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Use of Binding Energy in Catalysis Analyzed by Mutagenesis of the Tyrosyl-tRNA Synthetase†

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ABSTRACT: The utilization of enzyme–substrate binding energy in catalysis has been investigated by experiments on mutant tyrosyl-tRNA synthetases that have been generated by site-directed mutagenesis. The mutants are poorer enzymes because they lack side chains that form hydrogen bonds with ATP and tyrosine during stages of the reaction. The hydrogen bonds are not directly involved in the chemical processes but are at some distance from the seat of reaction. The free energy profiles for the formation of enzyme-bound tyrosyl adenylate and the equilibria between the substrates and products were determined from a combination of pre-steady-state kinetics and equilibrium binding methods. By comparison of the profile of each mutant with wild-type enzyme, a picture is built up of how the course of reaction is affected by the influence of each side chain on the energies of the complexes of the enzyme with substrates, transition states, and intermediates (tyrosyl adenylate). As the activation reaction proceeds, the apparent binding energies of certain side chains with the tyrosine and nucleotide moieties increase, being weakest in the enzyme–substrate complex, stronger in the transition state, and strongest in the enzyme–intermediate complex. Most marked is the interaction of Cys-35 with the 3′-hydroxyl of the ribose. Removal of the side chain of Cys-35 leads to no change in the dissociation constant of ATP but causes a 10-fold lowering of the catalytic rate constant. It contributes no net apparent binding energy in the E·Tyr·ATP complex and stabilizes the transition state by 1.2 kcal/mol and the E·Tyr–AMP complex by 1.6 kcal/mol. The preferential stabilization of products causes the unfavorable equilibrium constant for the formation of Tyr–AMP and PP_i from Tyr and ATP in solution (3.5×10^{-7}) to be displaced to a value of 2.3 for enzyme-bound reagents. These experiments thus show that (i) the binding energies of side chains remote from the seat of reaction can be used to increase catalytic rates and (ii) the structure of regions of the binding site of an enzyme can be closer in complementarity to an unstable enzyme-bound intermediate than to the transition state for its formation.

The distinctive characteristic of enzyme catalysis compared with solution catalysis is that the enzyme specifically binds its substrate and can use the binding energy to enhance catalytic rate. Pauling (1946) suggested that an enzyme should be complementary in structure to the transition state of the substrate rather than to the substrate itself so that the enzyme would tend to deform the substrate into the transition state. Current ideas on the utilization of binding energy in catalysis support the concept of enzyme–transition-state complementarity but do not demand that the substrate is distorted by the enzyme. The presence of binding sites on the enzyme that form better bonds with the transition state of the substrate than with the unreacted substrate is sufficient to increase the turnover number of the enzyme, k_{cat} (Fersht, 1974, 1985; Jencks, 1975). Evidence has been adduced for the differential binding of transition state and substrate ("transition-state stabilization") from experiments in which the structure of substrates or inhibitors is varied [see Fersht (1985) for review]. But it is now possible to examine directly the catalytic role of binding energy of groups on an enzyme by performing experiments in which the structure of the enzyme is varied by site-directed mutagenesis (Winter et al., 1982; Fersht et al., 1984).

The tyrosyl-tRNA synthetase is particularly suited for such an analysis. Apart from favorable properties in handling and producing accurate kinetic data (Wilkinson, 1983), it is known from the direct solution of the crystal structure of the enzyme-bound tyrosyl adenylate complex combined with mu-

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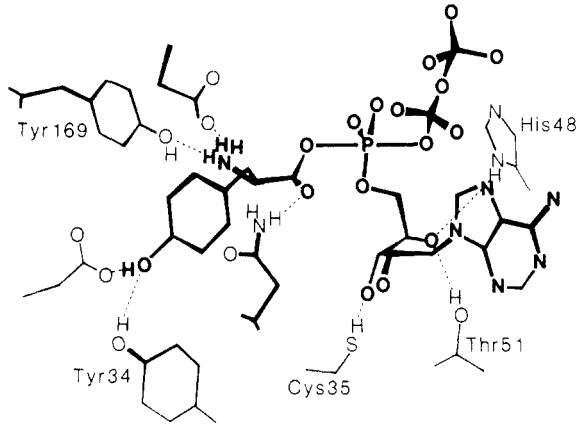

FIGURE 1: Sketch of transition state of tyrosine and ATP during the formation of tyrosyl adenylate illustrating hydrogen bonds made with the enzyme [from model building by extrapolation from the crystal structure of the enzyme–tyrosyl adenylate complex by Leatherbarrow et al (1985)].

tagenesis studies that nine hydrogen bonds are formed between substrates and the side chains of the enzyme (Figure 1). As the potential energy functions of hydrogen bonds are suitable for mediating differential binding effects (Fersht, 1974), analysis of mutants lacking hydrogen-bonding side chains will give the effects of small changes in structure on catalytic activity.

