Use of Binding Energy in Catalysis: Optimization of Rate in a Multistep Reaction†

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ABSTRACT: The role of binding energy in optimizing the overall rate of catalysis by the tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* has been investigated by measuring the rate constants for transfer of tyrosine from engineered mutants to tRNA. The residues chosen for mutation are those that were previously identified as binding tyrosyl adenylate and contributing to the rate constant for its formation. It was previously found that tighter binding of the tyrosyl adenylate was accompanied by an increase in the rate constant for its formation. A new linear free energy relationship is presented that links the two. We now find that the rate constant for transfer of Tyr from Tyr-AMP to tRNA decreases with increasing stability of the E·Tyr-AMP complex on mutation of Thr-51. Position 51 is the one that is found to be the most variable of the binding-site residues among the enzymes from different species. The tightness of binding of the intermediate is thus a compromise, since stabilizing the intermediate speeds up the first step but slows down the second. The rate constants for activation and transfer by wild-type enzymes are very similar, which is optimal for the rate of the overall reaction. Those for the mutants diverge so that the rate of overall catalysis is lower.

The aminoacylation of tRNA catalyzed by aminoacyl-tRNA synthetases is a two-step reaction: activation of the amino acid (eq 1) followed by its transfer to tRNA (eq 2):

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

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

Catalysis of the activation of tyrosine by the tyrosyl-tRNA synthetase (TyrRS) (*Bacillus stearothermophilus*) results solely from the use of binding energy [reviewed by Fersht (1987)]. Kinetic studies on engineered mutant enzymes revealed that different residues at the active site selectively stabilize the transition state and/or the intermediate tyrosyl adenylate, relative to the ground state. Catalysis is delocalized throughout the substrate binding region and can involve side chains far from the seat of the reaction. These studies may now be extended to step 2 of the reaction since we have obtained large quantities of $tRNA^{Tyr}$ and have found that the specific binding of tRNA and the subsequent transfer of tyrosine to the tRNA can be monitored in a stopped-flow spectrofluorometer (Avis et al., 1993). The side chains of TyrRS chosen for mutation are active-site residues whose roles in step 1 are known. Pre-steady-state kinetic analysis of step 2, catalyzed by the same mutants, will allow the free energy profile of the reaction to be extended up to the second transition state, thereby yielding information about the use of binding energy in overall catalysis. In this study, the roles of two active-site residues of TyrRS are investigated: cysteine 35 and threonine 51.

Pre-steady-state kinetic analysis of tRNA charging by TyrRS has confirmed that both the tyrosine activation and tRNA charging steps have similar activation barriers (Avis et al., 1993). Optimization of the overall rate during evolution could have involved alteration of the energy profile of both steps. There are nine polar residues in the active site of TyrRS known to provide hydrogen bonds for binding and catalysis during tyrosine activation. Thr51 and His48 are the only two of these that are not conserved in tyrosyl-tRNA synthetases from three bacterial species. Mutation of Thr51 has provided dramatic increases in the rate of activation (Wilkinson et al., 1984; Fersht et al., 1985). In the enzyme from *Bacillus caldotenax*, which shares 99% identity with the *B. stearothermophilus* enzyme, Thr51 is replaced by an alanine, and in the *Escherichia coli* enzyme (which shares 56% identity), proline is at this position (Jones et al., 1986). Mutation of Thr51 of the *B. stearothermophilus* TyrRS to alanine or proline gives improved catalysis of tyrosine activation due to formation of more favorable interactions with the transition state and E·Tyr-AMP intermediate (Fersht et al., 1985; Ho & Fersht, 1986). The *B. caldotenax* and *E. coli* enzymes also exhibit better catalysis of tyrosine activation (Ho & Fersht, 1986). However, the natural variants and the *B. stearothermophilus* TyrRS position 51 mutants differ in their rates of overall aminoacylation relative to that of the wild-type *B. stearothermophilus* enzyme. Whereas the natural variants have higher rates of overall aminoacylation, the TyrRS mutants exhibit reduced rates. It was proposed that the reduced aminoacylation rates of the mutants are due to a slower tyrosine transfer step caused by excessive stabilization of the tyrosyl adenylate intermediate (Ho & Fersht, 1986). It is possible that position 51 in TyrRS from *B. stearothermophilus* may have evolved so that such excessive stabilization is avoided, thereby reducing the activation energy for step II and optimizing overall catalysis. Position 51 does not have such a role in the *B. caldotenax* and *E. coli* enzymes, and the role may be filled by other active-site residues (Ho & Fersht, 1986).

Whereas Thr51 appears to form unfavorable interactions with the adenylate (via the ribose ring oxygen) in the transition state and intermediate, Cys35 forms a stabilizing interaction

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(directly or indirectly) with the 3′ OH of the adenylate ribose in the transition state and intermediate to aid catalysis (Wilkinson et al., 1983; Wells & Fersht, 1986; Brick et al., 1989). The two residues, therefore, having opposing effects during catalysis of step I. Their interactions with enzyme-bound species during step II are investigated here in order to establish their roles in optimization of overall catalysis of the two-step reaction.

