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Internal Thermodynamics of Position 51 Mutants and Natural Variants of Tyrosyl-tRNA Synthetase†

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ABSTRACT: Natural variation and evolution impose structural changes on an enzyme that can affect the energetics of catalysis. The energy profile of reaction could, in theory, be altered in three distinct ways: uniform binding changes, differential binding changes, and catalysis of elementary steps. Residue threonine-51 of tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* is subject to natural variation, being replaced by alanine and proline in the enzymes from *Bacillus caldotenax* and *Escherichia coli*, respectively. The consequences of this variation on the energetics of formation of tyrosyl adenylate have been investigated by constructing free energy profiles for wild-type and mutant enzymes constructed by introducing these amino acids into the *B. stearothermophilus* enzyme. Mutation of Thr-51 to alanine, proline, and cysteine by site-directed mutagenesis improves the stabilization of the transition state in the formation of tyrosyl adenylate. Most marked is the mutation Thr-51 → Pro-51 which stabilizes the transition state by 2.2 kcal/mol and accelerates the forward rate 20-fold to a level near that of the enzyme from *E. coli*. However, the improved transition-state binding is accompanied by an even greater stabilization of tyrosyl adenylate. This reduces the rate of pyrophosphorolysis of tyrosyl adenylate and/or weakens the binding of pyrophosphate in the reverse reaction, shifting the equilibrium between enzyme-bound reactants greatly in favor of the enzyme–intermediate complex. The more stable mutant enzyme–tyrosyl adenylate complexes have lower rates of aminoacylation, suggesting that mutations which stabilize the intermediate slow down the subsequent transfer of tyrosine from tyrosyl adenylate to tRNA. In contrast, the natural variants have apparently evolved additional mechanisms to bind the transition state preferentially without further stabilizing tyrosyl adenylate. The free energy profiles reveal all three classes of energetic changes on mutation.

The evolution of rates and specificity of enzyme-catalyzed reactions has been the subject of several theoretical studies (Fersht, 1974; Crowley, 1975; Cornish-Bowden, 1976; Albery & Knowles, 1976). It has been proposed that enzymes maximize rates by binding transition states strongly and substrates weakly (Pauling, 1946) and by avoiding disadvantageous accumulation of intermediates (Fersht, 1974). For enzymes following Michaelis–Menten kinetics, this is achieved by improving the specificity constant (k_{cat}/K_M) while maintaining the Michaelis constant (K_M) above the physiological concentration of substrate (Fersht, 1974). In an analysis of enzymes following Briggs–Haldane kinetics, improvements in rate are envisioned to proceed through three major types of structural changes that alter the free energies of enzyme-bound complexes until the rate becomes diffusion controlled (Albery & Knowles, 1976). These steps have been designated "uniform binding", "differential binding", and "catalysis of the elementary steps" according to their distinctive effects on the free energy profile of the catalyzed reaction.

Much of the supportive experimental evidence has centered on the kinetic constants of present-day enzymes. The hypotheses that K_M values tend to match in vivo levels of substrates and that k_{cat}/K_M increases to values expected for macromolecular diffusion are supported by the kinetic properties of several glycolytic enzymes (Fersht, 1985, pp 327–331). The free energy profile for one of these enzymes, triose-

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phosphate isomerase, has all the characteristics proposed for enzymes that have attained "catalytic perfection" (Albery & Knowles, 1976). Because information is obtained only for the highly refined products of enzyme evolution, this kind of analysis provides little empirical evidence concerning the changes in kinetic constants that occur during the process itself.

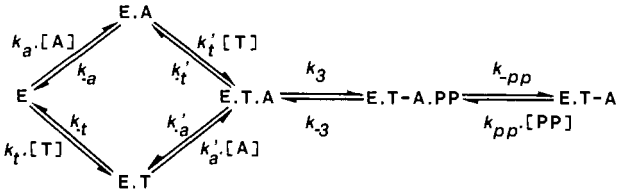
As a method for generating potential past forms of an enzyme, site-directed mutagenesis offers a way of testing proposals concerning the evolution of rates and specificity. With knowledge of the three-dimensional structure of the enzyme, mutants with large kinetic changes can be systematically generated. The application of site-directed mutagenesis to the study of enzyme evolution is being explored with the tyrosyl-tRNA synthetase from *Bacillus stearothermophilus*. The enzyme catalyzes the synthesis of tyrosyl adenylate from tyrosine and ATP, and the transfer of tyrosine from the enzyme-bound adenylate to $tRNA^{Tyr}$ (Fersht & Jakes, 1975b). Tyrosyl-tRNA synthetase is particularly suitable for this type of study because both reactions can be monitored in the pre-steady-state phase by stopped-flow and pulsed-quenched flow techniques (Fersht et al., 1975; Fersht & Jakes, 1975b). These methods provide all the kinetic parameters necessary to construct the free energy profile for the entire enzyme-catalyzed reaction.