The side chains we have chosen to modify in this study are those that are removed from the seat of reaction and have been shown previously from steady-state kinetics to form good hydrogen bonds with the transition state for the formation of tyrosyl adenylate (Fersht et al., 1985a). We have shown in a preliminary study that they can cause differential binding of substrate and transition state (Wells & Fersht, 1985). We therefore remove interactions that have arisen through evolution and so produce less efficient enzymes. In this study, by determining the free energy profiles of the reaction for wild-type and mutant enzymes, we build up a picture of the effects of adding side chains to the "less-evolved" enzymes. We calculate the contributions of the side chains to the binding energies of the substrates, transition states, and products from direct measurements by pre-steady-state kinetics of the rate and equilibrium constants for the formation of enzyme-bound tyrosyl adenylate and from the measured dissociation constants of the relevant substrates from their complexes with the enzyme.

Experimental Procedures

Materials

Reagents were obtained from Sigma (London) and radiochemicals from Amersham International.

Preparation and Purification of Tyrosyl-tRNA Synthetases. Mutant proteins were prepared with mutant M13mp93 phage constructed as described previously (Carter et al., 1984; Fersht et al., 1985b). An overnight culture of *Escherichia coli* JM101 in 2xTY medium was diluted 100-fold into 500 mL of fresh medium and infected with M13 at high multiplicity. Growth was continued at 37 °C in an orbital shaker, with aeration for 4–5 h. The starter culture was transferred to an MBR bioreactor fermentation system containing 10 L of 2xTY medium. The culture was fermented overnight at 37 °C and pH 6.8 with an aeration rate of 10 L s^{-1}. The cells were harvested by passage of the culture through a Pellicon filtration unit and then by centrifugation of the retentate at 8000 rpm for 20 min. Purification was continued as described previously (Winter et al., 1982), except that the final materials was spplied to a fast protein liquid chromatography (FPLC) Mono Q column (Pharmacia) equilibrated in 20 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.8, and eluted by using a gradient of 100–300 mM NaCl dissolved in the equilibration buffer. All enzymes were purified to electrophoretic homogeneity and were found to bind 1.0 mol of tyrosyl adenylate/mol of enzyme (Wilkinson et al., 1983).

Methods

All experiments were performed at 25 °C in a standard buffer containing 144 mM Tris-HCl (pH 7.78), 10 mM $MgCl_2$ (free), 14 mM 2-mercaptoethanol, and 0.1 mM phenylmethanesulfonyl fluoride. Additional $MgCl_2$ was added where necessary to compensate for its complexing with ATP and pyrophosphate.

Rate constants for formation of enzyme-bound tyrosyl adenylate and its pyrophosphorolysis were obtained by monitoring protein fluorescence by stopped flow, with excitation at 290 nm, and measuring emission at wavelengths greater than 325 nm after passage through a cutoff filter (Fersht et al., 1975). Typically, the final enzyme concentration was 0.25 μm although higher enzyme concentrations were occasionally used to improve the signal to noise ratio.

Forward Reaction (Tyrosyl Adenylate Formation): ATP Dependence. The forward reactions for all the mutants studied, except TyrTS(Phe-169), were monitored in the presence of saturating concentrations of tyrosine (0.2 mM). One syringe of the stopped-flow fluorometer contained enzyme plus 0.2 mM tyrosine and inorganic pyrophosphatase (2 units mL^{-1}) in the standard buffer. The other syringe contained ATP (the concentration of which was varied over a range from $K_a'/5$ to $5K_a'$) and 0.2 mM tyrosine in standard buffer.

The dissociation constant of tyrosine from the mutant Tyr–Phe-169 is so large that it is not possible to saturate the enzyme with tyrosine because of its low solubility. Rate data for this mutant were determined by saturating the enzyme with ATP (25 mM) and monitoring the reaction under various concentrations of tyrosine.

Reverse Reaction (Pyrophosphorolysis of Tyrosyl Adenylate): Pyrophosphate Dependence. The enzyme-bound tyrosyl adenylate complex was prepared in situ, and the excess ATP, tyrosine, and pyrophosphate were removed by desalting on a Sephadex G-25 column in the standard pH 7.78 buffer [apart from TyrTS(Phe-169) where a dilute pH 6 buffer was used because of the instability of the enzyme-bound tyrosyl adenylate]. The reverse rate constant was then measured by monitoring the fluorescence increase on mixing pyrophosphate with the enzyme–tyrosyl adenylate complex (Fersht et al., 1975). [For TyrTS(Phe-169), the solution of the complex in dilute pH 6 buffer was mixed with 2 × concentrated Tris, pH 7.78, buffer.]