MATERIALS AND METHODS

Materials. Molecular biological reagents are described by Avis et al. (1993). Chemical reagents were purchased from Sigma (London) and radiochemicals from Amersham International.

Preparation of Tyrosyl-tRNA Synthetase Mutants. The tyrosyl-tRNA synthetase gene in pYTS5 (E. A. First, unpublished) was mutated at positions 35 and 51 using the oligonucleotide-directed method of Kunkel et al. (1987). Mutants were verified by single-stranded DNA sequencing according to the Sequenase protocol supplied by U.S. Biochemical Corp. The template DNA for verified mutants was transformed into *E. coli* TG2, and mutant protein was expressed and purified as described by E. A. First (unpublished). Pure protein was obtained for the mutant enzymes Cys → Gly35, Cys → Ser35, Thr → Ala51, Thr → Pro51, and Thr → Cys51 in yields of 35–50 mg/L. The enzymes were flash-frozen and stored in liquid nitrogen in standard 144 mM Tris-HCl (pH 7.78) buffer containing 10 mM 2-mercaptoethanol, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), and 10% glycerol.

Preparation of $tRNA^{Tyr}$ Substrate. Unmodified *B. stearothermophilus* $tRNA^{Tyr}$ was obtained by *in vitro* transcription from a linearized double-stranded gene template as described previously (Avis et al., 1993).

Steady-State tRNA Charging Assays. Steady-state kinetic quantities for the charging of tRNA catalyzed by mutant enzymes were obtained as described by Avis et al. (1993).

Stopped-Flow and Quenched-Flow Experiments. The tRNA charging reaction catalyzed by mutants by TyrRS was observed in the pre-steady state by monitoring protein fluorescence in an Applied Photophysics stopped-flow fluorometer (Avis et al., 1993). The absolute rate constants for tyrosine transfer catalyzed by mutant enzymes were also obtained by measuring the rate of formation of [^{14}C]Tyr-tRNA upon mixing tRNA with E·[^{14}C]Tyr-AMP (25 °C, pH 7.78) in a pulsed quenched-flow apparatus as described in the preceding paper (Avis et al., 1993) for wild-type TyrRS. The rate constant for the release of product, k_5, was also estimated for the TyrRS mutant Thr → Pro51 using stopped-flow fluorometry as previously described (Avis et al., 1993).

RESULTS

Steady-State tRNA Charging Kinetics of Position 35 and 51 Mutants. The Pro51 mutant is the only one which has a significantly reduced K_M for tRNA, at about 4 times lower than that of wild type (Table I). This suggests that Pro51 may bind tRNA more tightly than wild-type TyrRS. There is also a 2-fold reduction in k_{cat} compared with wild-type enzyme.

Interaction of $tRNA^{Tyr}$ with Preformed Mutant E·Tyr-AMP Complexes Using Pre-Steady-State Kinetic Techniques. The in vitro-transcribed, unmodified *B. stearothermophilus* $tRNA^{Tyr}$ (Avis et al., 1993) was used as substrate in stopped-flow experiments with mutant E·Tyr-AMP complexes. When $k_{off} \gg k_4$, the observed rate constant (k_{2obs}) for the tyrosine transfer process catalyzed by the TyrRS mutants according to Scheme I, can be related to tRNA concentration by

$$k_{2obs} = \frac{k_4[tRNA]}{K_{tRNA} + [tRNA]} \quad (3)$$

where k_4 is the rate constant for tyrosine transfer and K_{tRNA} is the dissociation constant of tRNA from the E·Tyr-AMP complex. However, for the reaction of wild-type TyrRS, k_{off} is not considerably larger than k_4 and the use of the above equation, although providing an accurate k_4, overestimates K_{tRNA}. Consequently, the data are fitted to eq 4, which more accurately represents the situation (Avis et al., 1993):

$$k_{2obs} = \{k_{on}[tRNA] + k_{off} + k_4 - [(k_{on}[tRNA] + k_{off} + k_4)^2 - 4k_{on}[tRNA]k_4]^{1/2}\}/2 \quad (4)$$

When data are fitted to the above equation, the value of k_4 is fixed at that obtained from fitting data to eq 3 (identical values of k_4 are obtained from eqs 3 and 4 since k_{2obs} tends to k_4 as the tRNA concentration tends to infinity). The values obtained for k_{on} and k_{off} using this procedure were not reliable, having high standard errors, but their ratio gives values similar to those calculated from eq 3 ($k_{off}/k_{on} = K_{tRNA}$). The K_{tRNA} values calculated from this ratio are given in Table II for wild-type TyrRS and each of the mutant enzymes. As the important quantity in this paper is k_4, which is found accurately from either eq 3 or eq 4, and we found previously for the reaction of wild-type enzyme that the simple equation 3 gives values of K_{tRNA} that are within 25–30% of those obtained from eq 4 (Avis et al., 1993), we have not pursued the values of k_{off} and k_{on} further.

