesis to participate in catalysis via movement of flexible loops. The gross structure of the enzyme has also been probed. The crystalline enzyme is a symmetric dimer that binds 2 mol of substrates. The enzyme in solution, however, exhibits half-of-the-sites activity. Experiments on heterodimers constructed from various combinations of mutant and wild-type subunits have revealed that tRNA binds to the enzyme by spanning both subunits. There is also preexisting asymmetry in the enzyme in solution.

In 1982, the catalytic mechanism of the tyrosyl-tRNA synthetase and all other aminoacyl-tRNA synthetases was completely unknown. Many years of investigation of the 20 different aminoacyl-tRNA synthetases by classical protein chemistry and kinetics had failed to reveal acidic or basic groups involved in catalysis. The first examination of the crystal structure also gave no clues to catalytic mechanism, unlike the studies on lysozyme, carboxypeptidase, ribonuclease, and chymotrypsin in the late 1960s. Five years later, thanks to site-directed mutagenesis, we know far more about the mechanism and energetics of the activation of tyrosine than we do about most enzymatic reactions. Piecing together the clues about catalysis and the structure of the transition state from the minimal amount of structural information has required the introduction of powerful kinetic tools that were previously unavailable in protein chemistry. It has been possible to adapt for enzymology techniques that were used previously for studying structure–activity relationships in physical–organic chemistry. The opportunity of dissecting the structure and activity of an enzyme has provided much general

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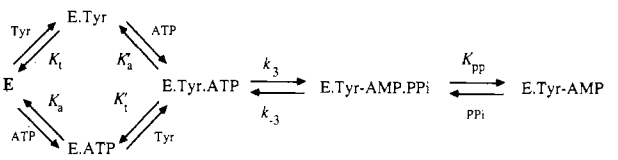
Scheme I



information on enzyme catalysis and molecular recognition as well as an answer to specific questions about an important class of enzymes.[1]

Underlying Philosophy and Goals

The fundamental difference between an enzyme-catalyzed reaction and its uncatalyzed counterpart in solution is the use of the binding energy between the enzyme and substrate to provide specificity and rate enhancement. Thus, to understand enzyme catalysis, we must know the interaction energies between the enzyme and substrate throughout the whole course of the reaction. We then see how binding energy is used to lower activation energies, juggle equilibrium constants, and determine specificity.

The magnitudes of many of the common interactions between biological molecules in solution are unknown because of the complicating effects of solvent water. Binding energies represent the differences between the ligand and receptor bound to water and the ligand and receptor bound to each other. One major goal of the protein engineering studies on the tyrosyl-tRNA synthetase was to develop methods of measuring the strengths of the common interactions and analyzing how they are used in catalysis and recognition.

The Tyrosyl-tRNA Synthetase

The tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* crystallizes as a symmetrical dimer of M_r 2 × 47 316 (Blow & Brick, 1985). It catalyzes the aminoacylation of $tRNA^{Tyr}$ in a two-step reaction (Fersht & Jakes, 1975). Tyrosine is first activated (eq 1) to form a very stable enzyme-bound tyrosyl adenylate complex. Tyrosine is then transferred to tRNA (eq 2).

$$E + Tyr + ATP \rightleftarrows E\cdot Tyr\text{-}AMP + PP_i \quad (1)$$

$$E\cdot Tyr\text{-}AMP + tRNA \rightarrow Tyr\text{-}tRNA + E + AMP \quad (2)$$

The crystal structures of the E·Tyr and E·Tyr-AMP complexes have also been solved (Brick & Blow, 1987; Rubin & Blow, 1981). Although 2 mol of tyrosine binds to the crystalline enzyme (Monteilhet & Blow, 1978), only 1 mol binds to the enzyme in solution (Fersht, 1975). Further, only 1 mol of tyrosyl adenylate is formed rapidly per mole of dimer (Fersht, 1975), and only 1 mol of tRNA is bound (Jakes & Fersht, 1975; Dessen et al., 1982). All mutants characterized so far exhibit this phenomenon of half-of-sites reactivity.

Activation of tyrosine is kinetically a random-order reaction (Scheme I). It seems from the crystal structure, however, that binding of ATP blocks access of tyrosine to its site and so the reaction is, in practice, ordered, with E → E·Tyr → E·Tyr·ATP. This does not affect the steady-state kinetic analysis as there is a virtual equilibrium between E·ATP and E·Tyr·ATP via the free enzyme.

