# Kinetic and Thermodynamic Properties of Wild-Type and Engineered Mutants of Tyrosyl-tRNA Synthetase Analyzed by Pyrophosphate-Exchange Kinetics

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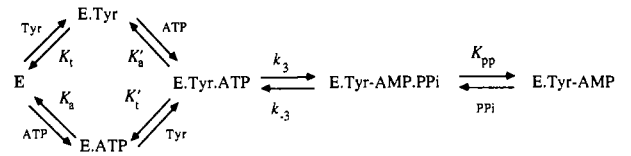
ABSTRACT: The first step of the reaction catalyzed by the aminoacyl-tRNA synthetases is the formation of enzyme-bound aminoacyl adenylate. The steady-state kinetics of this step has conventionally been studied by measuring the rate of isotopic exchange between pyrophosphate and ATP. A simple kinetic analysis of the pyrophosphate-exchange reaction catalyzed by the tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* is given in which all the observed rate and binding constants can be assigned to identifiable physical processes under a variety of limiting conditions. The free energies of binding to the enzyme of tyrosine, ATP, and the transition state for tyrosyl adenylate formation can be measured in relatively straightforward experiments. The excellent agreement between parameters measured in these experiments and those from earlier pre-steady-state kinetics confirms that the intermediates isolated in the presteady state are kinetically competent. The dissociation constant of ATP from the unligated enzyme, a constant that has previously been experimentally inaccessible, has been measured for wild-type and several mutant enzymes. The changes in enthalpy and entropy of activation on mutation have been measured by a rapid procedure for mutants that have altered contacts with tyrosine and ATP. Those mutants that have large changes of enthalpy and entropy of binding are likely to have structural changes and so warrant further examination by protein crystallography.

Dissection of the structure and activity of the tyrosyl-tRNA synthetase has relied heavily on analyzing engineered mutants by stopped-flow kinetics (Wells & Fersht, 1985, 1986; Ho & Fersht, 1986; Fersht, 1987). The first step of the reaction catalyzed by the tyrosyl-tRNA synthetase is activation, the formation of enzyme-bound tyrosyl adenylate (E·Tyr-AMP,[1] Scheme I). The rate constants for the formation of E·Tyr-AMP by the enzyme from *Bacillus stearothermophilus* may be monitored by stopped-flow measurements of the change in the intrinsic tryptophan fluorescence of the enzyme on forming the intermediate (eq 1; Fersht et al., 1975a).

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

An alternative approach is the use of steady-state kinetics. Often, however, this produces values of $k_{cat}$ and $K_m$ that are composed of combinations of several rate constants that must be deconvoluted. The general procedure for assaying activation by steady-state kinetics is the method of pyrophosphate exchange [see for example Duffield and Calvin (1946) and Cole and Schimmel (1970)], which is an example of chemical exchange at equilibrium whereby isotopically labeled pyrophosphate distributes between bulk pyrophosphate in solution and the $\beta,\gamma$ phosphates of the ATP in solution. Cole and Schimmel (1970) investigated a variety of possible mechanisms for the isoleucyl-tRNA synthetase on the basis of steady-state assumptions. Studies carried out monitoring the pre-steady-state kinetics of the reaction (Fersht et al., 1975a; Wells & Fersht, 1986) have shown that a simpler scheme is possible for the tyrosyl-tRNA synthetase from *B. stearothermophilus*. In this case, both the ATP and pyrophosphate dissociate from the relevant enzyme complexes at much faster rates than the chemical reaction on the enzyme. Pyrophosphate-exchange experiments can be designed that measure true dissociation constants and true rate constants, the parameters needed to construct free energy profiles (Fersht, 1985). We first show that the results of simple analysis of the activation reaction of the tyrosyl-tRNA synthetase measured by pyrophosphate exchange are identical with those from pre-steady-state kinetics and then use the scheme to produce novel data of interest.

Scheme I




## Experimental Procedures

### *Materials*

Reagents were obtained from Sigma, and radiochemicals were from Amersham International. Wild-type and mutant tyrosyl-tRNA synthetases (*B. stearothermophilus*) were expressed in *Escherichia coli* TG2 hosts (*recA* form of TG1; Gibson, 1984), with use of M13mp9 templates constructed as described previously (Carter et al., 1984), and purified to electrophoretic homogeneity according to Wells and Fersht (1986). Nitrocellulose discs, 2.5 cm with 0.22-$\mu$m pores, were obtained from Sartorius. The concentration of active enzyme was determined by active-site titration with $^{14}$C-labeled tyr-

[1] Abbreviations: T (Tyr), tyrosine; A (ATP), adenosine 5′-triphosphate; $PP_i$, pyrophosphate; T-A (Tyr-AMP), tyrosyl adenylate; [E·T-A]*, enzyme-bound transition state for the formation of tyrosyl adenylate; E (enzyme), tyrosyl-tRNA synthetase; E·T, enzyme-bound tyrosine etc.; Tris-HCl, tris(hydroxymethyl)aminomethane; BBOT, 2,5-bis(5′-*tert*-butylbenzoxazol-2-yl)thiophene; POPOP/PPO, 1,4-bis(5-phenyloxazolyl)benzene-2,5-diphenyloxazole; $Na_4PP_i$, tetrasodium pyrophosphate. Rate constants are defined according to Scheme I; for example, $K_a = k_a/k_{-a}$ = dissociation constant of ATP from free enzyme etc.