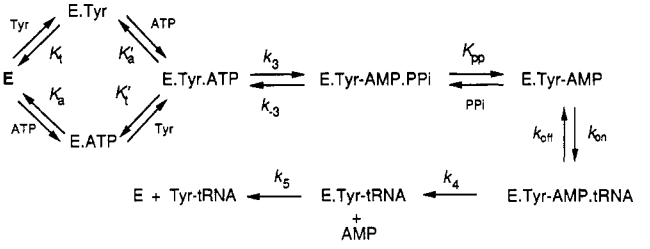
The values of k_4 obtained for wild-type TyrRS and each of the mutant enzymes are given in Table II. Table III shows

Table I: Steady-State tRNA Charging Kinetics of Wild-Type and Position 35 and 51 Mutants of TyrRS[a]

enzyme	k_{cat} (s^{-1})	K_M(tRNA) (μM)	$k_{cat}/K_{M(tRNA)}$ (s^{-1} M^{-1})
WT (+ *E. coli* $tRNA^{Tyr}$)	3.78 ± 0.15	1.20 ± 0.12	3.5×10^6
WT (+ *B. stearothermophilus* $tRNA^{Tyr}$)	3.70 ± 0.10	1.10 ± 0.1	3.36×10^6
WT (+ in vitro $tRNA^{Tyr}$)	4.00 ± 0.16	1.40 ± 0.22	2.90×10^6
Gly35 (+ *E. coli* $tRNA^{Tyr}$)	2.50 ± 0.09	0.50 ± 0.09	5.00×10^6
Ser (+ *E. coli* $tRNA^{Tyr}$)	3.00 ± 0.20	1.00 ± 0.20	3.00×10^6
Ala51 (+ *E. coli* $tRNA^{Tyr}$)	3.47 ± 0.06	1.05 ± 0.06	3.30×10^6
Pro51 (+ *E. coli* $tRNA^{Tyr}$)	1.80 ± 0.07	0.28 ± 0.06	6.43×10^6

[a] 25 °C in standard pH 7.78 buffer (see Materials and Methods) with 10 mM Mg·ATP, 100 μM tyrosine, and 0.5–10 μM $tRNA^{Tyr}$. Rate constants are quoted per mole of dimeric enzyme as measured by active-site titration. Results are means and standard errors from experiments performed in triplicate.

Scheme I[a]



[a] PP_i = inorganic pyrophosphate, K_t is the dissociation constant of Tyr from the E·Tyr complex, K'_t is the dissociation constant of Tyr from the E·Tyr·ATP complex, K_a is the dissociation constant of ATP from the E·ATP complex, K'_a is the dissociation constant of ATP from the E·Tyr·ATP complex, K_{pp} is the dissociation constant of pyrophosphate from E·Tyr-AMP, k_4 is the rate constant for tyrosine transfer, and k_5 is the rate of product (Tyr-tRNA) release from the enzyme, E.

Table II: Pre-Steady-State Kinetic Constants for Reaction of in Vitro-Transcribed $tRNA^{Tyr}$ with Wild-Type and Mutant Tyrosyl Adenylate Complexes[a]

E·Tyr-AMP	k_4 (s^{-1})	K_{tRNA}[b] (μM)
wild type	30.0 ± 0.2	0.43
Cys → Gly35	28.2 ± 0.6	0.59
Cys → Ser35	31.5 ± 1.4	1.35
Thr → Ala51	15.6 ± 0.4	0.27
Thr → Cys51	10.7 ± 0.25	0.12
Thr → Pro51	4.6 ± 0.15	0.089

[a] Stopped-flow experiments performed at 25 °C in a standard 144 mM Tris-HCl buffer (pH 7.78) using 0.25–12 μM $tRNA^{Tyr}$. [E·Tyr-AMP] was 0.5 μM with wild-type and position 35 mutant enzymes and 0.15 μM with position 51 mutants. Where [tRNA] is comparable to [enzyme], the free [tRNA] is adjusted for the proportion of tRNA bound to enzyme. k_4 is the rate constant for tyrosine transfer, and K_{tRNA} is the dissociation constant of tRNA from the E·Tyr-AMP complex. k_4 and K_{tRNA} are calculated using eqs 3 and 4 ($K_{tRNA} = k_{off}/k_{on}$). Results are means and standard errors from experiments performed in triplicate. [b] The standard error on K_{tRNA} obtained using eq 4 is high because of the high error on the k_{off} and k_{on} values (see text). Standard errors calculated on K_{tRNA} using eq 3 are ±10–15%.

Table III: Rate Constants for Tyrosine Transfer Measured by Quenched-Flow[a]

E·Tyr-AMP	k_4 (s^{-1})
wild type[a]	29.8 ± 2.10
Cys → Ser35[a]	28.9 ± 2.10
Thr → Ala51[b]	15.7 ± 1.40
Thr → Pro51[b]	4.86 ± 0.60

[a] Quenched-flow experiments performed at 25 °C in standard pH 7.78 buffer with 0.25 μM E·Tyr-AMP and 12 μM in vitro-transcribed $tRNA^{Tyr}$. Results are means and standard errors from experiments performed in triplicate. [b] As above, with 5 μM $tRNA^{Tyr}$.

the results of pre-steady-state quenched-flow experiments that measure specifically the rate of transfer of ^{14}C-labeled tyrosine from the mutant E·[^{14}C]Tyr-AMP complexes to $tRNA^{Tyr}$ at saturating concentrations of tRNA. There is very good agreement between the values of rate constants obtained using the two methods.