The dissociation constants of tyrosine from mutant enzymes other than TyrTS(Phe-169) were determined by equilibrium dialysis (Fersht et al., 1975). The dissociation constant (K_t) of tyrosine from TyrTS(Phe-169) was determined from the kinetics of pyrophosphate exchange as was K_a', the dissociation constant of ATP from E·Tyr·ATP. It can be shown from standard kinetic theory that the rate constant (k) for the loss of label from [γ-^{32}P]ATP at concentrations $\ll K_a'$ is given by $k = (k_3/K_a')([Tyr][E]_0/([Tyr] + K_t)$, where k_3 is the rate constant for the formation of tyrosyl adenylate, provided [ATP] $\ll$ [PP]. K_t is provided directly from the variation of k with [Tyr], and K_a' may be calculated from the measured values of k_3 from stopped-flow experiments. It was confirmed that the pyrophosphate does not inhibit the enzymes at the concentration used (2 mM). A control experiment on wild-

Scheme I

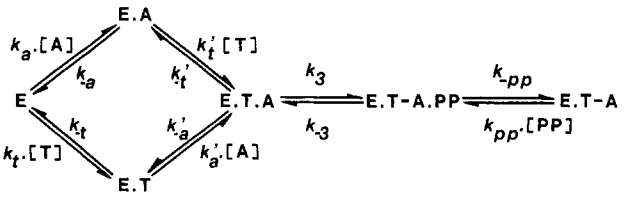

Table I: Rate and Dissociation Constants for the Formation of Enzyme-Bound Tyrosyl Adenylate[a]

enzyme	k_3 (s^{-1})	K_a' (mM)	K_t (μM)
wild type	38	4.7	12
Δ(319–419)[b]	34	5.2	12
tyrosine binding site mutants			
Tyr–Phe-34	35	4.4	29
Tyr–Phe-169[b]	35	4.6	1300
ATP binding site mutants			
Cys–Ser-35	4.7	4.8	8
Cys–Gly-35	4.0	4.5	11
His–Gly-48	9.9	9.9	23

[a] Experiments performed at 25 °C and pH 7.78 (144 nM Tris-HCl, 10 mM $MgCl_2$, and 14 mM 2-mercaptoethanol using stopped-flow fluorescence. Values of k_3 (see Scheme I) are extrapolated to saturating concentrations of ATP and tyrosine. K_a', the dissociation constant of ATP from the E·Tyr·ATP complex, was determined from stopped flow. K_t, the dissociation constant of tyrosine from the E·Tyr complex, was determined by equilibrium dialysis, apart for TyrTS (Phe-169) when kinetics was used. Standard errors are typically ±5% for k_3 and ±10% for dissociation constants. [b] Experiments performed on a truncated enzyme lacking the tRNA binding domain (residues 319–419) (Waye et al., 1983).

type enzyme gave a value of 11.4 μM for K_t from kinetics, compared with 11.6 μM from equilibrium dialysis.

Equilibrium concentrations of enzyme-bound tyrosyl adenylate complexes in mixtures of ^{14}C-labeled tyrosine (1 μM), ATP (400 μM), and pyrophosphate (6–500 μM) were measured by nitrocellulose disk filtration. A solution of wild-type enzyme (100 nM) was incubated at 25 °C in the standard buffer and other reagents were incubated for 1 min; samples (90 μL) were filtered through Schleicher & Schuell 0.45-μm cutoff filters and washed with 3 mL of cold buffer. The enzyme-bound tyrosyl adenylate, which is quantitatively retained by the filter, was assayed by scintillation counting.

Results

Calculation of Energy Levels of Intermediates and Apparent Binding Energies. The activation of tyrosine catalyzed by the tyrosyl-tRNA synthetase is described by Scheme I in which the relevant rate constants are defined ($K_t = k_{-t}/k_t$ = [E][Tyr]/[E·Tyr], $K_a' = k_{-a}'/k_a'$ = [E·Tyr][ATP]/[E·Tyr·ATP], etc.). The dissociation constant of tyrosine from E·Tyr, K_t, was measured directly by equilibrium dialysis [or kinetics for TyrTS(Phe-169)]. K_a' was determined by stopped-flow fluorometric measurements of the rate constants for formation of E·Tyr–AMP on mixing E·Tyr with ATP, which also gave k_3 (Table I). k_{-3} and K_{pp}, the dissociation constant of pyrophosphate from E·Tyr–AMP, were determined by stopped-flow fluorometry of the pyrophosphorolysis reaction on mixing E·Tyr–AMP with pyrophosphate (Table II).