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osine (Fersht et al., 1975b). In all cases, the enzymes were at least 90% active.

*Methods*

*Pyrophosphate Exchange.* The pyrophosphate-exchange reaction was measured in 144 mM Tris-HCl (100 mM Tris-HCl/44 mM Tris, pH 7.78) buffer at 25 °C. Tetrasodium [$^{32}$P]pyrophosphate was obtained as a solid from Amersham International and dissolved in water to give a 100 mM solution. It was diluted with unlabeled tetrasodium pyrophosphate (100 mM, pH 7.8) to produce a final specific activity of 1–4 cpm/pmol. Reactions contained 2 mM pyrophosphate as standard conditions in all experiments except those where pyrophosphate dependence was being studied. Enzyme concentrations were typically 0.2–1.0 $\mu$M and were adjusted to ensure a convenient experimental time range. $MgCl_2$ was added to a concentration 8 mM in excess of the total ATP and pyrophosphate concentrations. Magnesium pyrophosphate is insoluble at higher concentrations and so the addition of substrate to the reaction system was ordered to minimize the possibility of precipitation. In the initial rate studies, at least four 25-$\mu$L samples were withdrawn from the reaction mixture before the reaction was 5% complete. For the complete time course for the approach to equilibrium, the preferred method, nine samples of 25 $\mu$L were removed over three half-lives. Samples were quenched into 400 $\mu$L of 3.5% perchloric acid and 1% activated charcoal, vortexed, and filtered through Whatman GFC filters (2.5-cm diameter). The retained charcoal was washed three times with 4 mL of 10 mM tetrasodium pyrophosphate, pH 2, and then with 2 mL of ethanol. Radioactivity was measured by scintillation counting, with BBOT as a scintillant. BBOT, 9 g, was dissolved in 1.5 L of toluene and 0.5 L of methoxyethanol. The data were fitted to either a first-order exponential (approach to equilibrium) or a linear correlation, with use of a least-squares regression program, ENZFITTER (Elsevier-Biosoft).

*Tyrosine and ATP Dependence.* For each enzyme, the dependence of the reaction rate on both tyrosine and ATP was studied. One substrate was kept constant at a concentration much higher than its respective $K_m$ value. [Tyr] was $\sim 10K_m$ for variation of ATP, and [ATP] was $\sim 2K_m$ for variation of tyrosine. The concentration of the second substrate was varied over the range $K_m/5$ to $5K_m$ (Fersht, 1985).

For studies to determine $k_3/K_tK'_a$ and $K_a$, tyrosine concentrations were less than $K_t$, typically between $K_t/10$ and $K_t/100$. Enzyme was present at 1 $\mu$M final concentration, and MgATP (pH adjusted to 7.8) varied in the range $K_a/5$ to $5K_a$. Enzyme stocks were dialyzed against 144 mM Tris-HCl buffer, pH 7.8, 100 $\mu$M tetrasodium pyrophosphate, 10 mM $MgCl_2$, and 100 $\mu$M phenylmethanesulfonyl fluoride (PMSF) overnight, to remove enzyme-bound tyrosyl adenylate. Further overnight dialysis against 144 mM Tris-HCl buffer, pH 7.8, 10 mM $MgCl_2$, and 100 $\mu$M PMSF was necessary to remove pyrophosphate.

(The derivation of the conventional Michaelis–Menten equations assumes that the concentration of enzyme is much lower than that of the substrate. This is not necessary for the experiments here where [Tyr] and [ATP] are far below the dissociation constants from their complexes. The Michaelis–Menten or steady-state equations break down at high enzyme concentration because the concentration of unbound substrate is depleted by the accumulation of ES complexes. But, when [S] $\ll K_S$, the E·S complex does not appreciably accumulate.)

*Pyrophosphate Dependence.* The enzyme was incubated in the standard buffer at 25 °C, except with 16 mM $MgCl_2$, 6 mM ATP, 200 $\mu$M tyrosine, and 0.1–10 mM tetrasodium [$^{32}$P]pyrophosphate. The reaction was monitored by the appearance of [$^{32}$P]ATP by the charcoal binding assay (see below).

*Exchange at Low [ATP]/[Pyrophosphate].* At concentrations of ATP that are much lower than that of $PP_i$, it is difficult to monitor pyrophosphate exchange from the appearance of radiolabeled ATP. This is because at isotopic equilibrium only a small fraction of total label will be present as ATP, leaving a large background signal. It is easier to monitor the loss of label from [$\gamma$-$^{32}$P]ATP, essentially pyrophosphate exchange in the reverse direction. As the tyrosyl-tRNA synthetase shows a residual ATPase activity, which cannot be removed by ion-exchange chromatography or gel filtration, a low background rate of loss of label due to this hydrolysis was subtracted from the observed rate. Rates were measured in the standard buffer, with final concentrations of 1–2 $\mu$M [$\gamma$-$^{32}$P]ATP (600 Ci/mol), 1–200 $\mu$M tyrosine (200 $\mu$M standard), and 0.1–3.2 mM pyrophosphate (2 mM standard).

