Mutation of Lysine 233 to Alanine Introduces Positive Cooperativity into Tyrosyl-tRNA Synthetase†

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ABSTRACT: Tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* is a dimeric enzyme which displays half-of-sites reactivity with respect to the binding of both tyrosine and ATP. The binding of both substrates follows Michaelis–Menten kinetics. Mutation of lysine 233 to alanine (K233A) decreases the affinity of the active subunit for ATP at both saturating and subsaturating tyrosine concentrations (from the Hill plot, k_{cat} = 0.56 s^{-1}, n_H = 1.54, K_d = 372 mM at 50 μM tyrosine). In addition, this mutant displays sigmoidal kinetics (characteristic of positive cooperativity) with respect to the binding of ATP. These two effects can be reversed by the addition of NaCl (0.5 M final concentration) or by a second alanine mutation at either position K230 or T234. The effect of either NaCl or second site mutation is to increase the binding affinity of the K233A mutant for ATP (K_{ATP} values are 22 mM for the K233A mutant in the presence of 0.5 M NaCl, 0.16 mM for the K230A/K233A mutant, and 0.14 mM for the K233A/T234A mutant). With the restoration of the tight binding of ATP, Michaelis–Menten kinetics are restored since the kinetic analysis of tyrosyl adenylate formation involves only binding of ATP to the active subunit. It is likely that the physical mechanism for the positive cooperativity present in the K233A mutant actually exists in the wild-type enzyme but is not observed kinetically due to the initial binding of ATP to the active subunit. These results indicate that, in some cases, a decrease in substrate affinity is sufficient to introduce cooperativity into a noncooperative enzyme.

The tight regulation of substrate binding is achieved in multimeric proteins through cooperative interactions between the subunits. Recently, it has been found that mutation of a single residue is sufficient to introduce cooperativity into previously noncooperative oligomeric enzymes (Kuo et al., 1989; Stebbins & Kantrowitz, 1992; Scrutton et al., 1992). A caveat to two of these studies is that the enzymes investigated, ornithine transcarbamoylase from *Escherichia coli* (*E. coli;* Kuo et al., 1989) and aspartate transcarbamoylase from *Bacillus subtillus* (Stebbins & Kantrowitz, 1992) are evolutionarily related to aspartate transcarbamoylase from *E. coli* which, in its holoenzyme form, displays cooperativity in binding ligands. Consequently, cooperativity may have been present in the ancestors of ornithine transcarbamoylase from *E. coli* and aspartate transcarbamoylase from *B. subtillus*. In contrast, in glutathione reductase positive cooperativity has been introduced into an enzyme that is not known to be closely related to any enzyme displaying positive cooperativity (Scrutton et al., 1992). In the present study, tyrosyl-tRNA synthetase is shown to exhibit positive cooperativity on mutation of a single amino acid residue. As with glutathione reductase, positive cooperativity has not been previously observed in either this, or related, enzymes.

Tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* is a dimeric enzyme which is responsible for the charging of $tRNA^{tyr}$ by tyrosine. The process can be separated into two distinct reactions in vitro. The first reaction involves the binding of one molecule of tyrosine to the free enzyme followed by activation of this bound tyrosine by ATP to form an enzyme-bound tyrosyl adenylate intermediate. In the second reaction $tRNA^{tyr}$ binds to this enzyme·tyrosyl adenylate intermediate and is charged with tyrosine, and the $tRNA^{tyr}$–tyrosine complex is subsequently released. The formation of the product from the first reaction, the enzyme-bound tyrosyl adenylate intermediate, has been the subject of thorough investigation [for review, see Fersht (1987)]. One component of catalysis that has been found to be crucial for the normal function of the enzyme is the involvement of a mobile loop in the binding of the pyrophosphate moiety during the formation of the tyrosyl adenylate transition state. The decreased rate of catalysis on mutation of two of the residues in this loop to alanines, K230[1] and K233, implicates these residues in the binding of the pyrophosphate moiety of ATP (Fersht et al., 1988). In the process of investigating the role of K233 in catalysis, it was found that, in addition to decreasing the rate of catalysis, the K233A mutant also displays positive cooperativity with respect to the binding of ATP. This cooperativity is unique for several reasons. First, although negative cooperativity (with respect to the binding of tyrosine) has been observed in tyrosyl-tRNA synthetase, positive cooperativity has not been found in either the wild-type or other mutant forms of the enzyme. Second, the mutated residue, K233A, is located more than 30 Å away from the dimer