The key factors in making the tyrosyl-tRNA synthetase so amenable to kinetic analysis and for an extended study by site-directed mutagenesis stem from the stability of the E·Tyr-AMP complex. It accumulates on the reaction pathway

[1] Earlier work from steady-state kinetics was reviewed by Fersht et al. (1984).

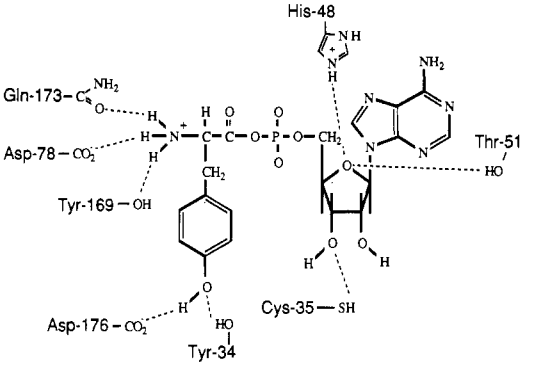


FIGURE 1: Interactions between hydrogen-bonding side chains from the tyrosyl-tRNA synthetase to Tyr-AMP that were implicated from early X-ray crystallographic work (D. M. Blow, personal communication) and provided the basis for mutagenic studies.

in the absence of tRNA, may be readily handled, and provides a means of accurate active-site titration. Further, the rate constants for its formation and pyrophosphorolysis are on the stopped-flow time range and may be conveniently measured by stopped-flow fluorescence. Absolute rate constants may be obtained by pre-steady-state kinetics, which are independent of enzyme concentration, unlike most measurements from steady-state kinetics (Fersht, 1985). It is thus possible to measure in a straightforward manner the individual rate and binding constants of Scheme I. K_t, the dissociation constant of Tyr from E·Tyr, is simply measured by equilibrium dialysis (or kinetics). K_a', the dissociation constant of ATP from E·Tyr·ATP, and k_3, the rate constant for formation of E·Tyr-AMP·PP_i, may both be measured by stopped-flow on mixing E·Tyr with ATP. k_{-3}, the rate constant for pyrophosphorolysis, and K_{pp}, the dissociation constant of PP_i from E·Tyr-AMP·PP_i, may also both be measured by stopped-flow by mixing E·Tyr-AMP with PP_i. K_a, the dissociation constant of ATP from E·ATP, may be measured from steady-state kinetics as can K_t', the virtual dissociation constant of Tyr from E·Tyr·ATP. The rate constant for transfer of tyrosine to tRNA is also in the stopped-flow time range and may also be measured by mixing tRNA with E·Tyr-AMP. Thus, one can construct a free energy profile for the enzymatic reaction.

The Basic Strategy

Equations had been derived earlier to analyze the effects of binding energy on catalysis and specificity, and the role of hydrogen bonding in mediating "strain" effects in catalysis had been emphasized (Fersht, 1974, 1977). As the crystal structure of the E·Tyr-AMP complex (Figure 1) revealed a large number and variety of hydrogen bonds between the enzyme and intermediate, the initial strategy was to mutate the hydrogen-bonding side chains to remove the donors or acceptors and so produce mutants lacking specific bonds. Comparison of the kinetics of tyrosine activation by wild-type and mutant enzymes would then give empirical measurements of the nature and magnitude of the contribution of hydrogen bonding to specificity and catalysis (Winter et al., 1982; Wilkinson et al., 1983).

The Hydrogen Bond and Specificity

In order for the results of mutagenesis to be interpreted in a simple manner, the mutation must not be disruptive and cause gross structural changes. The ideal case for attribution of a change in binding energy on mutation to a specific interaction is when removal of part of the side chain has no effect on the structure apart from leaving a small cavity. This class of mutation has been termed nondisruptive deletion (Fersht

et al., 1987a). Fortunately, crystal structural data on mutants of several enzymes [e.g., T4 lysozyme (Alber & Matthews, 1987), dihydrofolate reductase (Villafranca et al., 1987), subtilisin (Katz & Kossiakoff, 1986), tyrosyl-tRNA synthetase (Brown et al., 1987, and references cited therein)] and sensitive kinetic tests [double-mutant (Carter et al., 1984), linear free energy relationships (Fersht et al., 1987a)] have provided strong evidence that sensibly designed and other mutations are frequently nondisruptive and that the enzyme can often be treated as a scaffold that will tolerate small changes in structure.