The energy level, G, of each state of the enzyme was calculated from the usual thermodynamic equations by using standard states of 1 M for ATP, Tyr, and pyrophosphate (Table III and Figure 2). Relative to free enzyme (i.e., defining $G_E = 0$)

$$G_{E\cdot Tyr} = RT \ln K_t \quad (1)$$

$$G_{E\cdot Tyr\cdot ATP} = RT \ln (K_t K_a') \quad (2)$$

Table II: Pyrophosphorolysis of Enzyme-Bound Tyrosyl Adenylate Complexes[a]

enzyme	k_{-3} (s^{-1})	K_{pp} (mM)	K_{eq} (k_3/k_{-3})
wild type	16.6	0.61	2.29
Δ(319–419)	15.3	0.68	2.22
tyrosine binding site mutants			
Tyr–Phe-34	22.2	0.45	1.58
Tyr–Phe-169	13.8	0.74	2.52
ATP binding site mutants			
Cys–Gly-35	32.8	0.63	0.12
Cys–Ser-35	31.0	0.44	0.15
His–Gly-48	16.4	0.41	0.60

[a] Conditions as in Table I. Accuracy is ±5% for rate constants and ±10% for dissociation constants (standard error).

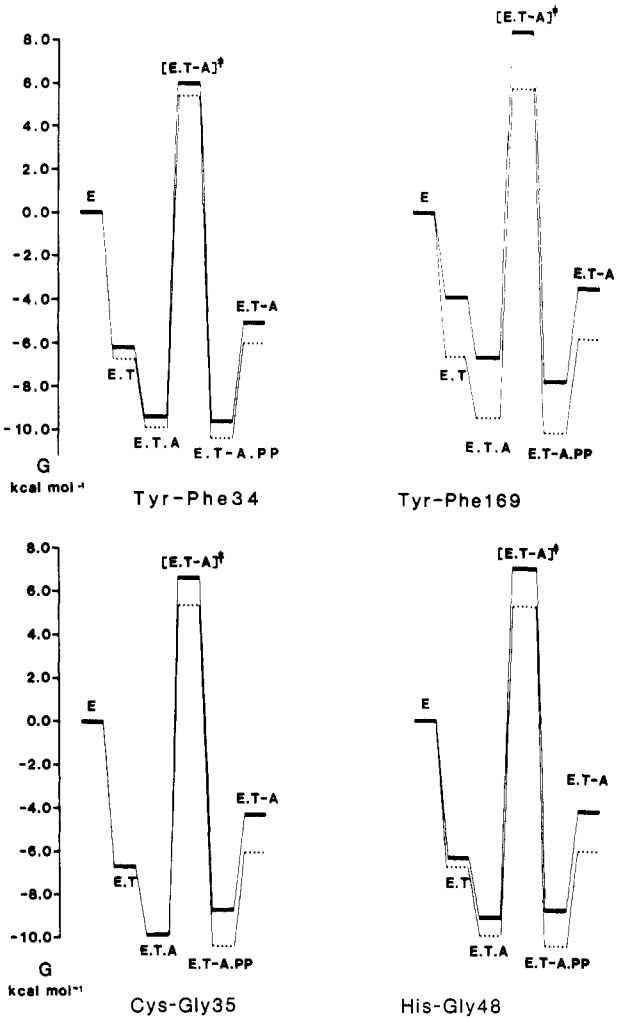

FIGURE 2: Gibbs' free energy profiles for the formation of tyrosyl adenylate and pyrophosphate, as defined in Scheme I, by wild-type (energy levels in broken lines) and mutant (energy levels in heavy lines) tyrosyl-tRNA synthetases, using standard states of 1 M for tyrosine, ATP, and pyrophosphate.

[note that the substrate binding is random ordered (Santi & Pena, 1971) and $K_a'K_t = K_aK_t'$. It is simple to measure $K_t{\cdot}K_a'$ but difficult to measure the individual components of K_aK_t' since the affinity of the free enzyme for ATP is too low to be measured by equilibrium dialysis and the binding of tyrosine to E·ATP involves highly complex pre-steady-state kinetics (Fersht et al., 1975)]

$$G_{[E\cdot Tyr\cdot ATP]^*} = RT \ln (k_B T/h) - RT \ln (k_3/K_a'K_t) \quad (3)$$

$$G_{E\cdot Tyr\text{–}AMP\cdot PP_i} = -RT \ln (k_3/k_{-3}K_a'K_t) \quad (4)$$

$$G_{E\cdot Tyr\text{–}AMP} = -RT \ln (k_3K_{pp}/k_{-3}K_a'K_t) \quad (5)$$

Table III: Gibbs Free Energies of Complexes of Wild-Type and Mutant Enzymes[a]