Two complementary approaches are being taken. First, favorable enzyme–substrate interactions can be deleted to create "less-evolved" enzymes, and the contribution of the missing side chains to the energetics of the reaction can be quantitated ["reverse evolution" (Wells & Fersht, 1986)]. Second, favorable contacts with the transition state can be introduced to assess the effects of adding improved interactions (Wilkinson et al., 1984). Since this type of mutation is more difficult to deduce by inspection, we are also investigating the kinetic properties of species variants of tyrosyl-tRNA synthetase that have improved rates.

This study focuses on the tyrosyl adenylate synthesis reaction catalyzed by tyrosyl-tRNA synthetases with mutations generated at threonine-51. This position is of interest because (i) the crystal structure of the enzyme-bound tyrosyl adenylate suggests that the hydrogen bond between the threonine –OH group and the oxygen in the ribose ring of ATP is not optimal and therefore can be improved (Fersht et al., 1985), (ii) of the nine polar side chains which are sufficiently close to polar groups of tyrosyl adenylate for hydrogen bonding to be possible, Thr-51 is the only nonconserved residue among the tyrosyl-tRNA synthetases from three species of bacteria, and (iii) position 51 appears to be highly variable, being replaced by alanine in the enzyme from *Bacillus caldotenax* [99% homologous (Jones et al., 1986)] and by proline in the enzyme from *Escherichia coli* [56% homologous (Winter et al., 1983)]. Both of these natural variants are more active than the wild-type *B. stearothermophilus* enzyme in the partial reaction of synthesis of tyrosyl adenylate and in the overall aminoacylation reaction (Jones et al., 1986; D. Lowe and A. R. Fersht, unpublished results). Accordingly, the mutants TyrTS(Ala-51)[1] and TyrTS(Pro-51) and the native enzymes from *B. caldotenax* and *E. coli* were investigated. Results are also presented for the mutant TyrTS(Cys-51) in which the hydrogen bond between the mutant –SH group and the ring oxygen in the ribose moiety of the transition state appears to be optimized (Fersht et al., 1985).

[1] Abbreviations: TyrTS, tyrosyl-tRNA synthetase; TyrTS(Xxx-Nn), mutant tyrosyl-tRNA synthetase (*Bacillus stearothermophilus*) with amino acid Xxx at position Nn; Tris, tris(hydroxymethyl)aminomethane; PP_i, inorganic pyrophosphate.

Scheme I



Materials and Methods

Purification of Tyrosyl-tRNA Synthetases. Mutant proteins were expressed from mutant M13mp93 phage templates constructed as described previously (Carter et al., 1984). Mutant enzymes from *B. stearothermophilus* and the enzyme from *B. caldotenax* were purified to electrophoretic homogeneity as described by Wells and Fersht (1986). The enzyme from *E. coli* had been purified according to Atkinson et al. (1979). TyrTS(Pro-51) and *E. coli* TyrTS were stored at −20 °C in 50% glycerol buffer containing 144 mM Tris-HCl (pH 7.8), 10 mM 2-mercaptoethanol, and 0.1 mM phenylmethanesulfonyl fluoride (PMSF). The glycerol was removed before assaying by desalting on a 1 × 10 cm Sephadex G-25 (fine) column equilibrated with 144 mM Tris-HCl (pH 7.8), 10 mM 2-mercaptoethanol, 10 mM $MgCl_2$, and 0.1 mM PMSF (standard buffer). TyrTS(Cys-51) was stored in small aliquots at −70 °C in the same buffer, except that 0.1 mM dithioerythritol replaced 2-mercaptoethanol. Because of the possible oxidation of Cys-51, the enzyme was assayed in the presence of 1 mM dithioerythritol. The other enzymes were stored in small aliquots at −70 °C in standard buffer after rapid freezing in liquid nitrogen.

Stopped-Flow Fluorescence and Equilibrium Dialysis. The pre-steady-state formation and pyrophosphorolysis of the enzyme-bound tyrosyl adenylate were monitored by changes in protein fluorescence in a stopped-flow fluorometer as described by Wells and Fersht (1986). The dissociation constant for the enzyme–tyrosine complexes (K_t) was determined by equilibrium dialysis (Fersht et al., 1975).

Results

Forward Reaction: Rate of Tyrosyl Adenylate Formation and Binding of ATP. Upon mixing of the enzyme–tyrosine complex with ATP, there is an exponential decrease in protein fluorescence (ca. 6%) corresponding to the formation of the enzyme-bound tyrosyl adenylate (Fersht et al., 1975). The observed rate constant is dependent on ATP concentration and follows Michaelis–Menten kinetics. The associated k_{cat} and K_M equal k_3 (rate of tyrosyl adenylate formation) and K_a' (dissociation constant of ATP), respectively (see Scheme I). Saturation kinetics appear to hold for TyrTS(Pro-51), but the rate constants (>400 s^{-1}) at ATP concentrations which approach saturation (>2 mM) are too high to be determined reliably. The same phenomenon has been previously observed for *E. coli* TyrTS ($k_{cat} \sim 1300\ s^{-1}$; $K_M \sim 18$ mM; Fersht & Jakes, 1975b). What is clear is that the mutation Thr-51 → Pro-51 accelerates the forward reaction by at least 1 order of magnitude above that of the wild-type *B. stearothermophilus* enzyme, and the rate is near that for the *E. coli* enzyme (Table I).