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[1] Abbreviations: YTS, tyrosyl-tRNA synthetase; PP_i, inorganic pyrophosphate; K_{Tyr}, dissociation constant for tyrosine; K_{ATP}, dissociation constant for ATP; K'_{ATP}, dissociation constant for ATP at saturating tyrosine concentrations; k_3, forward rate constant for the formation of tyrosyl adenylate. Wild-type amino acids are designated by the standard one-letter code, followed by their position in the peptide chain (e.g., K233). Mutations are designated by the wild-type amino acid, followed by the residue position, which is followed by the mutant amino acid (e.g., K233A refers to alanine replacing lysine at position 233). In complexes such as E·Tyr-AMP (where E refers to enzyme), the "·" indicates a noncovalent bond while the "-" indicates a covalent bond.

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interface (in contrast to glutathione reductase, where the mutated residue is located at the dimer interface). Finally, this positive cooperativity can be reversed either by increasing the ionic strength of the reaction or by mutating a second residue in the active site of the enzyme. The discovery of a tyrosyl-tRNA synthetase mutant that binds ATP in a cooperative manner is a consequence of our investigation into the role a mobile loop plays in catalyzing the formation of tyrosyl adenylate. Investigation of the mechanism of this cooperativity is therefore necessary to understanding the role of this mobile loop in catalysis. As the cooperative binding of ATP in this mutant could not have been predicted either from the X-ray crystal structure or from our knowledge of other aminoacyl-tRNA synthetases, site-directed mutagenesis has been used to probe the mechanistic basis of this cooperativity.

EXPERIMENTAL PROCEDURES

Materials. All enzymes were obtained from United States Biochemicals, chemicals from Sigma Chemicals (London), nitrocellulose filters from Sartorius, and radiochemicals from Amersham International.

Production of Mutants. All mutants were constructed by the method of Kunkel (1985) from a pTZ18u phagemid which contains the wild-type tyrosyl-tRNA synthetase gene from *B. stearothermophilus* preceded by a Trp-Lac promoter (pYTS5). Construction and *in vitro* mutagenesis of this phagemid vector is described in the preceding paper (First & Fersht, 1993). The following oligonucleotides were used to create the desired mutants (mismatches are shown in italics): 5′CT TTC CGT T*GC* CCC GAA TTT 3′(K233A) 5′CT TTC CGT T*GC* CCC GAA T*GC* CGT GCC GTC 3′(K230A/K233A) 5′CC GCT TTC CG*C* T*GC* CCC GAA TTT 3′(K233A/T234A).

Sequence Analysis. The mutant DNA templates were sequenced by the dideoxy method of Sanger et al. (1977) using Sequenase as the DNA polymerase (Sambrook et al., 1989).