Steady-state kinetic measurements have been made on a wide range of nondisruptive deletants of the tyrosyl-tRNA synthetase in which most of the hydrogen-bonding side chains in Figure 1 have been truncated. The following ranges were found for the contributions of hydrogen bonds to specificity (Fersht et al., 1985b): deletion of the partner of an uncharged donor or acceptor weakens binding by approximately 0.5–1.5 kcal/mol; losing a partner to a charged donor or acceptor weakens binding by some 3–6 kcal/mol (Fersht et al., 1985b, 1987b; Lowe et al., 1987). Similar results have been found for the removal of bonds from the substrate in glycogen phosphorylase–sugar complexes (Street et al., 1986). It must be emphasized that these data are strictly measurements of specificity of binding and do not represent the stabilization energy of the hydrogen bond in the complexes of the wild-type enzyme. Fortuitously, the stabilization energy of uncharged hydrogen bonds is also about 1 kcal/mol (Fersht, 1987).

The Hydrogen Bond in Catalysis: Difference Energy Diagrams and ΔG_{app}

The steady-state studies pinpointed which residues make interactions with the substrates and indicated their importance. The next stage was to measure the variation of the binding energy throughout the reaction profile. This was done by constructing free energy profiles for the activation of tyrosine by wild-type and mutant enzymes using pre-steady-state kinetics and equilibrium binding to measure the rate constants in Scheme I. The apparent contribution of the binding energy of a side chain that had been mutated, ΔG_{app}, was determined by subtracting the energy level of each complex of wild-type enzyme from that of mutant enzyme and then visualized by plotting as a difference energy diagram (Wells & Fersht, 1986; Ho & Fersht, 1986). Although ΔG_{app} is frequently interpreted as being equivalent to the stabilization energy of the interaction in the wild-type enzyme, this is not strictly true and ΔG_{app} seriously overestimates the binding energy of charged hydrogen bonds (unpublished work). Fortuitously, ΔG_{app} for the mutation of one partner of an uncharged hydrogen-bond pair is about as the same as its binding energy (Fersht, 1987). Changes in ΔG_{app} as the reaction proceeds from one intermediate to the next on the reaction pathway, $\Delta\Delta G_{app}$, can provide a direct measure of the changes in interaction energy of a particular side chain with the substrate (unpublished results).

The structure of the transition state of a reaction cannot be observed directly and so must be inferred from the structure of a ground state using kinetics or calculation. Difference energy diagrams are particularly important in providing qualitative and quantitative evidence on transition-state structure. Not only have difference energy diagrams quantified the importance of interactions known from the X-ray structure but they have also led to the discovery of unexpectedly important residues. The interaction energies throughout the activation reaction are summarized in Table I and the deduced stereochemical interactions in the transition state in Figure 2. The residues binding tyrosine and ATP may be classified functionally.

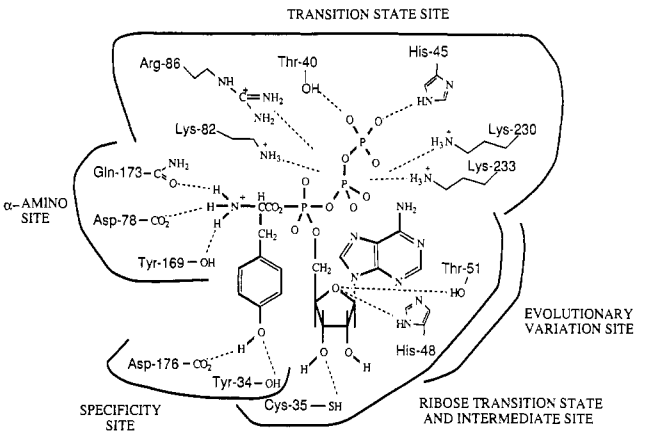


FIGURE 2: Interactions between the transition state for the formation of Tyr-AMP and hydrogen-bonding (or charged) side chains that were deduced from site-directed mutagenesis. The overall site may be classified in terms of subsites as indicated.