Previous steady-state measurements have shown that Pro-51 causes a 15-fold decrease in K_M(ATP) in the ATP–[^{32}P]PP_i exchange reaction (Wilkinson et al., 1984). Mutants displaying low steady-state K_M(ATP) values do not necessarily bind ATP strongly since the K_M reflects the apparent dissociation constants for *all* enzyme-bound complexes (Fersht, 1985, p 104). This is now shown to be the case for TyrTS-

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

enzyme	k_3 (s^{-1})	K_a' (mM)	k_3/K_a' (s^{-1} M^{-1})	K_t (μM)
wild-type[b] [TyrTS(Thr-51)]	38	4.7	8090	12
TyrTS(Ala-51)	75	4.7	15960	12
TyrTS(Pro-51)[c]	~700	~2	295000[e]	14
TyrTS(Cys-51)	41	0.34	122060	22
B. caldotenax TyrTS	35	1.9	18580	24
E. coli TyrTS[d]	~1300	~18	73000[e]	24

[a] Experiments performed at 25 °C, pH 7.78 (144 mM Tris-HCl, 10 mM $MgCl_2$, and 14 mM 2-mercaptoethanol). Constants are defined in Scheme I. Values of k_3 are extrapolated to saturating concentrations of ATP and tyrosine. K_a' is the dissociation constant of ATP from the E·Tyr·ATP complex determined from stopped-flow fluorescence. K_t is the dissociation constant of tyrosine from the E·Tyr complex determined by equilibrium dialysis. Standard errors are typically ±5% for k_3 and ±10% for dissociation constants. [b] Values for wild-type enzyme are from Wells and Fersht (1986). [c] Values for k_3 and K_a' were determined at below saturating concentrations of ATP. [d] Values for k_3, K_a', and K_t are from Jakes and Fersht (1975a,b). [e] Determined from the slope of rate vs. [ATP] at low substrate concentration.

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

enzyme	k_{-3} (s^{-1})	K_{pp} (mM)	k_{-3}/K_{pp} (s^{-1} M^{-1})	K_{eq} (k_3/k_{-3})
wild-type[b]	16.6	0.61	27210	2.29
TyrTS(Ala-51)	10.5	0.98	10710	7.14
TyrTS(Pro-51)	13.8	0.63	21900	~50
TyrTS(Cys-51)	17.4	0.89	19550	2.39
B. caldotenax TyrTS	45.5	0.33	137880	0.78
E. coli TyrTS	74.9	0.14	535000	~17

[a] Conditions as in Table I. [b] Values for wild-type enzyme are from Wells and Fersht (1986). k_{-3} is the rate of the attack of pyrophosphate on the enzyme-tyrosyl adenylate complex, and K_{pp} is the dissociation constant of pyrophosphate from the E·Tyr–AMP·PP_i complex determined from stopped-flow fluorescence.

(Ala-51) and TyrTS(Pro-51). The dissociation constant for ATP in these mutants is not significantly different from the wild-type *B. stearothermophilus* enzyme (Table I). K_a' for TyrTS(Pro-51) is an approximate measurement (for reasons discussed above), but it is clear that the large decrease in the steady-state K_M(ATP) is not paralleled by a similar large drop in K_a'. Rather, the reduction in K_M arises from stronger binding of the tyrosyl adenylate intermediate (see below).

For both TyrTS(Ala-51) and *B. caldotenax* TyrTS, the specificity constant (k_3/K_a') is elevated by 2-fold. The increase in the mutant arises from a 2-fold improvement in rate with no effect on ATP binding, whereas in the natural variant it results from stronger ATP binding with no change in rate. The 15-fold increase in specificity constant for TyrTS(Cys-51) is solely due to stronger binding of ATP (Table I).

Binding of Tyrosine by Equilibrium Dialysis. Tyrosine binding is not significantly affected in TyrTS(Ala-51) and TyrTS(Pro-51), indicating that the effects of the mutations are localized to the ATP binding site. K_t values for the other enzymes are greater by 2-fold (Table I). For *B. caldotenax* TyrTS (which differs from the wild-type *B. stearothermophilus* enzyme by four amino acids: His-48 → Asn-48 and Thr-51 → Ala-51 occur in the ATP binding pocket; Met-55 → Leu-55 and Ala-297 → Thr-297 are distant from the active site; Jones et al., 1986), the effect on tyrosine binding is probably solely due to the asparagine at position 48. Similar increases in K_t have been observed for position 48 mutants of the *B. stearothermophilus* enzyme (Lowe et al., 1985).

Reverse Reaction: Pyrophosphorolysis of Enzyme–Tyrosyl Adenylate Complexes and Binding of Pyrophosphate. Upon mixing of the enzyme–tyrosyl adenylate complex (isolated by gel filtration) with pyrophosphate, there is an increase in protein fluorescence as the complex reacts with pyrophosphate to re-form the enzyme–tyrosine–ATP ternary complex (Fersht et al., 1975). The observed rate constant for this process is pyrophosphate dependent and followed saturation kinetics for all enzymes studied with the associated k_{cat} and K_M equaling k_{-3} (rate of pyrophosphorolysis) and K_{pp} (dissociation constant for pyrophosphate), respectively (see Scheme I).