There is very little change in K_{tRNA} or k_4 upon mutation of Cys35 to Gly, suggesting that this residue is not involved in catalysis of step II. Mutation of Thr51 to Ala, Cys, and Pro causes progressively lower values for both K_{tRNA} and k_4. Thr51, therefore, appears to contribute to both the binding and catalytic steps of tRNA charging.

Measurement of the Rate of Product Release. The rate of the fluorescence change observed upon mixing isolated, preformed product, Tyr-tRNA, with free enzyme in a stopped-flow apparatus fit the relationship described by

$$k_{obs} = k_{off} + k_{on}[\text{Tyr-tRNA}] \quad (5)$$

The value of k_{off} (or k_5, referring to Scheme I), the rate constant for the release of product, could therefore be obtained. This value was calculated as 95 ± 10 s^{-1} for wild-type enzyme and 30 ± 11 s^{-1} for the Pro51 mutant.

Calculation of the Energy Levels of Enzyme-Bound Reaction Species Relative to Free Enzyme. The steps of tyrosine activation are defined in Scheme I. The energy levels of the reaction profile may be calculated for wild-type and mutant proteins by substituting pre-steady-state dissociation and rate constants into thermodynamic equations (Wells & Fersht, 1986; Avis et al., 1993). The Gibbs' free energy levels of the enzyme-bound tRNA complex and the subsequent transition state for tyrosine transfer are calculated relative to the energy levels of the respective mutant E·Tyr-AMP complex from eqs 6 and 7. The energy levels relative to free enzyme

Table IV: Gibbs' Free Energies of the Enzyme-Bound Complexes Involved in Step II of the TyrRS Reaction, Calculated Relative to the Free Enzyme ($G_E = 0$)

enzyme	$G_{E\cdot Tyr\text{-}AMP}$ (kcal mol^{-1})	$G_{E\cdot Tyr\text{-}AMP\cdot tRNA}$ (kcal mol^{-1})	$G_{[E\cdot Tyr\text{-}tRNA\cdot AMP]^*}$ (kcal mol^{-1})
wild type	−5.99 ± 0.1[a]	−14.70 ± 0.01	+0.76 ± 0.01
Cys → Gly35	−4.35 ± 0.1[a]	−12.84 ± 0.07	+2.63 ± 0.10
Cys → Ser35	−4.43 ± 0.1[a]	12.43 ± 0.07	+2.97 ± 0.10
Thr → Ala51	−6.95 ± 0.1[b]	−15.90 ± 0.06	−0.10 ± 0.08
Thr → Cys51	−7.43 ± 0.1[b]	−16.86 ± 0.06	−0.83 ± 0.08
Thr → Pro51	−8.29 ± 0.1[b]	−17.90 ± 0.06	−1.36 ± 0.07

[a] Energies of position 35 mutant E·Tyr-AMP complexes from Wells and Fersht (1986). [b] Energies of position 51 mutant E·Tyr-AMP complexes from Ho and Fersht (1986).

Table V: Gibbs' Free Energies of Complexes of Mutant Enzymes Relative to Wild Type

enzyme	$\Delta G_{E\cdot Tyr\text{-}AMP}$ (kcal mol^{-1})	$\Delta G_{E\cdot Tyr\text{-}AMP\cdot tRNA}$ (kcal mol^{-1})	$\Delta G_{[E\cdot Tyr\text{-}tRNA\cdot AMP]^*}$ (kcal mol^{-1})
Cys → Gly35	+1.64	+1.86	+1.87
Cys → Ser35	+1.56	+2.27	+2.21
Thr → Ala51	−0.96	−1.20	−0.86
Thr → Cys51	−1.44	−2.16	−1.59
Thr → Pro51	−2.30	−3.20	−2.12

$$G_{E\cdot T\cdot A\cdot tRNA} = RT \ln K_{tRNA} \quad (6)$$

$$G_{[E\cdot T\cdot tRNA]^*} = RT \ln [k_B T/h] - RT \ln [k_4/K_{tRNA}] \quad (7)$$

($G_E = 0$) are listed in Table IV. The standard state is 1 M for all substrates. The values of the differences in free energy between wild-type- and mutant-bound reaction complexes are shown in Table V. The net contribution of a mutated side chain toward stabilizing each enzyme-bound species on the reaction pathway is visualized in the form of a difference free energy diagram (Figure 1).

DISCUSSION

The most important novel data from this study are the values of k_4, the rate constant for the transfer of Tyr from E·Tyr-AMP to tRNA in the E·Tyr-AMP·tRNA complex, and K_{tRNA}, the dissociation constant of tRNA from the complex (Table II). Mutation of Cys35 has little effect on k_4. Mutation of the evolutionary "hot spot", position 51, causes parallel and significant decreases in the values of both k_4 and K_{tRNA}. Before analysis of these data, it is of interest to analyze the rate constants for the formation of E·Tyr-AMP complexes, compiled from published data from this laboratory on engineered mutants (Wells & Fersht, 1986, 1989; Wells et al., 1986, 1991; Fersht et al., 1987).