*Theory of Kinetic Methods*

The kinetic analysis is simplified as the release of ATP and $PP_i$ is fast enough not to contribute to the rate-determining step. Several lines of evidence support this assumption. Earlier stopped-flow kinetics indicated that the rate constants for the release of ATP and $PP_i$ are much higher than $k_3$ or $k_{-3}$; the relevant rate processes are all single exponentials with no indications of lags or biphasic steps (Fersht et al., 1975a). Further evidence for fast dissociation is that the second-order rate constant for reaction of E·Tyr with ATP to give products is slow at $8 \times 10^3$ s$^{-1}$ M$^{-1}$ as is that for the attack of $PP_i$ on E·Tyr-AMP at $25 \times 10^3$ s$^{-1}$ M$^{-1}$. Generally, slow dissociation of E·S complexes (as is found in Briggs–Haldane kinetics) is manifested in high values of the second-order rate constants ($k_{cat}/K_m$), which tend to the diffusion-controlled limit, as the rate-limiting step in the forward reaction at low concentrations of substrate is association of E and S (e.g., Fersht (1985) pp 101, 152). High dissociation rate constants are also consistent with the high equilibrium dissociation constants of ATP and $PP_i$, which are in the millimolar region. The dissociation rate constant of Tyr from the E·Tyr complex (24 s$^{-1}$; Fersht et al., 1975a) is lower than the rate constant $k_3$ (38 s$^{-1}$). However, the rate constants for the dissociation of tyrosine from E·Tyr·ATP or E·Tyr do not appear in the final rate equations for pyrophosphate exchange even if they are less than $k_3$. This is because the dissociation rate of tyrosine does not affect the pyrophosphate-exchange kinetics as only ATP and pyrophosphate have to dissociate from the enzyme to allow the distribution of radiolabel in the pyrophosphate exchange process. In effect, the upper route in Scheme I is used if the lower route has a slow dissociation step. The same is true for the forward reaction. (It appears, in any case, from examination of the crystal structure of the tyrosyl-tRNA synthetase that the prior binding of ATP blocks the binding of Tyr and so there is an obligatory prior binding of Tyr.)

It is unlikely that nonproductive enzyme–pyrophosphate or enzyme–tyrosine–pyrophosphate complexes are formed in addition to the enzyme-bound complexes in Scheme I as no inhibition was detected at saturating magnesium pyrophosphate (3.2 mM). These complexes may be considered thermodynamically insignificant for the tyrosyl-tRNA synthetase, unlike the case of the isoleucyl-tRNA synthetase from *E. coli* (Cole & Schimmel, 1970).

Applying standard kinetic analysis with the assumption that the release of ATP and $PP_i$ is fast and there are no nonproductive complexes gives eq 2 for the appearance of radiolabeled

ATP from tetrasodium [$^{32}$P]pyrophosphate. $v$ may be mea-

$$v = (k_3[E_0][T][A]/K_tK'_a)/(1 + [A]/K_a + \{[T]/K_t\} \times \{1 + [A]/K'_a + k_3[A]/K'_ak_{-3} + k_3K_{pp}[A]/K'_ak_{-3}[PP_i]\}) \quad (2)$$

sured either from following the complete approach to isotopic equilibrium or by monitoring the initial rate of pyrophosphate exchange. Although, traditionally, the reaction has been followed by monitoring the initial rate of transfer of radiolabel from pyrophosphate to ATP, the analysis is virtually identical for following the loss of $\gamma$ label from ATP. Experimental constraints as to substrate concentrations determine which method is the more suitable. Note that when the reaction velocity is determined by a time course for complete exchange, a first-order reaction is observed that has the rate constant $k_{obs}$ given by

$$k_{obs} = v([ATP] + [PP_i])/([ATP][PP_i]) \quad (3)$$

*Experimental Application of Kinetic Model To Produce True Binding and Rate Constants.* Experiments that are performed in the conventional manner by keeping two of the three substrates at saturating concentrations and varying the third give values of $k_{cat}$ and $K_m$ that are complex. For example, from eq 2

$$k_{cat} = (k_3k_{-3})/(k_3 + k_{-3}) \quad (4)$$

Equation 2 may be simplified, however, by reducing the number of dependent variables by choosing suitable concentrations of reagents. When the concentration of at least one of the three substrates is well below its true binding constant, the relevant term in the denominator of eq 2 will become insignificant and can be ignored. Under conditions where one substrate is saturating, the concentrations of all enzyme-containing species that do not contain that substrate become insignificant and can be ignored.

*Determination of $k_3/K'_a$.* The value of $k_{cat}/K_m(A)$ determined at saturating [Tyr] is $k_3/K'_a$, irrespective of the concentration of $PP_i$. $k_3/K'_a$ is the second-order rate constant for E·Tyr + ATP → E·Tyr-AMP.

*Determination of $k_3/K'_t$.* The value of $k_{cat}/K_m(T)$ determined at saturating [ATP] is $k_3/K'_t$, irrespective of the concentration of $PP_i$. $k_3/K'_t$ is the second-order rate constant for E·ATP + Tyr → E·Tyr-AMP.