It is seen from Table II that the specificity constants for the reverse reaction (k_{-3}/K_{pp}) for all three mutants are lower than that for wild-type enzyme. The decrease reflects a combination of effects from slightly lower rates and/or weaker binding of pyrophosphate. TyrTS(Ala-51) had the lowest specificity constant (2.5-fold decrease). In contrast, the values for the same constant are severalfold greater for the *B. caldotenax* (5-fold) and *E. coli* (20-fold) variants. This arises from both faster rates of pyrophosphorolysis and stronger binding of pyrophosphate. Thus, improved kinetic constants for pyrophosphorolysis is a distinguishing feature of the natural variants.

Calculation of Energy Levels of Enzyme-Bound Intermediates. The steps of the tyrosyl adenylate synthesis reaction and corresponding rate constants are described by Scheme I in which the dissociation constants are defined: $K_t = k_{-t}/k_t = [E][Tyr]/[E\cdot Tyr]$, $K_a' = k_{-a}/k_a' = [E\cdot Tyr][ATP]/[E\cdot Tyr\cdot ATP]$, etc. Gibbs' free energy of each enzyme-bound complex can be calculated from the measured rate and binding constants (summarized in Tables I and II) according to the thermodynamic equations (Wells & Fersht, 1986)

$$G_E = 0 \text{ (reference state)} \quad (1)$$

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

$$G_{E\cdot Tyr\cdot ATP} = RT \ln (K_t K_a') \quad (3)$$

$$G_{[Tyr\text{–}AMP]^*} = RT \ln (k_b T/h) - RT \ln (k_3/K_a' K_t) \quad (4)$$

$$G_{E\cdot Tyr\text{–}AMP\cdot PP_i} = -RT \ln (k_3/k_{-3} K_a' K_t) \quad (5)$$

$$G_{E\cdot Tyr\text{–}AMP} = -RT \ln (k_3 K_{pp}/k_{-3} K_a' K_t) \quad (6)$$

where R is the gas constant, T is the absolute temperature, k_b is Boltzmann's constant, and h is Planck's constant. The standard state is 1 M for ATP, tyrosine, and pyrophosphate and the reference state is the free enzyme. The net contribution of a mutated residue toward stabilizing each enzyme-bound complex along the reaction pathway is determined from the difference in Gibbs' free energy ($\Delta G_{E\cdot X}$) by subtracting the free energy of the mutant enzyme–X complex from that of the corresponding complex with wild-type enzyme. Energy differences are readily visualized by comparing the free energy profiles for wild-type enzyme and mutant (or species variant) with the external (unbound) states superimposed (Figure 1). For cases in which the enzyme may contain major structural differences (e.g., *E. coli* TyrTS), ΔG represents the global contribution of multiple mutations to the binding energy.

Previous steady-state kinetic measurements have shown that Ala-51 and Pro-51 stabilize the activated complex by 0.38 and 1.9 kcal/mol (Wilkinson et al., 1984). Our results confirm this (the corresponding differences in free energy calculated from pre-steady-state data are 0.41 and 2.17 kcal/mol) and also reveal that there is greater stabilization of the E·Tyr–AMP·PP_i and E·Tyr-AMP complexes of both mutants (Table III). Ala-51 and Pro-51 stabilize the enzyme–tyrosyl adenylate complex by 0.96 and 2.30 kcal/mol, respectively. Ala-51 contributes no net stabilization to the E·Tyr·ATP ternary complex, and Pro-51 stabilizes the ternary complex by ~0.4 kcal/mol. The net effect of Pro-51 is an incremental

Table III: Differences in Free Energy between Bound Complexes of Mutant or Natural Variant Tyrosyl-tRNA Synthetases and Wild-Type Enzyme from *B. stearothermophilus*[a]

enzyme	ΔG (kcal mol^{-1})			
	$G_{E\cdot Tyr\cdot ATP}$	$G_{[E\cdot Tyr\cdot ATP]^*}$	$G_{E\cdot Tyr-AMP\cdot PP_i}$	$G_{E\cdot Tyr-AMP}$
TyrTS(Ala-51)	0	−0.41	−0.67	−0.96
TyrTS(Pro-51)	~−0.4	−2.17	−2.27	−2.30
TyrTS(Cys-51)	−1.19	−1.24	−1.21	−1.44
B. caldotenax TyrTS	−0.11	−0.08	+0.52	+0.88
E. coli TyrTS	+1.21	−0.89	−0.01	+0.88

[a] These are the differences in energy levels between the broken and solid horizontal lines in Figure 1. The values for the natural variants (*B. caldotenax* and *E. coli*) are not readily interpretable in absolute terms because of the multitude of amino acid replacements, but the relative values for the different complexes show the differential stabilization effects of the structural changes.