Linear Free Energy Relationships for the Formation of E·Tyr-AMP and Tyrosine Transfer to tRNA. It has been found that there are linear free energy relationships (LFERs) between rate constants for the chemical step in activation and binding energy changes when comparing different mutants of the tyrosyl-tRNA synthetase (Fersht et al., 1986, 1987; Wells & Fersht, 1989). These are primarily for mutants in the side chains that are involved in binding the ribose ring of ATP [Cys35, His48, Thr51; see Fersht (1987)]. These side chains were found to contribute essentially no binding energy to ATP in the E·Tyr·ATP complex but significant energy when the transition state was bound for the formation of Tyr-AMP ([Tyr-AMP]*) and even more energy in the E·Tyr-AMP complex (Wells & Fersht, 1986; Ho & Fersht, 1986). The following is an improved analysis that relates changes in rate

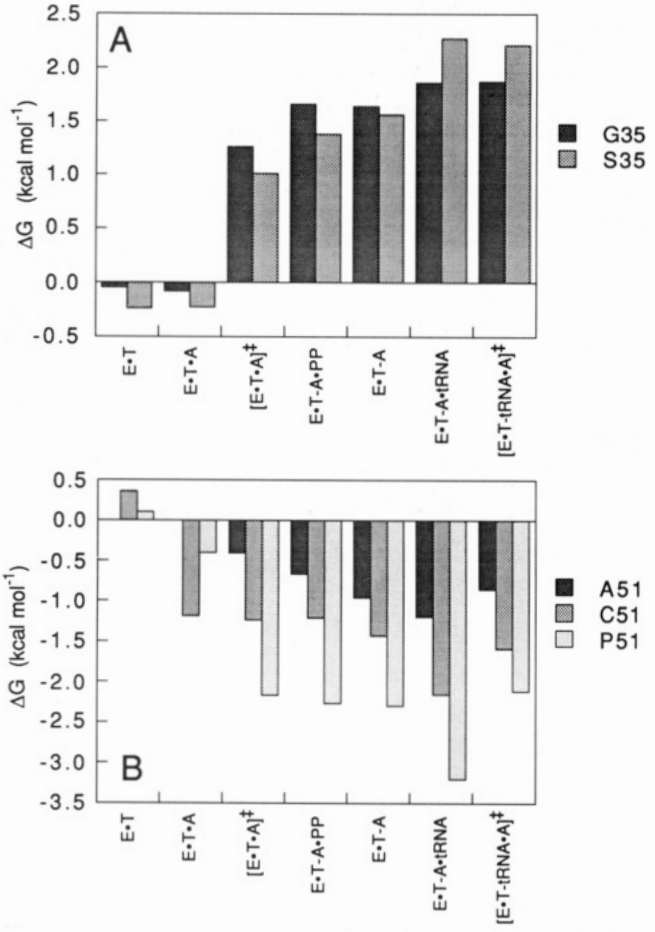


FIGURE 1: Difference free energy diagrams between the Gibbs' free energies of mutant enzyme-bound complexes, as defined in Scheme I, relative to wild type. (A) Catalysis by position 35 mutants; (B) catalysis by position 51 mutants.