Enzyme–Substrate Interactions

Tyrosine Binding Site: Specificity of Amino Acid Binding. Residues Asp-78, Tyr-169, and Gln-173 form a binding site for the α-amino group of Tyr. Mutation of any one weakens binding by about 3 kcal/mol. The interaction energy for Tyr-169 remains unchanged throughout the reaction, but the other residues are more difficult to analyze as they form part of extensive hydrogen-bonding networks that are disturbed on mutation (Lowe et al., 1987). The specificity site for tyrosine against phenylalanine is composed of Asp-176 and Tyr-34. The key group in specificity is the carboxylate of Asp-176 that functions as a hydrogen-bond acceptor of the substrate hydroxyl. Unfortunately, mutation of this residue has never yielded an active enzyme. Tyr-34 binds the substrate uniformly up to the transition state but binds Tyr-AMP slightly more tightly by a small, but real, factor (Wells & Fersht, 1987).

Ribose Binding Site: Enzyme–Substrate versus Enzyme–Transition-State versus Enzyme–Intermediate Complementarity. The crystal structure of the E·Tyr-AMP complex implicated residues Cys-35, Thr-51, and possibly His-48 as binding with the ribose of the nucleotide. Interestingly, mutagenesis revealed that these residues contribute little or no interaction energy with ATP in the E·Tyr·ATP ground-state complex (Wells & Fersht, 1985, 1986; Ho & Fersht, 1986). Cys-35 and His-48 contribute significant stabilization energy with ATP in the transition state, however, and thus contribute to catalysis by their binding energy being used to lower the energy difference between the ground state and transition state. This provides direct evidence for the classic idea of enzyme–transition-state complementarity of Haldane (1930) and Pauling (1946). The binding energies of groups relatively far from the seat of reaction are used for catalysis. But they contribute even more stabilization energy in the E·Tyr-AMP complex. That is, in apparent contradiction to Pauling, there is enzyme–intermediate complementarity. Transition-state complementarity always optimizes rate for reactions that involve a single important transition state, and the substrates and products diffuse rapidly into solution. Other criteria, however, can apply for multistep processes or when products or intermediates remain bound to the enzyme. Maximization of the rate of a single step of a reaction may then not necessarily lead to the optimization of the overall rate of the process. Enzyme–product complementarity had been sug-

Table I: Interaction Energies of Side Chains of Tyrosyl-tRNA Synthetase with Reagents

residue	interaction energy of side chains[a] in complex with				
	Tyr	ATP	[Tyr-ATP]‡	PP_i	Tyr-AMP
	Tyrosine Binding Site				
Tyr-34	+	0	+	0	+
Asp-78	++++	++*	++++	++*	++++
Tyr-169	++++	0	++++	0	++++
Gln-173	++++	++*	++++	+*	++++
	Nucleotide and Pyrophosphate Site				
Cys-35	0	0	++	0	+++
Thr-40	0	0	++++	++++	0
His-45	0	0	++++	++++	0
His-48	0	0	+++	0	+++
Thr-51	0	0	0	0	-
Lys-82	0	++	++++	++++	0
Arg-86	0	0	++++	++++	-
Asp-194	0	0	++++	+	+++
Lys-230	0	0	++++	++++	0
Lys-233	0	++++	++++	++++	0

[a] Apparent stabilization energy from the side chain in kcal/mol: 0 = −0.5 to +0.5; + = 0.5–1.0; ++ = 1.0–1.5; +++ = 1.5–2.0; ++++ = >2.0; - = −0.5 to −1.0; * = evidence for some disruption of protein structure on mutation.

gested as a means of changing the equilibrium constant for highly unfavorable reactions (Jencks, 1969) and enzyme–intermediate complementarity as a means of sequestering unstable intermediates that would otherwise diffuse from the enzyme (Fersht, 1974). Both reasons are probably important in the energetics of binding Tyr-AMP (Wells & Fersht, 1986).

ATP Phosphates and Pyrophosphate: Identification from Energy Diagrams of Residues Involved in Transition-State Stabilization. All residues mutated so far were implicated directly from the X-ray structure. However, there were no obvious clues implicating residues more directly involved in catalysis or binding the ATP phosphates in the transition state. Mutation of His-45 provided evidence for transition-state binding (Fersht et al., 1984). This was confirmed by model building and subsequent mutagenesis that suggested His-45 and Thr-40 form a binding site for the γ-phosphate of ATP in the transition state (Leatherbarrow et al., 1985; Fersht et al., 1986a; Leatherbarrow & Fersht, 1987). The difference energy diagrams for mutation of Thr-40 and His-45 (Table I) provide clean and clear evidence for their binding ATP in the transition state and PP_i in the E·Tyr-AMP·PP_i complex: mutations has negligible effects on the E·Tyr, E·Tyr·ATP, and E·Tyr-AMP complexes but considerably weakens binding in the E[Tyr-ATP]‡ and E·Tyr-AMP·PP_i complexes. A similar site has been found by analogous mutagenesis of the valyl-tRNA synthetase from *B. stearothermophilus* (Borgford et al., 1987).