enzyme	kcal mol^{-1}				
	$G_{E\cdot Tyr}$	$G_{E\cdot Tyr\cdot ATP}$	$G_{[E\cdot Tyr\cdot ATP]^{\ddagger}}$	$G_{E\cdot Tyr\text{–}AMP\cdot PP}$	$G_{E\cdot Tyr\text{–}AMP}$
wild type	−6.71	−9.89	5.41	−10.38	−5.99
Δ(319–419)[b]	−6.71	−9.82	5.53	−10.31	−5.99
TyrTS(Phe-34)	−6.19	−9.40	5.94	−9.67	−5.11
TyrTS(Phe-169)[b]	−3.93	−7.12	8.23	−7.66	−3.39
TyrTS(Gly-35)	−6.76	−9.97	6.67	−8.72	−4.35
TyrTS(Ser-35)	−6.95	−10.12	6.42	−9.00	−4.43
TyrTS(Gly-48)	−6.32	−9.06	7.04	−8.76	−4.14
standard error	±0.06	±0.08	±0.08	±0.09	±0.10

[a] Standard state = 1 M Tyr, 1 M ATP, 1 M PP_i, and free enzyme ($G_E = 0$). [b] Truncated enzyme (Waye et al., 1983).

Table IV: Relative Gibbs Free Energies of Complexes of Mutant Enzymes Equal Apparent Binding Energies of Side Chains[a]

enzyme	kcal mol^{-1}				
	$\Delta G_{E\cdot Tyr}$	$\Delta G_{E\cdot Tyr\cdot ATP}$	$\Delta G_{[E\cdot Tyr\cdot ATP]^{\ddagger}}$	$\Delta G_{E\cdot Tyr\text{–}AMP\cdot PP}$	$\Delta G_{E\cdot Tyr\text{–}AMP}$
tyrosine binding site mutants[b]					
TyrTS(Phe–34)	0.5	0.5	0.5	0.7	0.9
TyrTS(Phe–169)	2.8	2.7	2.7	2.6	2.6
ATP binding site mutants[c]					
TyrTS(Gly-35)	0.0	0.0	1.2	1.6	1.6
TyrTS(Gly-48)	0.0	0.4	1.2	1.2	1.5
standard error	±0.08	±0.11	±0.11	±0.12	±0.14

[a] The apparent binding energies of the side chains (–OH, –OH, $-CH_2SH$, and imidazole) are equivalent to the negative value of ΔG. [b] Energies relative to those of wild type. Free enzyme is the standard state ($G_E = 0$). [c] The E·Tyr complex is the standard state ($G_{E\cdot Tyr} = 0$) to isolate effects of mutation in ATP site.

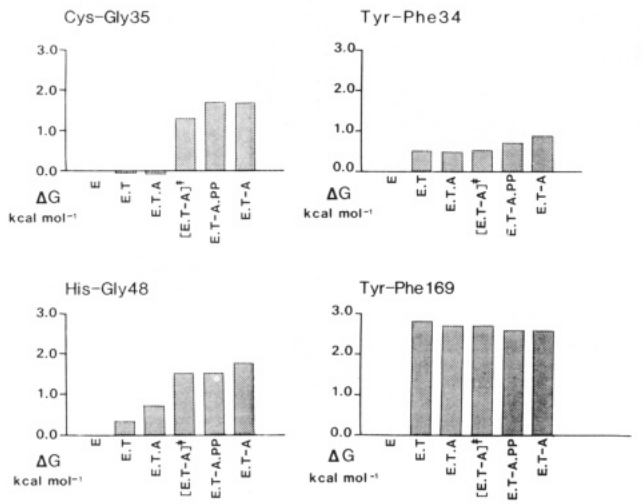

FIGURE 3: Gibbs' free energies of enzyme-bound complexes of mutant enzymes relative to those of wild-type enzyme. These are the global free energy differences, but the negative values of G may be considered as the apparent binding energies of the side chains with the various forms of the substrates during the reaction. Equilibrium concentrations of enzyme-bound tyrosyl adenylate complexes as determined by nitrocellulose disk filtration (100 nM enzyme, 1 μM ^{14}C-labeled tyrosine, 0.4 mM ATP, and indicated concentrations of pyrophosphate in standard buffer; see text). The solid curve is calculated from measured values of K_t, K_a', k_3, k_{-3}, and K_{pp} in Scheme I.