Table IV: Comparison of Kinetic Constants for Activation of Tyrosine Determined by Steady-State (Pyrophosphate Exchange) and Pre-Steady-State Kinetics

enzyme	k_{cat} in steady state (s^{-1})	
	observed[a]	calculated[b]
wild-type	8.4	9.5
TyrTS(Ala-51)	8.8	6.4
TyrTS(Pro-51)	12.4	10.3
TyrTS(Cys-51)	12.0	9.3
B. caldotenax TyrTS	16.2	18.5
E. coli TyrTS	71	66

[a] Values of k_{cat}, determined from the ATP dependence of the ATP–[^{32}P]PP$_i$ exchange reaction in the presence of 50 μM tyrosine and extrapolated to infinite concentration, are from Wilkinson et al. (1984), Fersht et al. (1985), Jones et al. (1986), and C. K. Ho and A. R. Fersht (unpublished results). [b] Calculated from eq 8 using pre-steady-state data from Tables I and II.

rise in binding energies as the reaction proceeds (Table III; Figure 2).

The predominant effect of Cys-51 is a 15-fold lowering of K_a' with no changes in the subsequent rate and binding constants (Tables I and II). The net result is the uniform stabilization of all complexes in which enzyme is bound to the ATP moiety (Table IV; Figure 1). The bound tyrosyl adenylate is stabilized by 1.44 kcal/mol.

Relative to the wild-type *B. stearothermophilus* enzyme, the binding energy in complexes of the *B. caldotenax* TyrTS decreases as the reaction proceeds. A similar trend occurs for *E. coli* TyrTS except that the initial E·Tyr·ATP complex is destabilized by 1.21 kcal/mol. The net result is the *destabilization* of the enzyme–tyrosyl adenylate complex by 0.88 kcal/mol for both enzymes (Table III).

Comparison of Steady-State and Pre-Steady-State Kinetic Parameters. The steady-state phase of the adenylate synthesis reaction is conventionally measured by the ATP–PP$_i$ isotope exchange method (Calendar & Berg, 1966). Because the exchange process occurs under equilibrium conditions, the steady-state kinetic parameters are functions of both the forward and reverse reactions. For the mechanism

$$E\cdot Tyr + ATP \overset{K_a'}{\rightleftharpoons} E\cdot Tyr\cdot ATP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E\cdot Tyr{-}AMP + PP_i \quad (7)$$

(where $K_a' = [E\cdot Tyr][ATP]/[E\cdot Tyr\cdot ATP]$) it can be shown that, in the presence of a fixed concentration of PP in the pyrophosphate exchange assay and at saturating concentrations of tyrosine and ATP

$$k_{cat} = k_3k_{-3}'/(k_3 + k_{-3}') \quad (8)$$

Table V: Comparison of the Free Energy of Enzyme–Tyrosyl Adenylate Complexes[a] and Rate of Aminoacylation[b]

enzyme	$G_{E\cdot Tyr-AMP}$ (kcal mol^{-1})	k_{cat} amino-acylation (s^{-1})
B. caldotenax TyrTS	−5.11	6.9[c]
E. coli TyrTS	−5.11	6.3[d]
wild-type TyrTS (*B. stearothermophilus*)	−5.99	4.7[e]
TyrTS(Ala-51)	−6.95	4.0[e]
TyrTS(Cys-51)	−7.43	2.9[f]
TyrTS(Pro-51)	−8.29	1.8[e]

[a] Standard state = 1 M Tyr, 1 M ATP, 1 M PP$_i$, and free enzyme ($G_E = 0$). [b] Steady-state rates determined at 25 °C, pH 7.78 (144 mM Tris-HCl, 10 mM $MgCl_2$, 14 mM 2-mercaptoethanol, and 0.1 mM phenylmethanesulfonyl fluoride). [c] From Jones et al (1986). [d] From A. R. Fersht, unpublished data. [e] From Wilkinson et al. (1984). [f] From Fersht et al. (1985).

where

$$k_{-3}' = k_{-3}[PP_i]/([PP_i] + K_{pp}) \quad (9)$$

When the pre-steady-state rate and dissociation constants are substituted into these equations, the calculated and observed steady-state kinetic parameters are in good agreement (Table IV). When the forward rate is fast ($k_3 \gg k_{-3}$) the relationships predict that the reverse reaction is rate limiting. TyrTS(Ala-51), TyrTS(Pro-51), and the *E. coli* enzyme confirm that the steady-state turnover number (k_{cat}) approaches the rate of pyrophosphorolysis (k_{-3}) (see Tables II and IV). For cases in which both forward and reverse processes are partially rate limiting [TyrTS(Cys-51), *B. caldotenax* TyrTS] k_{cat} has the expected intermediate value. The excellent agreement between pre-steady-state and steady-state rates gives us greater confidence in the model and in the accuracy of the values obtained from stopped-flow fluorescence.