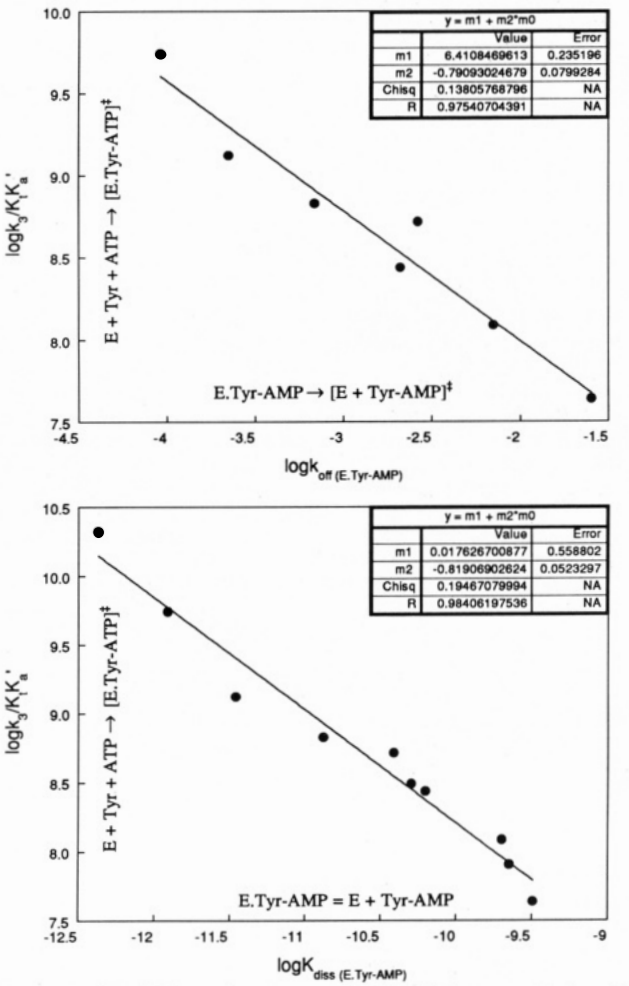


FIGURE 2: (Top) Linear free energy relationship between the log of the complex third-order rate constant $k_3/K_tK'_a$ and that of rate constant $k_{off(E\cdot Tyr\text{-}AMP)}$. The former is the apparent rate constant for the formation of E·Tyr-AMP from free enzyme and Tyr and ATP. According to transition-state theory, the log of the rate constant $k_3/K_tK'_a$ and its free energy of activation are directly proportional to the free energy of binding of the transition state for formation of Tyr-AMP from Tyr and ATP relative to the energies of Tyr and ATP in solution (Fersht et al., 1987). The value of log $k_{off(E\cdot Tyr\text{-}AMP)}$ is similarly proportional to the binding energy of Tyr-AMP in its transition state for dissociation compared with its binding energy to the enzyme in the ground state. The enzymes are wild type, Tyr → Phe34, Cys → Ser35, His → Gly48, Thr → Ala51, Thr → Cys51, and Thr → Gly51. (Bottom) Linear free energy relationship between log $(k_3/K_tK'_a)$ and the log of the dissociation constant for Tyr-AMP from E·Tyr-AMP $[K_{diss(E\cdot Tyr\text{-}AMP)}]$. This compares the binding energy of the transition state for the formation of Tyr-AMP from Tyr and ATP with that of Tyr-AMP. The enzymes are as above plus Cys → Gly35, His → Asn48 and Thr → Pro51.

constants for the chemical steps with changes in the binding energy of Tyr-AMP that have been measured more directly in subsequent experiments (Wells & Fersht, 1989). The bottom plot in Figure 2 shows that there is a linear relationship between the logarithm of the compound rate constant $k_3/K_tK'_a$ and the log of $K_{diss(Tyr\text{-}AMP)}$ (where $K_{diss(Tyr\text{-}AMP)}$ is the dissociation equilibrium constant of the E·Tyr-AMP complex, equal to [E][Tyr-AMP]/[E·Tyr-AMP]). The quantity $k_3/K_tK'_a$ is the third-order rate constant for the reaction E + Tyr + ATP to give E·Tyr-AMP. According to transition-state theory, log $(k_3/K_tK'_a)$ is directly proportional to the free energy of activation of the reaction. This has as a component the binding energy of the transition state [Tyr-ATP]‡ with the enzyme relative to the solvation energies of E, Tyr, and ATP separately in solution (Fersht et al., 1987). That is,

$$RT \ln (k_3/K_tK'_a) = \ln (k_BT/h) - \Delta G^{\ddagger} - \Delta G_S \qquad (8)$$

where k_B is the constant of Boltzmann, h is that of Planck, $\Delta G^{\ddagger}$ is the free energy of activation of the chemical (covalent step), and ΔG_S is the binding energy of the transition state (relative to Tyr and ATP in water; Fersht, 1985). Further,

$$RT \ln [K_{diss(Tyr\text{-}AMP)}] = \Delta G_{(Tyr\text{-}AMP)} \qquad (9)$$

where $\Delta G_{(Tyr\text{-}AMP)}$ is the free energy of binding of Tyr-AMP to the enzyme, relative to Tyr-AMP in solution. The slope of log $k_3/K_tK'_a$ vs log $K_{diss(E\cdot Tyr\text{-}AMP)}$ for the mutants is –0.82 ± 0.085 (standard error). That is, there is a LFER for variation of the groups involved in mutation which shows that they exhibit only 82% of their energy of binding in the transition state relative to that in the E·Tyr-AMP complex [see Fersht et al. (1992), pp 781–782, for a deeper analysis of the interpretation of such slopes]. Previous work (Wells & Fersht, 1989) has shown that all the binding energy of the E·Tyr-AMP complex is lost in the transition state for its dissociation (i.e., the plot of log $k_{off(Tyr\text{-}AMP)}$ vs log $K_{diss(Tyr\text{-}AMP)}$ has a slope of 1.0, where $k_{off(Tyr\text{-}AMP)}$ is the dissociation rate constant). Accordingly (Figure 2, top), there is also an LFER between log $k_3/K_tK'_a$ and log $k_{off(E\cdot Tyr\text{-}AMP)}$ with a very similar slope of –0.79 ± 0.08 (standard error). Although the two plots give identical information, there is an important difference between the two. Some of the values of $K_{diss(Tyr\text{-}AMP)}$ were calculated using $k_3/K_tK'_a$ as a component (Wells & Fersht, 1989) and so are statistically correlated, but the values of $k_3/K_tK'_a$ and $k_{off(E\cdot Tyr\text{-}AMP)}$ were measured entirely independently and so are uncorrelated. However, both give very similar results.