Whereas the effects of mutation of His-45 could easily be rationalized by model building, random mutagenesis of charged sided chains by Bedouelle and Winter (1986) revealed a set of residues that were found to be crucial in catalysis but whose roles were not immediately obvious as they are either remote from the bound reagents or so disordered that they were initially difficult to localize (Fersht et al., 1987b). Mutagenesis of Arg-86 and Lys-230 alters the free energy profile of the reaction (Table I) in the manner expected if they bind to ATP only in the transition state and to pyrophosphate in the E·Tyr-AMP·PP_i complex. Difference energy profiles of mutants of Lys-82 and Lys-233 suggest the same interactions but that these residues also bind ATP in the E·Tyr·ATP complex. The side chains of Lys-82 and Arg-86 have high *B* values (i.e., are mobile) but are within hydrogen-bonding distance of the β-phosphate and β- and γ-phosphate oxygens, respectively, in our model of the transition state. Lys-230 and Lys-233 are in a loop of very mobile backbone and their side chains are so disordered that they cannot be located at all in the X-ray map. Modeling these side chains in even their most extended conformations, pointing from the backbone to the transition state, cannot locate the ϵ-NH_3^+ to closer than about 8 Å from the phosphates of ATP in the transition state. The very mobility of the backbone loop containing 230 and 233 is a component of catalysis. The loop must move to wrap itself around the transition state in an induced-fit mechanism (Fersht et al., 1987b).

Evolutionary Variation Site. Residue Thr-51 is the only hydrogen-bonding residue in the active site of the tyrosyl-tRNA synthetase from *B. stearothermophilus* that is not conserved in the enzyme from *Escherichia coli*, which contains a proline at this position (Winter et al., 1983). The enzyme from *Bacillus caldotenax* differs from that from *B. stearothermophilus* by just four amino acids, one of them being alanine at position 51 (Jones et al., 1986). Mutation of position 51 in the enzyme from *B. stearothermophilus* gives an opportunity of studying the effects of evolution on overall and individual rate constants. Mutation of position 51 generates a family of tyrosyl-tRNA synthetases, each one of which is the most active in aminoacylation at different concentration ranges of ATP (Fersht et al., 1985a). The changes in kinetic constants on mutation nicely illustrate that selective pressure in evolution is to maximize the term $k_{cat}[S]/(K_M + [S])$ in the Michaelis–Menten equation.

Measurement of the individual values of rate and dissociation constants of Scheme I for the mutants shows that the types of evolutionary changes classified by Albery and Knowles (1976) do occur (Ho & Fersht, 1986). Some mutations cause uniform changes in binding energy, i.e., change the energy levels of all bound complexes by the same amount, others cause differential changes in energy, while some may be classified as causing catalysis of the elementary step.

Analysis and Classification of Binding Energy Changes Using Linear Free Energy Relationships

A classical method of probing transition-state structure in physical–organic chemistry is the use of linear free energy relationships, such as the Brønsted equation. This relates changes in rate constant for a reaction to changes in equilibrium constant as the structure of the reagents is varied. Rate and equilibrium constants for the activtion of tyrosine have been found to follow the Brønsted equation for variation of residues that principly bind the ribose ring (Fersht et al., 1986b). Theory shows that the Brønsted equation when applied to enzymatic reactions measures changes in binding energy (Fersht et al., 1987a). The appeal of such plots is that they systematize and order a large amount of data into a simple plot that shows trends and notes exceptions. The slopes give the fractional binding energy change for the process. Most importantly, the observation that the kinetics of a large number of mutant enzymes can be fitted to such plots shows that genuine trends are being observed in the mutagenesis experiments and that they are hardly likely to be the results of a series of artifacts.