Discussion

Mutation of threonine-51 in the tyrosyl-tRNA synthetase from *B. stearothermophilus* to alanine, proline, and cysteine introduces favorable interactions with the transition state of the tyrosyl adenylate synthesis step, as is demonstrated by faster rates and/or stronger ATP binding in the forward reaction. Do these mutations therefore improve catalysis? Because both the activation and transfer reactions are partially rate limiting (Fersht & Jakes, 1975b; Fersht & Kaethner, 1976), selective pressure will be exerted on both steps to maximize the overall rate. Under physiological conditions the mutant enzymes exist mainly as the enzyme–tyrosyl adenylate complex (most of the tRNA in *E. coli* is aminoacylated and bound to elongation factor Tu; Mulvey & Fersht, 1977). Therefore, the rate of catalysis will depend on the rate of transfer of tyrosine from tyrosyl adenylate to tRNA^{Tyr} and the turnover of tyrosyl-tRNA^{Tyr}. Although this study concentrates on the first half-reaction and provides no information about the rates of transfer, steady-state aminoacylation rates have been measured for each of the enzymes (Wilkinson et al., 1984; Fersht et al., 1985; Jones et al., 1986). Comparing the k_{cat} values for aminoacylation with the free energy of the enzyme–tyrosyl adenylate complex (Table V), we find that the more stable complexes have lower turnover numbers. For this reason the mutant enzymes are poorer physiological catalysts despite the improvement in transition-state binding.

Because the rate for the partial adenylate synthesis reaction is unchanged or improved in the mutant enzymes, the apparent explanation for slower charging is that the mutations reduce the subsequent rate of transfer of tyrosine from tyrosyl ade-

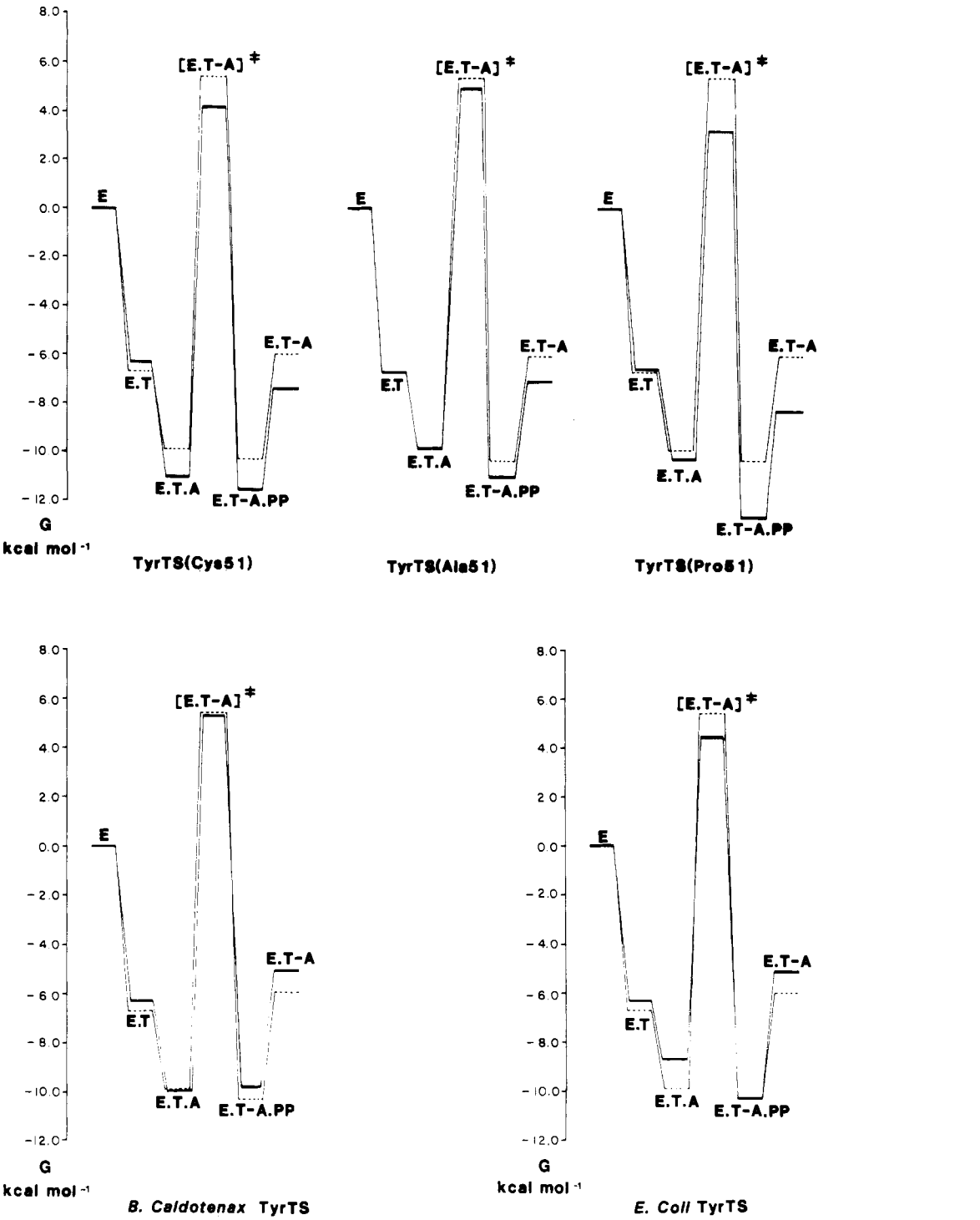


FIGURE 1: Gibbs' free energy profiles for the formation of tyrosyl adenylate (as defined in Scheme I) by wild-type (energy levels in broken lines) and indicated mutant or natural variant (energy levels in solid lines) tyrosyl-tRNA synthetases, using standard states of 1 M for tyrosine, ATP, and pyrophosphate. Values for the free energy differences between mutant and wild-type enzyme–substrate complexes are given in Table III.

nylate to tRNA. The ease of transfer will depend on the free energy difference between the enzyme–tyrosyl adenylate complex and the transition state of the transfer step. It seems probable that the mutants bind tyrosyl adenylate too strongly and create a thermodynamic pit out of which the intermediate must climb. On the other hand, the natural variants have evolved to bind the intermediate *less* strongly and to avoid thermodynamic traps. This difference illustrates the catalytic disadvantage of accumulating intermediates.