*Determination of $k_{-3}/K_{pp}$.* At saturating concentrations of tyrosine, eq 2 may be rearranged to show the dependence of rate upon pyrophosphate concentration.

$$v = ([E_0]k_3k_{-3}[A][PP_i])/((k_3[A] + K'_ak_{-3} + k_{-3}[A]) \times [PP_i] + k_3[A]K_{pp}) \quad (5)$$

This equation predicts the normal Michaelis–Menten hyperbolic kinetics for dependence of rate upon pyrophosphate concentration at constant ATP concentration. This equation can be rearranged into the form $v = k_{cat}[E_0]/(K_m(PP) + [PP_i])$ to give the observed values of $k_{cat}$ and $K_m(PP_i)$. The ratio of eqs 6 and 7 shows that $k_{cat}/K_m(PP_i)$ under the above conditions equals $k_{-3}/K_{pp}$.

$$k_{cat} = (k_3k_{-3})[A]/(k_3[A] + K'_ak_{-3} + k_{-3}[A]) \quad (6)$$

$$K_m(PP_i) = (K_{pp}k_3)[A]/(k_3[A] + K'_ak_{-3} + k_{-3}[A]) \quad (7)$$

*Determination of $k_3/K_tK'_a$ ($=k_3/K'_tK_a$).* It is possible to measure the termolecular rate constant for E + Tyr + ATP → E·Tyr-AMP in a single experiment. Provided that the intrinsic ATPase activity of the enzyme is small in comparison to the observed rate, the reaction rate can be monitored under conditions where the concentrations of tyrosine and ATP are much less than $K_t$ and $K'_a$ but the concentration of $PP_i$ is appreciable. Under these conditions, the enzyme is almost totally unligated and the reaction velocity is given by $v = k_3[E_0][Tyr][ATP]/K_tK'_a$. Since $K_tK'_a = K'_tK_a$, $k_3/K_tK'_a = k_3/K'_tK_a$. $k_3/K_tK'_a$ is obtained by dividing $v$ by $[E_0][Tyr][ATP]$.

The experiments at low concentrations of ATP are performed by following exchange to equilibrium rather than initial rates. Under these conditions (i.e., $[ATP] \ll [PP_i]$), eq 3 reduces to $k_{obs} = v/[ATP]$, that is, $k_{obs} = k_3[E_0][Tyr]/K_tK'_a$. The calculation of $k_3/K_tK'_a$ from $k_{obs}$ is thus independent of [ATP] and so increases the accuracy of the experiment as the concentration of ATP is not always easy to determine accurately because the specific activity is high and radiolysis may be significant.

*Determination of $K_a$.* At concentrations of tyrosine much lower than $K_t$, the enzyme is present mainly as unligated enzyme or, depending on the concentration of ATP, as enzyme–ATP complex. The term $[T]/K_t$ in the denominator of eq 2 becomes insignificant and the equation reduces directly to

$$v = (k_3[E_0][T][A]/K_tK'_a)/(1 + [A]/K_a) \quad (8)$$

At a constant concentration of tyrosine, the variation of observed reaction velocity, $v$, with varying ATP concentrations follows Michaelis–Menten kinetics, with $K_m = K_a$, the dissociation constant of ATP from the free enzyme.

*Determination of $K_t$.* Similarly, at concentrations of ATP much lower than $K_a$, the enzyme is present mainly as unligated enzyme. The term $[A]/K_a$ in the denominator of eq 4 becomes insignificant and the equation reduces directly to

$$v = (k_3[E_0][T][A]/K_tK'_a)/(1 + [T]/K_t) \quad (9)$$

The variation of $v$ with [T] at constant [A] follows a Michaelis–Menten equation with $K_m = K_t$. It is emphasized, as discussed above, that even when the rate constant for the dissociation of Tyr from the E·Tyr·ATP and E·Tyr complexes is lower than $k_3$, the measured value of $K_t$ is that of the true dissociation constant.

*Measurement of Activation Energy Parameters.* The following procedure may be used to compare the activation energy parameters of wild-type and mutant enzymes. It avoids calculating the individual energies of activation and minimizes the problems of curvature in Arrhenius plots that are often found for enzymatic reactions. Suppose the rate constant ($k_{wt}$) of the reaction catalyzed by wild-type enzyme has an energy of activation of $\Delta H_0^*$ and an entropy of activation of $\Delta S_0^*$. Then the equivalent values for a mutant ($k_{mut}$) may be written as $\Delta H_0^* + \Delta\Delta H^*$ and $\Delta S_0^* + \Delta\Delta S^*$, where $\Delta\Delta H^*$ and $\Delta\Delta S^*$ are the changes on mutation. From transition state theory

$$k_{wt} = (k_BT/h)\exp(\Delta S_0^*/R - \Delta H_0^*/RT) \quad (10)$$

$$k_{mut} = (k_BT/h)\exp(\{\Delta S_0^* + \Delta\Delta S^*\}/R - \{\Delta H_0^* + \Delta\Delta H^*\}/RT) \quad (11)$$

and so

$$\ln(k_{mut}/k_{wt}) = \Delta\Delta S^*/R - \Delta\Delta H^*/RT \quad (12)$$

Measurement of the ratio of rate constants as a function of temperature gives directly $\Delta\Delta H^*$ and $\Delta\Delta S^*$. If $\Delta H_0^*$ and $\Delta S_0^*$ change with temperature or there are small changes in pH with changes in temperature, the effects are likely to be the same on wild type and mutant and so cancel out. We have verified that $k_3$, $K'_a$, and $K_t$ are essentially pH-independent around pH 7.8 (pH 6–8.4, unpublished results), and so the small changes