Thirteen nondisruptive mutants at positions predominantly affecting the binding of the ribose ring are found to fit to several Brønsted plots. These show that, on average, 12% of the binding energy of these interactions is realized on the binding of ATP to the E·Tyr complex, 84% on binding the transition state [Tyr-ATP]‡, and 91% on binding Tyr-AMP and PP_i simultaneously—all measured relative to 100% for binding Tyr-AMP (Fersht et al., 1987a). This approach nicely

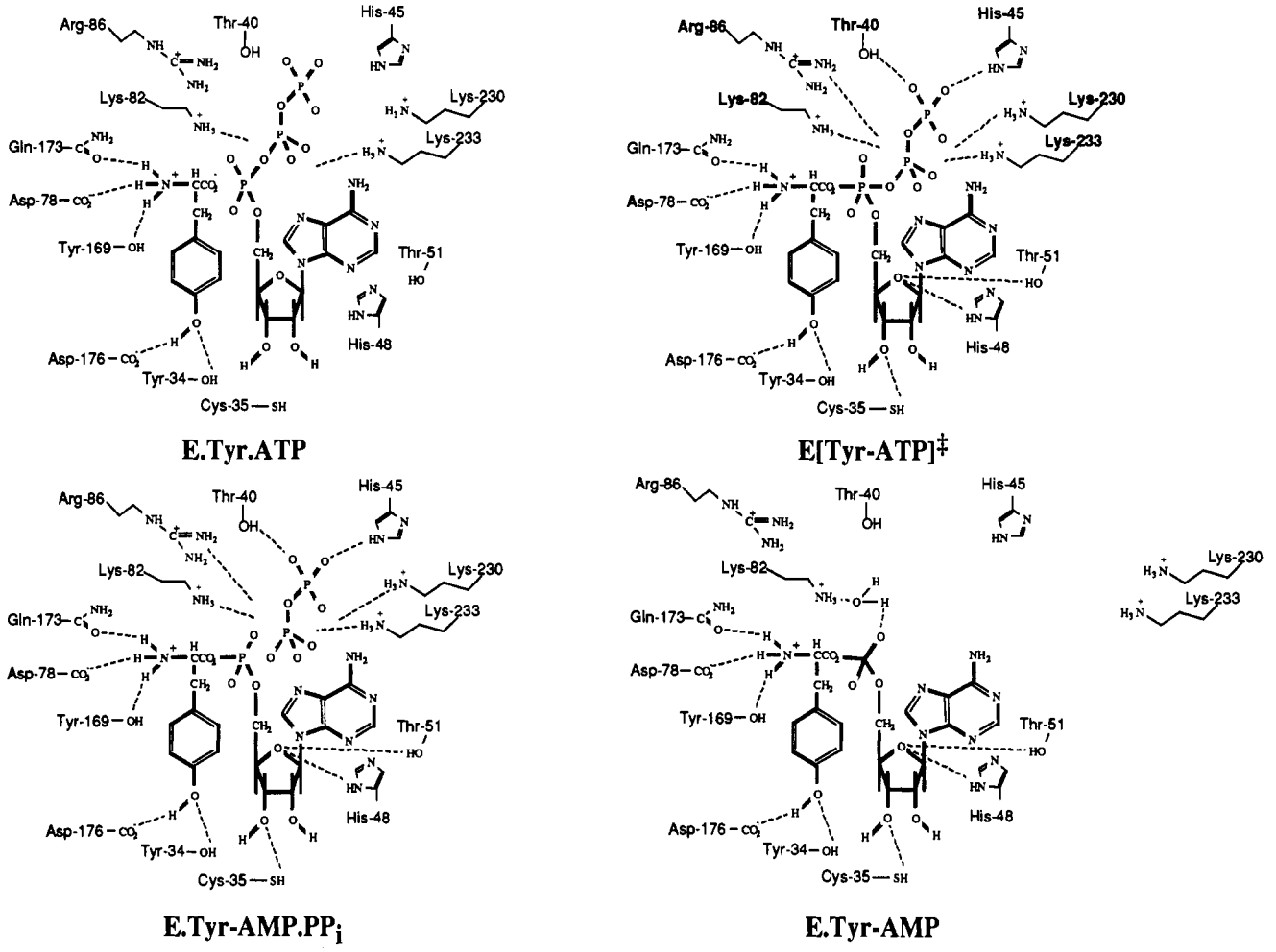


FIGURE 3: Steps in the formation of E·Tyr-AMP from E·Tyr·ATP (see text).

shows the trends in how the binding energy of apparently noncatalytic residues contributes to altering the catalytic rate constant (k_3) and the equilibrium constant between enzyme-bound Tyr and ATP on one side of the equation and enzyme-bound Tyr-AMP and PP_i.