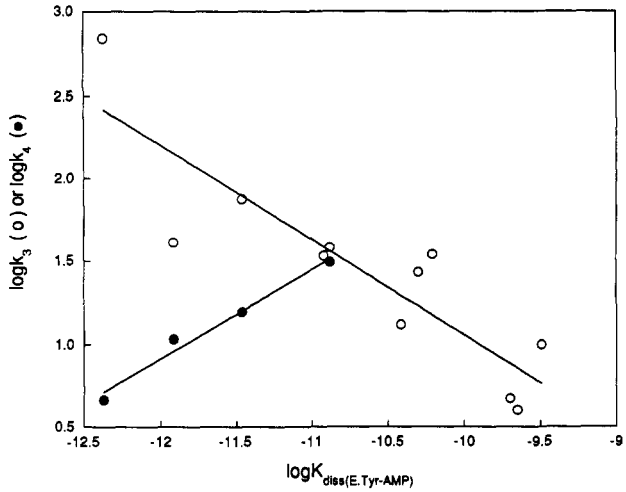


FIGURE 3: Plots of log k_4 (log of the rate constant for the transfer of Tyr from Tyr-AMP to tRNA) and log k_3 (log of the rate constant for the formation of E·Tyr-AMP·PPi from E·Tyr·ATP) against log $K_{diss(Tyr\text{-}AMP)}$.

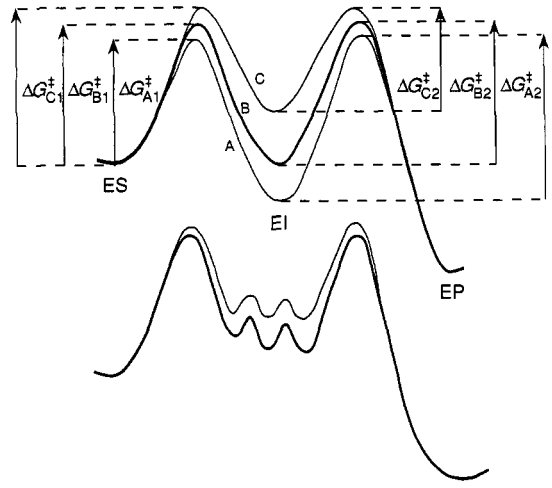


FIGURE 4: (Top) Illustration of the energetic consequences of altering the energy levels of an intermediate, EI, where its energy affects the energy levels of the transition states for its formation from ES and its breakdown to EP. (Bottom) In the reaction of TyrRS, there are several intermediates between ES and EP that have their energy levels raised or lowered by mutations. The first chemical step is the reaction of E·Tyr·ATP to give E·Tyr-AMP·PPi, and the next is E·Tyr-AMP·tRNA to give E·Tyr-tRNA·AMP. In between, there are intermediates that do not involve changes in covalent bonds.

Figure 3 contains a similar LFER between k_3, which is the rate constant for the process of E·Tyr·ATP → E·Tyr-AMP·PPi, and $K_{diss(Tyr\text{-}AMP)}$. The slope is -0.6 ± 0.1. log k_3 is proportional to the difference in binding energy between the transition state [Tyr-ATP]‡ bound to the enzyme minus Tyr and ATP being bound in the E·Tyr·ATP complex. The slope of the LFER indicates that 60% of the energy of binding of Tyr-AMP is utilized to give an increase in the rate constant k_3. There is also a LFER between k_4, the rate constant of E·Tyr-AMP·tRNA → E·Tyr-tRNA·AMP, and $K_{diss(Tyr\text{-}AMP)}$, with slope 0.54 ± 0.06 (Figure 3). The linear correlation does not extend to position 35.

The data for k_3 and k_4 may be explained qualitatively by Figure 4. The top three energy profiles illustrate a reaction ES → EI → EP in which the energy of the intermediate is changed by variation of its structure. The classical LFER behavior is that raising or lowering of G_{EI} is reflected in the raising or lowering of the energies of the flanking transition states. The ratios of changes in activation energies to ground-state energies is the Brønsted β value. The bottom pair of profiles illustrates the case for the TyrRS where the two rate-determining chemical reactions (E·Tyr·ATP = E·Tyr-AMP·PPi and E·Tyr-AMP·tRNA = E·Tyr-tRNA·AMP) are separated by reactions of lower energy barriers involving association and dissociation steps. For the position 51 mutants, there is an apparent Brønsted β value of –0.6 for the first step and +0.5 for the second with decreasing stability of the intermediate.

It must be emphasised that although much of the above is described in terms of LFERs, the existence of *linear* relationships is not essential—it is of importance merely to find that there is a correlation between the stability of the intermediate and the rate constants for the chemical steps that precede and follow it on the reaction pathway.

Relationship between k_{cat} for Aminoacylation of tRNA and the Individual Rate Constants in the Tyrosyl-tRNA Synthetase Reaction. The turnover number, k_{cat}, for the aminoacylation of tRNA in Scheme I at saturating concentrations of substrates and in the absence of pyrophosphate (or the presence of inorganic pyrophosphatase) is given by