Table I: Comparison of Values of $K_t$ and $k_3/K'_a$ Measured by Pyrophosphate Exchange with Those from Equilibrium Dialysis and Stopped-Flow Fluorescence[a]

| enzyme | $k_3/K'_a$ ($M^{-1}$ $s^{-1}$ × $10^{-3}$) | | $K_t$ (μM) | |
|---|---|---|---|---|
| | $PP_i$ exchange | stopped flow | $PP_i$ exchange | equilibrium dialysis |
| wild type | 8.25 | 8.08 | 11.4 | 11.6 |
| Cys → Gly-35 | 0.92 | 0.88 | 8.0 | 11 |
| His → Gly-48 | 1.02 | 1.00 | 22.6 | 23 |
| His → Asn-48 | 8.21 | 7.10 | 22.0 | nd[b] |
| Thr → Gly-51 | 5.22 | 6.20 | 11.9 | nd |
| Tyr → Phe-169 | 7.64 | 7.61 | 1320 | nd |

[a] Data taken from Wells and Fersht (1986), except for His → Asn-48, which was taken from Fersht et al. (1987). The standard errors of the data are all better than ±5–10%. [b] Not determined.

in pH with change in temperature are unimportant here in any case.

## Results

*Values of $k_{-3}/K_{pp}$.* The Eadie–Hofstee plot for the variation of $v$ versus $v/[PP_i]$ is linear over a wide range of concentrations, indicating that the enzyme–pyrophosphate complex does not form to any detectable level. The measured value 25.2 × $10^3$ $s^{-1}$ $M^{-1}$ for $k_{cat}/K_m$ compares with values of 27.2 × $10^3$ $s^{-1}$ $M^{-1}$ measured by stopped flow by Wells and Fersht (1986). The value of $k_{cat}$ measured at saturating concentrations of ATP and Tyr given by eq 6 is $k_3k_{-3}/(k_3 + k_{-3})$. This is the same value as $k_{cat}$ for the ATP dependence of the "forward" pyrophosphate-exchange process (eq 4). The value of 8.6 $s^{-1}$ for $k_{cat}$ when pyrophosphate is varied at high concentrations of Tyr (200 μM) and ATP (6 mM) compares with the value of 8.4 $s^{-1}$ measured previously for the reaction when ATP is the substrate whose concentration is varied at near-saturating Tyr and $PP_i$ (Wilkinson et al., 1983). The calculated value for $k_{cat}$ at 200 μM Tyr and 6 mM ATP with use of eq 5 and the data from Wells and Fersht (1986) is 9.0 $s^{-1}$.

*Values of $k_3/K'_a$.* Values of $K_3/K'_a$ determined by pyrophosphate-exchange kinetics of wild-type enzyme and mutants (Table I) agree very well with those determined previously from stopped flow.

*Values of $K_t$.* Values of $K_t$ from kinetics for wild-type enzyme and for the mutant His → Gly-48 are in remarkable agreement with those measured by equilibrium dialysis (Table 1), but the value for Cys → Gly-35 is about 30% lower. The values for the other mutants in Table I are new. The value of 1320 μM for Tyr → Phe-169 is too high to have been measured by equilibrium dialysis.

*Values of $K_a$.* Values of $K_a$ have not been determined previously. $K'_a$, the dissociation constant of ATP from the E·Tyr·ATP complex, was always measured in the past (Wells & Fersht, 1986). Values of $K_a$ and $K'_a$ are similar but differ slightly (Table II). The differences between $K_a$ and $K'_a$ result from the stabilization or destabilization of ATP binding caused by the presence of tyrosine, which could result from van der Waals' or electrostatic interactions between the two substrates or induced conformational changes. Structural changes on mutation can leave the interaction unchanged (Thr → Ala-51), increased (His → Gly-48), or decreased (Cys → Gly-35, His → Asn-48, and Thr → Gly-51). Most noticeable is the increase of $K_a$ for the mutant Cys → Gly-35.

*Thermodynamics of Activation of $k_3/K'_aK_t$ for Wild-Type and Mutant Enzymes.* The value of $k_3/K'_aK_t$ can be determined from a single pyrophosphate-exchange experiment. The values of $k_3/K'_aK_t$ measured by this procedure are ~30% higher than those measured by determining $k_3$, $K'_a$, and $K_t$ separately. The simplicity of the direct measurement of $k_3/K'_aK_t$ enables the rapid determination of the enthalpy and entropy of the activation process. In these experiments, the variation of $k_3/K'_aK_t$ with temperature can be measured without the complexities of stopped-flow pre-steady-state kinetics and with precise temperature control. There is up to a 10-fold variation of $k_3/K'_aK_t$ over the 20–30 °C temperature range. The Arrhenius plot log $(k_3/K'_aK_t)_{wt}$ versus $1/T$ (Figure 1, top panel) is very curved, as frequently seen in Arrhenius plots with enzymes. However, as described in the Theory of