Catalytic Mechanism for the Activation of Tyrosine

Catalysis of formation of tyrosyl adenylate appears to involve just the use of binding energy, with no classical general acid–base or covalent catalysis. It is as if catalysis is delocalized over the whole binding site. The events are summarized in Figure 3 (Fersht et al., 1987b). In the ground-state E·Tyr·ATP complex (top left), ATP binds to Lys-82 and Lys-233. There is insignificant binding energy with Cys-35, Thr-40, His-45, His-48, Thr-51, Lys-82, Arg-86, and Lys-230. In the transition state (top right), the charged and other groups interact with the pyrophosphate moiety of ATP and its ribose ring. In the E·Tyr-AMP·PP_i complex (bottom left), the groups still interact with the reagents. In the E·Tyr-AMP complex (bottom right), the Lys-230/233 loop moves away from the adenylate after dissociation of PP_i, and Lys-82 binds to the α-phosphate of Tyr-AMP via a bridging water molecule. It is possible that there is an additional E′·Tyr·ATP complex between the ground-state E·Tyr·ATP complex and the transition state in which the enzyme and ATP have geometries closer to that in the transition state.

ATP and PP_i react as their magnesium salts. The position of the Mg^{2+} in the complexes is not yet known. Preliminary evidence from mutagenesis and model building is implicating residue Asp-194 in binding the metal ion (M. Fothergill, J. W. Knill-Jones, and A. R. Fersht, unpublished results).

Probing the Gross Structure of the Enzyme by Mutagenesis

Domain Structure. Deletion mutagenesis has shown that the enzyme is organized into two major domains, the N-terminal solely responsible for the activation step and the C-terminal primarily responsible for binding of tRNA. Only the N-terminal 319 amino acids are seen in the X-ray map of the enzyme; 100 residues of the C-terminus are too disordered. The N-terminal fragment was constructed by mutagenesis and found to be kinetically identical with wild-type enzyme in the activation step but unable to bind or aminoacylate tRNA (Waye et al., 1983). The crystal structure of the truncated enzyme is very similar to that of the N-terminal domain of the wild-type enzyme (Brick & Blow, 1987).

Heterodimers containing one full-length and one truncated subunit have been constructed and separated from parental full-length and truncated homodimers. In one procedure, the subunits are linked across the subunit interfaces by a salt bridge buried in a hydrophobic region (Ward et al., 1986). Phe-164 is on the symmetry axis such that the two side chains of the symmetry-related partners interact. Phe-164 was mutated to Lys or Arg on one subunit and to Asp or Glu on the other. Measurements of the dissociation constants of the salt-bridged heterodimers have given information on the strengths of salt bridges that are buried in a hydrophobic environment: the bridge is very weak but does serve to give specificity of binding (Ward et al., 1987). These artificial salt bridges are unlike those that occur naturally. The artificial make just an electrostatic interaction whereas the natural

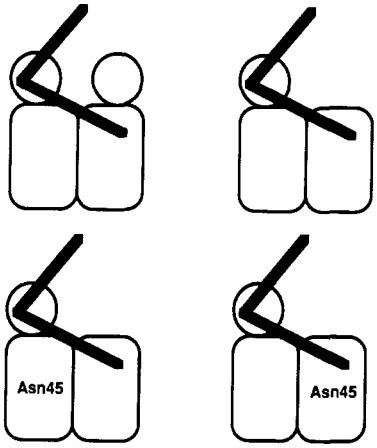


FIGURE 4: Mode of binding of tRNA deduced by Carter et al. (1986). tRNA binds predominantly to the domain indicated by the circle, which may be removed by mutagenesis (Waye et al., 1983). The heterodimer between the wild-type and truncated dimer charges tRNA at half the value of V_{max} for the wild-type homodimer. Mutating His → Asn-45 lowers the rate of formation of tyrosyl adenylate by some 10^4-fold. Putting this mutation into the full-length subunit in the wild-type/truncated heterodimer does not affect the rate of aminoacylation whereas putting the mutation into the small subunit virtually abolishes aminoacylation activity.

always fulfill all possible hydrogen-bonding interactions with neighboring groups. This is consistent with our measurements on the contribution of hydrogen bonds to specificity: loss of a hydrogen bond to a charged group weakens binding by 3–6 kcal/mol (Fersht et al., 1985b).