Classification of Mutations. Albery and Knowles (1976) have suggested that mutations that improve the rate of the catalyzed reaction can be grouped according to their effects on the free energies of enzyme-bound complexes. It was proposed that mutations that equally stabilize all bound complexes (uniform binding) are the easiest to achieve, since the stabilization can be mediated by many types of binding interactions and is nonspecific. Differential binding is harder to achieve in as much as the mutation must discriminate among several bound species that resemble one another. Lastly, mutations that preferentially stabilize the transition state of the highest remaining barrier (catalysis of the elementary steps) are the most difficult; these require subtle interactions with features of the transition state not present in the ground-state complexes. Our analysis of the energetics

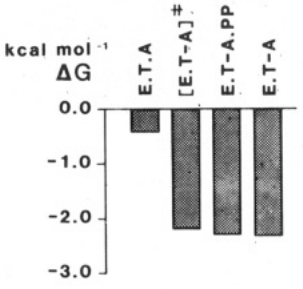


FIGURE 2: Illustration of the effects of the mutation Thr-51 → Pro-51 on the Gibbs' free energies of enzyme-bound complexes. Free energy differences are relative to those of the wild-type *B. stearothermophilus* enzyme.

of the reaction catalyzed by mutants and natural variants of tyrosyl-tRNA synthetase provides the first experimental evidence for mutations in all three classes.

Mutation of Thr-51 → Cys-51 uniformly lowers the free energy of all states in which the enzyme is bound to the ATP moiety by 1.2–1.4 kcal/mol and thus represents a uniform binding mutant. TyrTS(Ala-51) forms stronger interactions with ATP than does the wild-type enzyme when the nucleotide moiety is in the activated and intermediate complexes but exhibits no additional binding energy in the initial E·Tyr·ATP complex. Because the mutation distinguishes between the unreacted and reacted forms of ATP, TyrTS(Ala-51) is a differential binding mutant (Table III; Figure 1). Because the rate of the chemical step of the reaction is also increased, there is also catalysis of the elementary step. Mutation of Thr-51 → Pro-51 also leads to mixed effects, but the changes are more dramatic. TyrTS(Pro-51) stabilizes the initial ternary complex by ~0.4 kcal/mol, but the greatest stabilizations occur in the [E·Tyr·ATP]‡, E·Tyr–AMP·PP_i, and E·Tyr–AMP complexes (2.2–2.3 kcal/mol). It is interesting that mutation of a single position which is far removed from the seat of reaction can lead to all three classes of binding energy changes. A pure catalysis of the elementary step effect has been demonstrated in other experiments from this laboratory (Leatherbarrow et al., 1985). A site was located that binds the γ-phosphate of ATP only when it is in the transition-state structure. Mutation of that site affects just the rate constant k_3 and not the binding of tyrosine and ATP.

The *B. caldotenax* and *E. coli* enzymes are not simply "position-51" mutants of the *B. stearothermophilus* TyrTS but are natural variants containing other mutations. Their free energy profiles may still be compared but are more difficult to classify because of several mixed effects on the reaction energetics (Table III). However, it is informative to note that (i) the forward and reverse rates (k_3 and k_{-3}) are faster in *E. coli* TyrTS than in the wild-type *B. stearothermophilus* enzyme and (ii) the specificity constants for tyrosyl adenylate formation and pyrophosphorolysis (k_3/K_a' and k_{-3}/K_{pp}) for both *E. coli* TyrTS and *B. caldotenax* TyrTS are greater than those for the *B. stearothermophilus* enzyme (Tables I and II). The former shows that transition-state stabilization is improved relative to the innermost bound species (E·Tyr·ATP and E·Tyr–AMP·PP_i). The latter shows that it is also improved relative to the outermost bound species (E·Tyr and E·Tyr–AMP). Thus, irrespective of any mixed effects from uniform binding and differential binding, the natural variants stabilize the transition-state complex relative to any of the ground-state complexes (Figure 1).