Table II: Comparison of Dissociation Constants of ATP from the E·ATP and E·Tyr·ATP Complexes[a]

| enzyme | $K_a$ (mM) | $K'_a$ (mM) | $K_a/K'_a$ |
|---|---|---|---|
| wild type | 3.5 | 4.7 | 0.74 |
| wild type[b] | 3.5 | 4.7 | 0.74 |
| Cys → Gly-35 | 9.5 | 4.0 | 2.38 |
| His → Gly-48 | 4.5 | 9.9 | 0.45 |
| His → Asn-48 | 6.4 | 3.8 | 1.69 |
| Thr → Ala-51 | 3.6 | 4.7 | 0.77 |
| Thr → Gly-51[c] | 3.0 | 2.1 | 1.43 |

[a] All experiments were carried out with use of 2 mM $Na_4PP_i$, 1.0 μM tyrosine, and 10 μM enzyme. [b] In this experiment, 0.1 μM tyrosine was used instead of 1.0 μM tyrosine. Standard errors are better than ±10%. [c] Significant inhibition was seen with concentrations greater than 12 mM MgATP. This may be caused by competitive inhibition by ATP binding in the tyrosine pocket that has been observed crystallographically (Monteihet & Blow, 1978) in the wild-type enzyme.

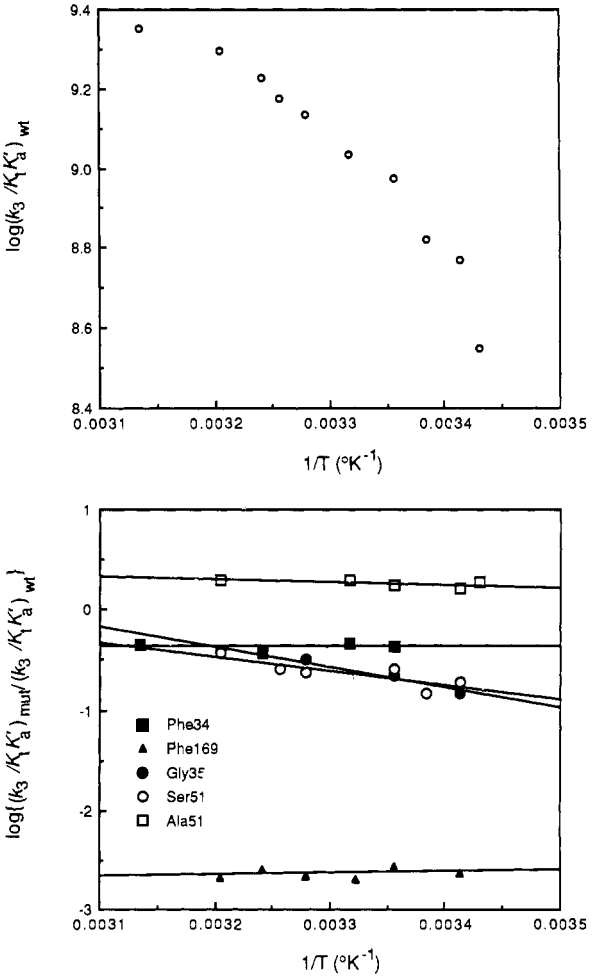


FIGURE 1: (Top) Arrhenius plot for the variation of $k_3/K_tK'_a$ for wild-type enzyme with temperature. (Bottom) Modified Arrhenius plot of log $[(k_3/K_tK'_a)_{mut}/(k_3/K_tK'_a)_{wt}]$ vs $1/T$ (eq 17) whereby $\Delta\Delta H^*$ and $\Delta\Delta S^*$ may be determined without their being obscured by the curvature in the normal plots.

Table III: Thermodynamic Changes in Binding Energy of Tyrosine and ATP on Mutation[a]

| mutation | $\Delta\Delta G^{\ddagger}$[b] (kcal/mol) | $\Delta H^{\ddagger}$ (kcal/mol) | $\Delta\Delta S^{\ddagger}$ (cal/K) |
|---|---|---|---|
| Tyr → Phe-34 | 0.50 | −0.11 ± 0.15 | −2.04 ● 4.9 |
| Tyr → Phe-169[c] | 3.59 | −0.70 ± 1.56 | −14.4 ± 4.8 |
| Cys → Gly-35 | 0.90 | 8.8 ± 0.9 | 26.4 ± 3 |
| Thr → Ser-51 | 0.91 | 6.3 ± 2.1 | 18 ± 7 |
| Thr → Ala-51 | −0.35 | 1.2 ± 0.8 | 5.3 ± 2.6 |
| Ala → Ser-51 | 1.31 | 5.1 ± 2.2 | 12.7 ± 7.5 |

[a] Calculated from the ratios of $k_3/K_tK_a$ for activation of tyrosine. [b] The values of $\Delta\Delta G^{\ddagger}$ have standard errors of less than ±0.08 kcal/mol. [c] In truncated enzyme (Δwt).