The other procedure simply involves reversibly denaturing and renaturing an equimolar mixture of truncated and full-length enzymes when hybridization occurs to give a 1:2:1 mixture of full-length homodimer, heterodimer, and truncated homodimer (Carter et al., 1986). These heterodimers have given the opportunity of studying the symmetry of the enzyme and its complexes in solution.

Symmetry of tRNA Binding from Experiments on Heterodimers. Heterodimers of full-length and truncated enzyme have the great utility of providing a means of "tagging" the individual subunits so that a second mutation may be specifically introduced into one, predefined, subunit. For example, (Figure 4), the mutation His → Asn-45 was introduced into one subunit to reduce drastically the rate constant for the activation step by the subunit (Carter et al., 1986; see above and Table I). When Asn-45 is in the large subunit of the heterodimer and the truncated subunit has the wild-type site, the rate of aminoacylation of tRNA is half that of the wild-type enzyme. But, when the heterodimer has Asn-45 in the small subunit, the rate of aminoacylation of tRNA is undetectably low (Carter et al., 1986). This shows that, although tRNA binds predominantly to the full-length subunit, amino acid acceptance occurs from the small subunit. tRNA must bind to the wild-type enzyme in a similar manner, spanning both subunits.

The heterodimer of truncated and full-length enzyme thus provides an asymmetric system for testing the effects of mutations on catalytic and binding subunits in aminoacylation. This enabled Bedouelle and Winter (1986) to assign by random mutagenesis residues that are crucial to either catalysis or binding. Patches of lysine and arginine residues were implicated in binding tRNA, which enabled a low-resolution model of the enzyme–tRNA complex to be built.

Symmetry of Enzyme in Solution. Use has been made of the catalytic asymmetry of heterodimers to show that the wild-type enzyme is asymmetrical in solution (Figure 5; Ward & Fersht, 1987). The enzyme is frozen into two populations: 50% are active in one subunit and 50% in the other. For example, heterodimers containing Asn-45 on one subunit form 0.5 mol of Tyr-AMP/mol of dimer rapidly at the wild-type rate and a further 0.5 mol very slowly at the rate expected for the mutant. Any interconversion of active and inactive subunits is on a much slower time scale than the several minutes half-life for formation of E·Tyr-AMP at the mutated site (Asn-45). There is no evidence that the behavior of the heterodimers is different from that of the wild-type enzyme.

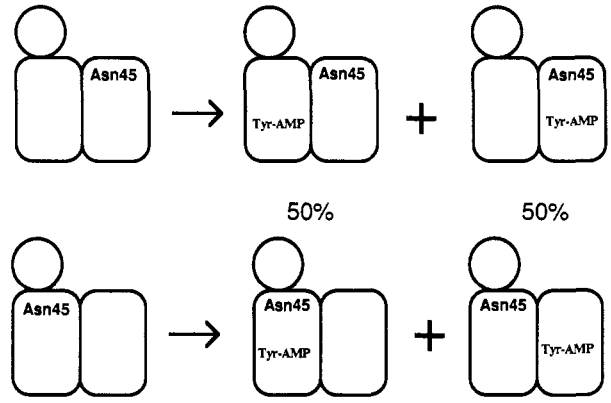


FIGURE 5: Formation of bound Tyr-AMP by heterodimers between full-length and truncated enzyme that contain the mutation His → Asn-45 in one subunit. Despite the 10^4 lower activity of the mutant subunit, the heterodimers show half-of-the-sites activity in which Tyr-AMP is formed in equal amounts at each subunit. There is, therefore, a preexisting asymmetry in the enzyme that determines which subunit is active.

The half-of-the-sites activity appears to be the result of a preexisting asymmetry in the enzyme in solution. There are thus important differences between the crystal and solution structures in terms of both overall symmetry and positions of mobile side chains, such as that containing Lys-230 and Lys-233.

CONCLUSION

There is a powerful procedure in physical–organic chemistry of making small perturbations in the structure of a reagent and then measuring the changes in reactivity on the changes in structure. The studies on the tyrosyl-tRNA synthetase show that the same approach may be applied to the analysis of proteins. The conventional physical–organic experiments are frequently aimed at investigating bonding in transition states, probed by the inductive effects of substituents (field effects). The site-directed mutagenesis studies described here tend to map the interactions of the reagents with their environment, i.e., the enzyme. Electrostatic field effects can also be studied by site-directed mutagenesis, and experiments have been initiated in this area on subtilisin (Russell et al., 1987; Russell & Fersht, 1987).

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