Purification of Enzymes. Mutant enzymes were expressed in *E. coli* TG2 hosts and purified to electrophoretic homogeneity by a modification of the procedure described by Fersht et al. (1988). An overnight culture of *E. coli* TG2 cells containing the mutant plasmid was grown in 2×TY medium (16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl per liter) containing 50 μg/mL ampicillin. This overnight culture was diluted 100-fold into 500 mL of fresh 2×TY medium containing 50 μg/mL ampicillin and shaken at 37 °C until the A_{595} was approximately 1.0 (2–3 h), at which time 1 mL of 10 mM isopropylthio-β-D-galactopyranoside was added and the culture growth was continued at 37 °C overnight. The cells were harvested by centrifugation at 7000 rpm for 15 min, resuspended in a buffer containing 50 mM tris-(hydroxymethyl)aminomethane hydrochloride (TrisHCl, pH 7.8), 1 mM ethylenediaminetetraacetic acid (EDTA), and lysed immediately by sonication. The lysate was heated at 56 °C for 40 min to precipitate endogenous *E. coli* enzymes and clarified by centrifugation at 15 000 rpm for 30 min. The supernatant was then mixed with 5 mL of Whatman DE-52 cellulose anion exchange resin to bind the protein. This resin was washed once with 20 mL of TE8 buffer (10 mM Tris, pH 8, 1 mM EDTA), followed by removal of the protein from the resin by three 8-mL elutions with TE8 containing 1 M NaCl. The eluates were pooled and dialyzed over a period of 48 h at 4 °C against four changes of 2 L of 50 mM potassium phosphate (pH 6.5), 1 mM EDTA, 5 mM β-mercaptoethanol, 0.1 mM phenylmethanesulfonyl fluoride, and 0.1 mM tetrasodium pyrophosphate (to remove any enzyme-bound tyrosyl adenylate). The protein was then purified on a Pharmacia FPLC Mono Q column using a gradient from 20 mM 2-bis-(2-hydroxyethyl)-amino-2-(hydroxymethyl)-1,3-propanediol (Bis-Tris), pH 6.0, to 20 mM Bis-Tris, pH 6.0, and 1 M NaCl. After the eluted fractions for tyrosyl adenylate formation were assayed (Wilkinson et al., 1983), a peak eluting at approximately 200 mM NaCl was collected and dialyzed overnight against 20 mM Tris, pH 7.78, and 5 mM β-mercaptoethanol. This protein was then repurified on a Pharmacia FPLC Mono Q column using a gradient from 20 mM Tris, pH 7.78, to 20 mM Tris and 1 M NaCl, pH 7.78. After the eluted fractions for tyrosyl adenylate formation were assayed, a peak eluting at approximately 240 mM NaCl was collected and dialyzed overnight against 20 mM Tris, pH 7.78, 5 mM β-mercaptoethanol, and 10% glycerol (v/v). Purified proteins were stored frozen at −70 °C.

Purification of the K233A mutant enzyme from M13mp93 has been previously described (Fersht et al., 1988).

Kinetic Procedures. All experiments were performed at 25 °C in a standard buffer containing 144 mM Tris-HCl (pH 7.78) and 10 mM $MgCl_2$. ATP was added as the magnesium salt to maintain the free Mg^{2+} at 10 mM. The final pH values of the reaction solutions were adjusted to pH 7.78 with NaOH.

Activation. For measurements where tyrosine was present at saturating concentrations (200 μM), the rates for the formation of enzyme-bound tyrosyl adenylate by the K233A mutant were followed by stopped-flow fluorescence as described in the previous paper.

The rate of formation of enzyme-bound tyrosyl adenylate by the K233A mutant at subsaturating concentrations of tyrosine (1 μM tyrosine) was monitored by filtration through nitrocellulose filters using a modification of the procedure described in the preceding paper (First & Fersht, 1993). Assay solutions (260 μL) were made up such that, after addition of enzyme (40 μL, 4 mM), the assay solutions contained 144 mM Tris-HCl, pH 7.78, 10 mM $MgCl_2$, 0.001 units/mL inorganic pyrophosphatase, 50 mM [^{14}C]tyrosine (5–15 Bq/mmol), and various concentrations of MgATP (from 0.02 to 75 mM). When NaCl was present, the assay solution was made up such that, after addition of enzyme, the assay solution contained 0.5 M NaCl. Rate constants were calculated by nonlinear regression to a first-order theoretical curve using Kaleidagraph 2.1.1 (Abelbeck Software). The observed rate constant for formation of E·Tyr-AMP (k_{obs}) was corrected where necessary for the loss of Tyr-AMP by dissociation or hydrolysis (Leatherbarrow & Fersht, 1987). The kinetic constant K_{ATP} was calculated from the variation of the initial rate for the formation of E·Tyr-AMP with ATP concentration, at subsaturating concentrations of tyrosine. The kinetic constants K'_{ATP} and k_3 were calculated from the variation of the initial rate for the formation of E·Tyr-AMP with ATP concentration, at saturating concentrations of tyrosine.