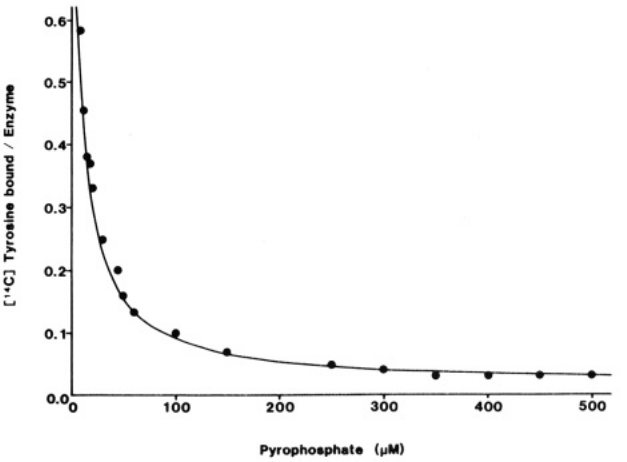

FIGURE 4: Equilibrium concentrations of enzyme-bound tyrosyl adenylate complexes as determined by nitrocellulose disk filtration (100 nM enzyme, 1 μM ^{14}C-labeled tyrosine, 0.4 mM ATP, and indicated concentrations of pyrophosphate in standard buffer; see text). The solid curve is calculated from measured values of K_t, K_a', k_3, k_{-3}, and K_{pp} in Scheme I.

where R is the gas constant, T is the absolute temperature, k_B is Boltzmann's constant, h is Planck's constant, and [E·Tyr·ATP]‡ is the transition state for the formation of tyrosyl adenylate.

The apparent contribution of an individual side chain to the stabilization energy of each state (enzyme-bound complex) can be calculated by subtraction of the energy of the state of the mutant enzyme lacking the relevant side chain from the energy of the same state of wild-type enzyme (Table IV and Figure 3). (The energy differences are, of course, the global properties of the enzymes. But, for analytical purposes, it is often convenient to attribute these to the specifically modified interactions. In many cases, this attribution will be correct. Model building by computer graphics suggests this is so here.) As mutations in the ATP binding site can cause small effects on the dissociation constants of tyrosine, we sometimes use the E·Tyr complex as the standard state (i.e., $G_{E\cdot Tyr} = 0$) for the ATP-binding site mutants as this isolates the effects of the interactions on the binding of the nucleotide moiety. This does not affect the relative energy levels of the other states or the following discussion.

Detailed Effects of Mutations on Energetics. Gibbs Free Energy Profile of Activation of Tyrosine by Wild-Type Enzyme. The equilibrium constants for the formation of enzyme-bound tyrosyl adenylate complexes were determined, in part, from measurements in the pre steady state of forward and reverse reactions. The validity of this approach was confirmed by measuring the concentrations of these intermediates at equilibrium (Figure 4). The equilibrium concentrations are in good agreement with those calculated from

the measured values of K_t, K_a', k_3, k_{-3}, and K_{pp}. The equilibrium constant between the ternary complexes K_3 ($=k_3/k_{-3}$, = [E·Tyr–AMP·PP_i]/[E·Tyr·ATP]) is 2.3.

Removal of Groups Interacting with Tyrosine. Removal of side chains within the tyrosine binding pocket (Tyr–Phe-34 and Tyr–Phe-169) does not significantly alter k_3 or K_a' but increases the dissociation constant for tyrosine. It is thus inferred that the hydroxyl moieties of Tyr-34 and Tyr-169 interact equally well with tyrosine when it is in the unreacted form and when it is in the transition state of the reaction (Figure 3). The value of k_{-3} for TyrTS(Phe-34) is significantly increased so that K_3 is lower for the mutant. Removal of the –OH group destabilizes the E·Tyr–AMP complex more than any other state. Removal of the –OH group from Tyr-169 alters the energy levels of all states equally, within experimental error.

Removal of Groups Interacting with ATP: TyrTS(Cys–Ser-35) and TyrTS(Cys–Gly-35). Mutation of Cys-35 to Gly-35 removes a hydrogen bond to the 3'-OH of the ribose (Figure 1), and mutation to Ser-35 leads to a too-long bond (Winter et al., 1982; Wilkinson et al., 1983). In both cases, this causes a 10-fold lowering of k_3 with no significant changes in K_a' or K_t (Table I). That is, compared with the mutants, the side chain of Cys-35 in wild-type enzyme makes no apparent binding energy contribution in the E·Tyr·ATP complex but does improve the binding in the transition state [E·Tyr·ATP]‡ (Tables III and IV). The pyrophosphorolysis rate constants are increased 2-fold on mutation. The net result is a 15-fold lowering of the equilibrium constant K_3. Removal of the side chain of Cys-35 destabilizes the E·Tyr–AMP and E·Tyr–AMP·PP complexes more than the transition state [E·Tyr·ATP]‡, so the side chain makes an even greater binding energy contribution to the enzyme–product complex.