Mechanistic Interpretation. Not enough mutants have been analyzed to allow us to decide which of the three classes of mutations are easier to achieve. Among ATP-binding mutants, however, those which stabilize transition-state and intermediate

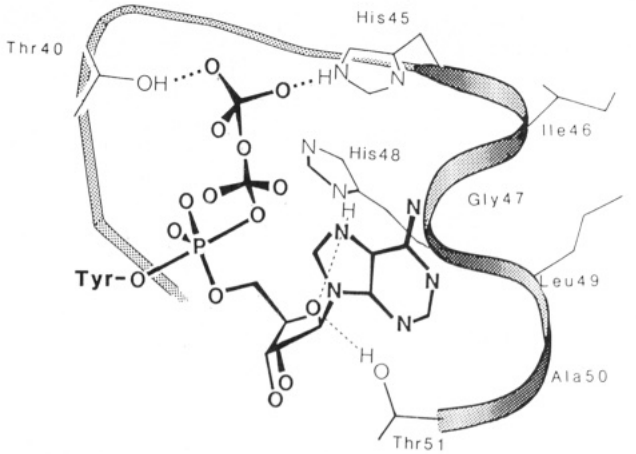


FIGURE 3: Sketch of the transition state of the ATP moiety during the formation of tyrosyl adenylate illustrating hydrogen bonds made with the enzyme [from model building by extrapolation from the crystal structure of the enzyme–tyrosyl adenylate complex by Leatherbarrow et al. (1985)].

complexes more than the E·ATP·Tyr ternary complex [Gly-35, Ser-35, Gly-48 (Wells & Fersht, 1986), Ala-51, and Pro-51 mutants] are apparently more prevalent than uniform binding mutants (Cys-51). This is probably because the mutations are specifically targeted at the active site to alter the interaction with the ribose ring of ATP, and the configuration of ribose in the transition state more closely resembles that in the enzyme–tyrosyl adenylate complex than that in the unreacted ternary complex. This supports the proposal that ATP and enzyme move relative to one another during the reaction (Wells & Fersht, 1986). That TyrTS(Cys-51) is the exception implies that the optimal contact between the –SH group and ribose is maintained throughout the reaction or that a favorable interaction with unreacted ATP is exchanged for another equally favorable one with reacted ATP as the reaction proceeds. This could be facilitated by the longer hydrogen bond between the mutant –SH and the ribose moiety and by freer rotation about the C_α–C_β bond upon deleting the γ-CH_3 group in mutating threonine to cysteine.

Mechanistic Basis for the Preferential Transition-State Stabilization by Natural Variants. The enzymes from *E. coli* and *B. caldotenax* are more effective than the enzymes from *B. stearothermophilus* in selectively stabilizing the transition state of tyrosine and ATP without undue stabilization of the intermediate tyrosyl adenylate. Selective binding may, in principle, arise from either substrate destabilization and/or transition-state stabilization. One way to achieve this is by improving interactions with features in the transition-state configuration that are absent in the ground-state complexes. A likely target is the pyrophosphate moiety of ATP. That the *E. coli* and *B. caldotenax* enzymes bind pyrophosphate more strongly in the reverse reaction supports this proposal.

The enzymes from *B. caldotenax* and *B. stearothermophilus* differ by four amino acids, two of which are at the active site: His-48 → Asn-48 and Thr-51 → Ala-51 (the other two are distant) (Jones et al., 1986). Although neither of the side chains at positions 48 and 51 interact directly with pyrophosphate, both lie in a helix (residues 46–61) near the pyrophosphate binding pocket (Figure 3) (Leatherbarrow et al., 1985). In the *B. stearothermophilus* enzyme neither mutation alone improves binding of pyrophosphate [unpublished data for TyrTS(Asn-48) from T. Wells and A. R. Fersht], so that the stronger pyrophosphate binding in the *B. caldotenax* enzyme arises from the coupled interactions of Asn-48 and Ala-51. Improved pyrophosphorolysis therefore may result

		40					45		48		51	
B.St.	TyrTS	T	A	D	S	L	H	I	G	H	L	A	T
B.c.	TyrTS	T	A	D	S	L	H	I	G	N	L	A	A
E.c.	TyrTS	T	A	D	S	L	H	L	G	H	L	V	P

FIGURE 4: Comparison of the amino acid sequence of positions 40–51 in the tyrosyl-tRNA synthetases from *B. stearothermophilus* (*B.st.*), *B. caldotenax* (*B.c.*), and *E. coli* (*E.c.*). Nonhomologous positions are boxed. Additionally, residues 33–39 (not shown) are identical among the three species variants.

from indirect effects on side chains that directly interact with pyrophosphate (e.g., His-45).

Identifying analogous structural differences that account for improved pyrophosphorolysis and transition-state binding in *E. coli* TyrTS is complicated by the lesser degree of homology with the *B. stearothermophilus* enzyme. Although the mutation Thr-51 → Pro-51 in the *B. stearothermophilus* enzyme accelerates the forward rate to that of the *E. coli* enzyme, Pro-51 has little effect on the kinetic constants for the reverse reaction so that it alone cannot account for improved pyrophosphorolysis in the *E. coli* enzyme. Searching elsewhere around the active site, we find that all of the amino acids in the loop–helix segment which surrounds the pyrophosphate binding pocket (residues 33–49; see Figure 3) are conserved in the *E. coli* enzyme except for Ile-46, which is replaced by leucine (Figure 4). The contribution of this residue and of other nonhomologous amino acids toward pyrophosphate binding and preferential transition-state stabilization can be assessed in further heuristic rounds of mutagenesis.

Registry No. TyrTS, 9023-45-4; ATP, 56-65-5; L-Thr, 72-19-5; L-Ala, 56-41-7; L-Pro, 147-85-3; L-Cys, 52-90-4; L-Tyr, 60-18-4.

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