Kinetic Methods, use of eq 12, which measures the ratio of rate constants for the reaction of wild-type and mutant enzymes, appears to minimize the factors causing curvature. The plots of log $\{(k_3/K'_aK_t)_{mut}/(k_3/K'_aK_t)_{wt}\}$ versus $1/T$ (eq 12) are indeed satisfactory (Figure 1, bottom panel) and so give measurable values of $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$ (Table III). The mutant Tyr → Phe-34 has a minimal change in enthalpy and a very small change in entropy. Noticeable again are the high values for Cys → Gly-35.

## Discussion

Pre-steady-state kinetics has provided a powerful way of determining some of these rate and binding parameters for the tyrosyl-tRNA synthetase and has allowed the reaction pathway to be dissected by direct measurement of the formation of the enzyme-bound tyrosyl adenylate (Fersht et al., 1975b; Wells & Fersht, 1986). Steady-state kinetics is, in general, not as useful in elucidating enzyme mechanisms since it does not give direct information about intermediates. However, when an intermediate has been shown by pre-steady-state kinetics to be on a reaction pathway, steady-state kinetics combined with active-site titration can measure true rate and binding constants [e.g., chymotrypsin, reviewed by Fersht (1985)]. This is possible here because of the formation of a stable enzyme-bound tyrosyl adenylate.

A simple analysis of pyrophosphate-exchange kinetics gives values for the dissociation constants of the complexes in Scheme I. The values of $K_t$, the dissociation constant of E·Tyr, and $K'_a$, the dissociation constant of ATP from E·Tyr·ATP are in excellent agreement with those determined previously by equilibrium dialysis and stopped-flow kinetics. Values of the second-order rate constants, $k_3/K'_a$ and $k_{-3}/K_{pp}$, from pyrophosphate-exchange and stopped-flow kinetics are also in excellent agreement. The steady-state kinetic analysis is reliable and so can be used to investigate novel phenomena.

The dissociation constants of ATP from the E·ATP complexes, $K_a$, were previously unknown: values of $K_a$ are too high to be measured by equilibrium dialysis, and there is no change in the spectral properties of the enzyme for monitoring nucleotide binding. By comparing this value with that for $K'_a$ measured from pre-steady-state experiments, it has been possible to show that there is some synergism in the binding of the substrates of tyrosyl-tRNA synthetase. The occurrence of a thermodynamically significant E·ATP complex and measurement of its dissociation constant do not necessarily imply that the complex is on the reaction pathway. It is, in fact, likely that the E·ATP complex is nonproductive. Examination of the crystal structure suggests that the binding of ATP blocks the access of tyrosine to its binding pocket and so tyrosine must bind first in the forward direction and dissociate last in the reverse. The equilibrium between E·ATP and E·Tyr·ATP is a virtual equilibrium, the dissociation constant of Tyr from the E·Tyr·ATP is defined by the equilibrium thermodynamics of the system, but the kinetic pathway involves the prior dissociation of ATP followed by reassociation. This would cause inhibition at very high concentrations of ATP, and this has been observed for some mutants.

*Changes in Thermodynamics of Substrate Binding on Mutation.* According to transition-state theory, the third-order rate constant $k_3/K'_aK_t$ represents the process of free ATP and Tyr binding to E to give the transition state for the reaction $k_3$ (eq 13). Changes in the values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$($\Delta\Delta H^{\ddagger}$

$$E + Tyr + ATP \xrightarrow{k_3/K'_aK_t} [E\cdot Tyr\text{-}ATP]^{\ddagger} \qquad (13)$$

and $\Delta\Delta S^{\ddagger}$) between wild-type and mutant enzymes are related to the changes in apparent binding energy of the substrates in the transition state (Fersht et al., 1987). Study of the rate constant $k_3/K'_aK_t$ for wild-type and mutants in the Tyr or ATP binding sites provides a convenient measure of the effects of mutation on the combined binding of both ATP and Tyr.

There is curvature in the Arrhenius plot for the reaction of wild-type enzyme (Figure 1, top panel). Curvature is frequently found in Arrhenius plots for enzymatic reactions and can arise from changes in the rate-determining step with temperature or changes in the gross properties of the enzyme and its interaction with solvent. We have tried the following approach to determine changes in thermodynamic properties of substrate binding on mutation. If the thermodynamics of binding of one particular element of structure can be separated from bulk changes, then, as discussed in Theory of Kinetic Methods, the binding enthalpy of a mutant enzyme and substrate can be written as $\Delta H_0^{\ddagger} + \Delta\Delta H^{\ddagger}$ and the entropy as $\Delta S_0^{\ddagger} + \Delta\Delta S^{\ddagger}$, where $\Delta H_0^{\ddagger}$ and $\Delta S_0^{\ddagger}$ are the values for the wild-type enzyme and $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$ are the changes on mutation. Comparing wild type and mutant by eq 11 causes $\Delta H_0^{\ddagger}$ and $\Delta S_0^{\ddagger}$ to cancel out along with the factors causing extreme curvature. Reasonable plots to give $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$ are obtained by eq 12 (Figure 1, bottom panel).