$$1/k_{cat} = 1/k_3 + 1/k_4 + 1/k_5 \qquad (10)$$

An alternative way of viewing eq 10 [Fersht (1985), pp 118–119] is that the transit time for the overall reaction, $1/k_{cat}$, is the sum of the residence times for each step ($1/k_3$ is the average time a reagent molecule spends in step 3, etc.). It is seen in Figure 3 that the plots of log k_3 and log k_4 cross at wild-type enzyme so that $k_3 \sim k_4$. This means that the sum of $1/k_3 + 1/k_4$ is at a minimum for wild type; that is, the reciprocal of $1/k_3 + 1/k_4$ is at a maximum. The values of $1/(1/k_3 + 1/k_4)$ are as follows: wild type, 17 s^{-1}; Thr → Ala51, 13 s^{-1}; Thr → Cys51, 8.5 s^{-1}; Thr → Pro51, 4.6 s^{-1}; Cys → Ser35, 4 s^{-1}; and Cys → Gly35, 3.5 s^{-1}. Wild-type enzyme is the most efficient at saturating substrate concentrations in forming E·Tyr-tRNA^{Tyr}. Data on k_5 for the dissociation of Tyr-tRNA^{Tyr} (*E. coli*) from wild-type and Pro51 enzymes give values of 95 ± 10 s^{-1} and 30 ± 11 s^{-1}, respectively. Again, wild type is the more efficient.

The measured k_{cat} appears low when compared to the values of the individual rate constants calculated by pre-steady-state kinetic techniques. The discrepancy, in the case of the wild-type enzyme, is not due to slow product release. The steady-state studies are complicated because there are biphasic kinetics in the steady state with respect to the concentration of tyrosine. At high concentrations of tyrosine, k_{cat} is enhanced as much as 5-fold (Jakes & Fersht, 1975; Wilkinson et al., 1983). The data from the pre-steady-state experiments do not suffer from such complications and correspond more with reality. The rate constant for the release of product by the Pro51 mutant enzyme has been measured (30 ± 11 s^{-1}). Again, for this enzyme, product release is not the rate-determining step (the rate constant for tyrosine transfer by this mutant is 4.6 s^{-1}). However, the rate of product release is slower than for the wild-type enzyme and may partially contribute to a further lowering of the measured k_{cat}.

The residue in position 51 of TyrRS is of particular interest because it is the most variable of the residues that bind Tyr-AMP in the active sites of the enzymes from *B. stearothermophilus*, *B. caldotenax*, and *E. coli*. The latter two enzymes have alanine and proline, respectively, in the equivalent position. The rate constant for transfer of Tyr to tRNA (k_4) for the *E. coli* enzyme is about the same as that for the *B. stearothermophilus* enzyme (Fersht & Jakes, 1975), so that

there must be changes elsewhere in the *E. coli* enzyme to compensate for its having a proline at position 51.

Evolution of the Tyrosyl-tRNA Synthetase To Optimize Overall Rate of Catalysis. Albery and Knowles (1976) proposed that an enzyme evolves toward optimum catalytic efficiency by three types of alteration to the free energy profile of the reaction: "uniform binding", "differential binding", and "catalysis of the elementary steps". An enzyme that evolves toward "catalytic perfection" uses all three methods to minimize the energy barriers between kinetically important intermediates and transition states on the reaction pathway. All three types of binding have been identified as contributing to catalysis of tyrosine activation by tyrosyl-tRNA synthetase (Wells & Fersht, 1986; Ho & Fersht, 1986; Fersht, 1987) by applying site-directed mutagenesis to "revert" the enzyme to a "less-evolved" structure. The major features are that (*i*) the side chains that bind ATP do so weakly in the E·Tyr·ATP complex but more strongly in the transition state and most strongly in the E·Tyr-AMP complex (differential binding), (*ii*) there are residues (e.g., Thr40 and His45) that bind strongly to the pyrophosphate moiety in the transition state for the formation of Tyr-AMP but not at all to ATP or Tyr-AMP (catalysis of the elementary steps), and (*iii*) several side chains bind Tyr equally well throughout the reaction (uniform binding). Catalysis of activation is due entirely to the use of binding energy (Fersht, 1987). Mutagenesis of Thr51 in the *B. stearothermophilus* enzyme and kinetic analysis of further steps of the reaction has revealed that the enzyme has evolved to balance the activation energy barriers of the two chemical steps, and probably that of product dissociation also, and optimize overall catalysis. The reaction of the tyrosyl-tRNA synthetase differs in one important aspect from that of triosephosphate isomerase, which has been extensively studied as a paradigm for evolution of catalysis (Albery & Knowles, 1976; Knowles, 1991). Triosephosphate isomerase catalyzes a reaction that is close to being reversible. The tyrosyl-tRNA synthetase catalyses a reaction that is closer to being irreversible because one of the products, PP_i, is hydrolyzed to pyrophosphate in vivo. The energy levels of intermediates in the reaction of triosephosphate isomerase tend to be at the same level in order that intermediates do not accumulate (Fersht, 1974), whereas those in the reactions of the tyrosyl-tRNA synthetase descend from substrates to products.

Evolutionary pressure, however, need not always maximize the value of k_{cat}. For example, the aminoacyl-tRNA synthetases are under selective pressure to reduce errors of misincorporation to a tolerable level and this may be at the expense of rate. Further, evolutionary pressure depends on the concentrations of substrates in vivo (Fersht, 1974, 1985). The present study emphasizes, however, that the optimization of one step in a multistep reaction can have deleterious effects elsewhere in the pathway.

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