His–Gly-48. In contrast with the above mutants, the change His–Gly-48 leads to a mixture of effects: a 4-fold lowering of k_3; a 2-fold increase in K_a'; a small change in K_t. The side chain of His-48 causes a net stabilization of the bound adenosine of 0.44 kcal/mol in the E·Tyr·ATP complex, 1.24 kcal/mol in the transition state, and 1.47 kcal/mol in E·Tyr·AMP. The increase in K_t is typical of position 48 mutants and may just reflect the loss of an electrostatic interaction with the carboxylate of the tyrosine (Lowe et al., 1985). It seems unlikely from the double mutant studies of Carter et al. (1984) that the Gly-48 mutation causes any large structural changes within the active site.

Accuracy of Data. Data were collected to a comparable accuracy for all enzymes: the standard error of the mean for dissociation constants is about ±10%, while rate constants are ±5%. It is calculated from theory of errors that the standard error for calculations of $\Delta G_{E\text{-}Tyr}$ in Table IV is ±0.08 kcal mol^{-1}. The errors are propagated through the calculations and are calculated to be ±0.11, ±0.11, ±0.12, and ±0.14 kcal mol^{-1} for ΔG in the E·Tyr·ATP, E·[Tyr·ATP]‡, E·Tyr–AMP·PP_i, and E·Tyr–AMP complexes, respectively.

Certain of the important conclusions from the data, namely, those concerning the differential contributions of the side chains to the binding energies in the E·Tyr·ATP, E·[Tyr–ATP]‡, and E·Tyr–AMP·PP_i complexes, are based on even higher precision. This is because the relative energies of these complexes may be calculated from just k_3 and k_{-3}, which are measured with an accuracy of ±5%. For example, k_3 for TyrTS(Cys–Ser-35) is lowered by a factor of 9.5 ± 0.07 compared with wild-type enzyme. Thus, relative to wild-type enzyme, the complex E·[Tyr–AMP]‡ in the reaction of mutant is raised by 1.33 ± 0.04 kcal mol^{-1} over the energy of the E·Tyr·ATP complex. Further, as k_{-3} for the mutant is ±5% times higher than for wild-type enzyme, the value of K_3 is (19 ± 2)-fold lower for the mutant. Thus, the energy of the mutant E·Tyr–AMP·PP_i complex relative to the E·Tyr·ATP complex is raised by 1.74 ± 0.06 kcal mol^{-1} compared with wild type. This energy is significantly higher than the change in energy of the transition state. The value of k_{-3} for TyrTS(Tyr–Phe-34) is significantly higher (37%) than that for wild-type enzyme and so indicates that there is a real change in the relative energy levels for that mutant.

Discussion

We have applied a sort of "reverse evolution" to measure the apparent contributions of the binding energies of side chains in the binding site of the tyrosyl-tRNA synthetase to catalysis: removal of the side chains in each case produces a poorer enzyme by either weakening the binding of the substrates or lowering the catalytic rate constant and so represents a backward step in evolution. We are not suggesting that the mutants are the ancestral forms of the present wild-type enzyme, but merely forms of the enzyme that have presumably been tested by evolution and rejected. The rate of spontaneous point mutation is sufficiently high that the mutants must be formed in vivo.

Apparent or Observed Group Binding Energies. The removal of a side chain from an enzyme can have a purely localized effect on binding due to the absence of a specific interaction between the enzyme and substrate or a more global effect due to subtle or gross changes in enzyme conformation. ethe free energy of the unligated enzyme may also be altered on mutation by changes in hydration (Fersht et al., 1985a,b). What is phenomenologically important, however, is the actual effect on catalysis, and this is precisely what we measure in the mutagenesis studies. Where it seems likely from model building and other experiments that the effect on catalysis is caused just by a localized effect, the mutagenesis experiments give empirically the contribution of the side chain to binding energy. This energy contains the contributions from the direct interactions with the substrate that are altered and any additional energy terms due to local changes in structure in the free enzyme and in its complexes. We suggest that the binding energies that are measured from directed mutagenesis be termed *apparent* or *observed* group binding energies, by analogy with "apparent" or "observed" pK_a values. It is stressed that none of the following discussion depends crucially on the effects of mutation being localized.

Differential Stabilization of Substrates, Transition States, and Products. Irrespective of detailed structural changes, we find that removal of side chains can affect the binding energies of substrates, transition states, and products by different amounts. Conversely, the presence of a side chain can contribute to the differential binding of substrates, transition states, and products.