Table III lists values of $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$ for deletion of groups on the enzyme, which, in the main, make hydrogen bonds with groups on Tyr or ATP. These are not the values of $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$ for hydrogen-bond formation. Changes in binding energy that are measured on mutation are not true binding energies, $\Delta G_{bind}$, but are apparent binding energies, $\Delta G_{app}$ (Wells & Fersht, 1986). Suppose a hydrogen-bond donor −XH is deleted by mutation from a wild-type enzyme E-XH that forms a hydrogen bond with an acceptor B on a substrate S-B. Then $\Delta G_{app}$, the measured difference in binding energies of S-B to E-XH and mutant E′, is related to $\Delta G_{bind}$, the true binding energy of the hydrogen bond between B and XH by

$$\Delta G_{app} = \Delta G_{bind} - G_{WW} + G_{SB\cdot W} - G_{E'/BS} + G_{E'/W} + \Delta G_{reorg} - \Delta G_R \qquad (14)$$

where $G_{WW}$ is the hydrogen-bond dissociation energy of $H_2O\cdots HOH$, $G_{SB\cdot W}$ that of $SB\cdots OH_2$, $G_{E'/BS}$ is the dissociation energy of the new interaction in the mutant enzyme–substrate complex, $G_{E'/W}$ is the new bond energy between E′ and water, and $\Delta G_R$ is an energy term that contains any entropic or other energetic changes that accompany the formation of the hydrogen bond in wild-type enzyme [for example, the favorable entropy change accompanying the release of bound water (Fersht, 1988)]. To allow for the possibility of the mutation causing energetic changes because of reorganization of the solvent shell of the enzyme or local structure in the enzyme, there is an additional term, $\Delta G_{reorg}$, the "reorganization energy". This contains all the spurious factors arising from

the rearrangement of the enzyme and solvent, including any perturbations of the binding of the rest of S-B to enzyme. Also

$$\Delta G_{app} = (G_{E'/W} - G_{EXH\cdot W}) - (G_{E'/BS} - G_{EXH\cdot BS}) + \Delta G_{reorg} \quad (15)$$

where $G_{EXH\cdot BS}$ is the hydrogen-bond dissociation energy of E-XH····B-S, and where $G_{EXH\cdot W}$ is the hydrogen-bond dissociation energy of E-XH····$OH_2$ (Fersht, 1988).

It is difficult, therefore, to interpret $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$ because they relate to the temperature dependence of $\Delta G_{app}$, which has contributions from a complexity of factors, including $\Delta G_{reorg}$. There do appear to be, however, some patterns that offer clues. The –OH of Tyr-34 makes a hydrogen bond with the –OH of the substrate tyrosine. Mutation of Tyr → Phe-34 weakens the binding of Tyr and of the transition state by only 0.5 kcal/mol (Table III; Wells & Fersht, 1986), and there are only very small values of $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$ (Table III). It is seen in the following paper that the mutation appears to have no structural changes associated with it (Fothergill & Fersht, 1991). It is expected in this case from eq 20 that there will be low values of $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$ for the following reasons. The reorganization energy terms are negligible. The $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ components of $(G_{E'/W} - G_{EXH\cdot W}) - (G_{E'/BS} - G_{EXH\cdot BS})$ will tend to cancel since EXH·W and EXH·B are both –OH····O– hydrogen bonds and the dispersion energies that give rise to $G_{E'/W}$ and $G_{E'/BS}$ are expected to be relatively low because of the small areas of contact.

The –SH of Cys-35 is in the ATP binding site, and mutation of Cys → Gly-35 weakens the combined binding of ATP and Tyr in the transition state by 0.90 kcal/mol. This small value hides a relatively large enthalpy change of 8.8 kcal/mol and an entropy change of 26.4 cal per K per mol. This is, perhaps, an indication that there is a reorganization energy term that is itself small but hides larger compensating changes in $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$. Structural work on this mutant shows that there are changes in structure on mutation (Fothergill & Fersht, 1991). The mutant Cys → Gly-35 also has an anomolously high ratio of $K_a/K'_a$. The mutant Thr → Ser-51 appears to warrant further examination as although the loss of the methyl group loses about the expected amount of free energy, there are larger changes in enthalpy and entropy.

The –OH of Tyr-169 makes a hydrogen bond with the $\alpha$-$NH_3^+$ of the substrate tyrosine. Loss of this bond in the complex of Tyr with the mutant Tyr → Phe-169 should lose a large enthalpic contribution. Yet, the observed change in apparent binding energy is 3.59 kcal/mol, which is composed of a very small enthalpy change of $-0.70 \pm 1.56$ kcal/mol but a larger entropy term of −14.4 kcal mol. This is consistent with the $\alpha$-$NH_3^+$ of the substrate retaining a bound water molecule on binding to the mutant to replace the mutated –OH group. There is an unfavorable entropy term because of the loss of translational and overall rotational entropy of the water molecule that is bound plus compensating changes in enthalpy.

Measurements of enthalpy and entropy changes on mutation are thus useful in identifying mutants that could have undergone structural changes and warrant further detailed structural investigation.

**Registry No.** ATP, 56-65-5; Tyr, 60-18-4; Cys, 52-90-4; His, 71-00-1; Thr, 72-19-5; Gly, 56-40-6; Asn, 70-47-3; Phe, 63-91-2; Ser, 56-45-1; Ala, 56-41-7; tyrosyl-tRNA synthetase, 9023-45-4.

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