Mutation of the tyrosine binding and ATP binding sites leads to two different types of effects (Figure 3). Removal of the hydroxyl groups of tyrosine residues 34 and 169 that form hydrogen bonds with the substrate tyrosine does not affect the rate constant k_3 for activation but just weakens the binding of tyrosine. Thus, the side chains bind tyrosine equally well in the ground and transition states. Conversely, mutation of Cys-35, which interacts with ATP, has no effect on the dissociation constant of ATP from the enzyme–substrate complex but causes a 10-fold lowering of k_3. Thus, the side chain of Cys-35 contributes no binding energy to the enzyme–substrate complex but binds well to the transition state. Mutation of His-48, which also interacts with ATP, gives a mixture of

effects, leading to a 4-fold decrease in k_3 and 2-fold increase in dissociation constant. This side chain thus binds to both the substrate and transition state but binds better to the transition state. In all cases, the side chains contribute most binding energy to the tyrosyl adenylate complex (Figure 3).

Enzyme Complementary to Intermediates. Removal of certain side chains destabilizes the enzyme–intermediate complex more than any other complex. This implies that the structure of those parts of the enzyme that we have modified in this study is closest in complementarity to the structure of the products of the reaction. There is somewhat lower complementarity toward the transition state, and there is least complementarity to the substrates (the predominant effects being on the nucleotide moiety).

It has been suggested that an enzyme could be complementary in structure to enzyme-bound intermediates or products rather than transition states when (i) unfavorable equilibria need to be overcome (Jencks, 1969, p 313) or (ii) a highly reactive intermediate has to be sequestered (Fersht, 1974). Both reasons could be important here. The equilibrium constant for the formation of Tyr–AMP and PP from Tyr and ATP in solution is indeed very unfavorable at 3.5×10^{-7} (T. N. C. Wells, C. K. Ho, and A. R. Fersht, unpublished results). The value for the enzyme-bound species is 2.3 (Table II). The enzyme thus stabilizes E·Tyr–AMP·PP with respect to E·Tyr·ATP by some 9 kcal/mol. The residues mutated in this study account for 3 kcal/mol of this (Table IV). The favorable equilibrium constant for the enzyme-bound reagents means that activation is not inhibited by high concentrations of pyrophosphate and that pyrophosphorolysis of tyrosyl adenylate does not compete with transfer to tRNA. This could well be of phyisological importance since the flux of pyrophosphate produced in protein biosynthesis is so high that pyrophosphate accumulates to a concentration of ~0.5 mM in *E. coli* and some other organisms (Kukko & Heinonen, 1982).

It is also likely that the enzyme–product complementarity is required to slow down the rate of dissociation of Tyr–AMP from the enzyme. The aminoacylation of tRNA in vivo catalyzed by such enzymes as the tyrosyl-tRNA synthetase is probably stepwise: most of the tRNA in *E. coli* is aminoacylated and bound to elonation factor Tu so that the rate-determining step in aminoacylation is the release of discharged tRNA during protein synthesis [summarized by Mulvey & Fersht (1977)]. The enzyme thus exists mainly as the enzyme–tyrosyl adenylate complex in vivo.

Two points must be emphasized. First, enzyme–product or enzyme–intermediate complementarity should only be important for *enzyme-bound* species and not for reactions that require the products or intermediates to diffuse into solution. Second, although the residues modified here are responsible for enzyme–intermediate complementarity, there are residues elsewhere that are responsible for a large factor of enzyme–transition-state complementarity. Leatherbarrow et al. (1985) have located a binding site for the γ-phosphoryl group of ATP that contributes no stabilization energy to the E·Tyr·ATP or E·Tyr–AMP complexes but stabilizes the transition state by some 4–5 kcal/mol. The effect of the residues modified in this study is to fine tune the catalysis.

Mechanistic Interpretation. During the changes in substrate geometry that occur on formation of the transition state and products, the interactions of important side chains with tyrosine remain relatively unaltered, whereas those with ATP are markedly optimized. This suggests that tyrosine moves relatively little with respect to its binding site on the enzyme during the reaction but that there is a significant displacement of the ribose moiety of ATP. The latter could be accomplished either by a movement of the ATP relative to a rigid enzyme, since there are changes in bond lengths and angles during the reaction, or by a conformational change or by a combination of these two processes. It is even possible that there could be a good hydrogen bond between the enzyme and, say, Cys-35 in the E·Tyr·ATP complex but that it causes a distortion of the enzyme or conformational strain in the ATP that is relieved as the reaction proceeds. Resolution of these possibilities will need the direct solution of the structure of the enzyme–ATP complex, which has not proved possible so far by X-ray crystallography (Monteilhet et al., 1984). It is also likely that the position of the ribose moiety in the transition state is closer to its position in the E·Tyr–AMP complex rather than in the E·Tyr·ATP complex since the energy contributions from the side chains are more similar in the transition states and products than in the transition states and substrates.

Registry No. 5′-ATP, 56-65-5; PP_i, 14000-31-8; TyrTS, 9023-45-4; L-Tyr, 60-18-4.

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