For the K230A/K233A and K233A/T234A double mutants, the formation of enzyme-bound tyrosyl adenylate from [^{14}C]tyrosine and ATP was monitored by filtration through nitrocellulose filters as described in the preceding paper (First & Fersht, 1993). K_{ATP} for wild-type enzyme in the presence of 0.5 M NaCl was determined from steady-state pyrophosphate exchange kinetics (Calender & Berg, 1966). K'_{ATP} for the wild-type enzyme in the presence of 0.5 M NaCl was determined from pre-steady-state kinetics for the formation of the enzyme-bound tyrosyl adenylate species as previously described (Fersht et al., 1988).

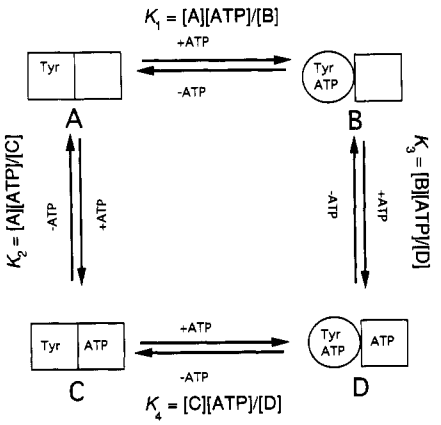

FIGURE 1: Thermodynamic cycle for the binding of ATP to tyrosyl-tRNA synthetase in the presence of saturating amounts of tyrosine. A model for the binding of two molecules of ATP to the E·Tyr complex is shown. Monomers of each subunit in tyrosyl-tRNA synthetase are represented by either a square (if the subunit is inactive) or a circle (if the subunit is active). The binding of tyrosine to a subunit is denoted by Tyr, and the binding of ATP to a subunit is denoted by ATP. K_1, K_2, K_3, and K_4 correspond to the dissociation constants for each step in the thermodynamic cycle. A, B, C, and D denote each of the four possible E·Tyr species in the cycle.

Analysis of Kinetics. The analysis of the Michaelis–Menten kinetics normally observed for both wild-type and mutant tyrosyl-tRNA synthetases have been described previously (Fersht et al., 1988). For analysis of the sigmoidal kinetics at saturating tyrosine concentrations, the binding of tyrosine and ATP is assumed to be ordered, with tyrosine binding before ATP. In this model, tyrosine is allowed to bind to only one subunit (designated the "active subunit"). ATP is allowed to bind to either subunit of tyrosyl-tRNA synthetase. As indicated in Figure 1, the addition of ATP to tyrosyl-tRNA synthetase can be considered as a thermodynamic cycle. A consequence of this is that only three of the four dissociation constants are independent (i.e. $K_1K_3 = K_2K_4$).

In the model proposed above, the relative k_{cat} values for the monoligated and biligated species (B and D in Figure 1) are not known. Consequently, it is not possible to derive an Adair equation which will give meaningful estimates for the four dissociation constants. In order to emphasize the cooperative nature of the K233A mutant, rather than to obtain precise values of each dissociation constant, we have chosen to fit the data to the following Adair equation:

$$k_{obs} = k_{cat}(\alpha[ATP] + \beta[ATP]^2)/(1 + (\alpha + \gamma)[ATP] + \beta[ATP]^2) \quad (1)$$

where k_{obs} is the observed rate constant for the formation of tyrosyl adenylate, k_{cat} is the maximum rate constant for tyrosyl adenylate formation, and α, β, and γ are fitting constants. If the k_{cat} values for the monoligated and biligated species are assumed to be identical, then α corresponds to K_1, β corresponds to K_2, and γ corresponds to K_1K_3. Following the initial curve fitting, in which all parameters were allowed to vary, the curves were refit using k_{cat} values that were fixed at the observed maximum values of 0.55 s^{-1} ([Tyr] = 200 μM) and 0.067 ([Tyr] = 1 μM).

RESULTS

K'_{ATP} for the Dissociation of the K233A·ATP Complex. Two independent but identical clones containing the K233A mutation, designated K233A-1a and K233A-13a, were constructed. Sequencing of the entire tyrosyl-tRNA synthetase gene confirms that, with the exception of the K233A mutation, the nucleotide sequence of these two clones is identical to that of wild-type enzyme.

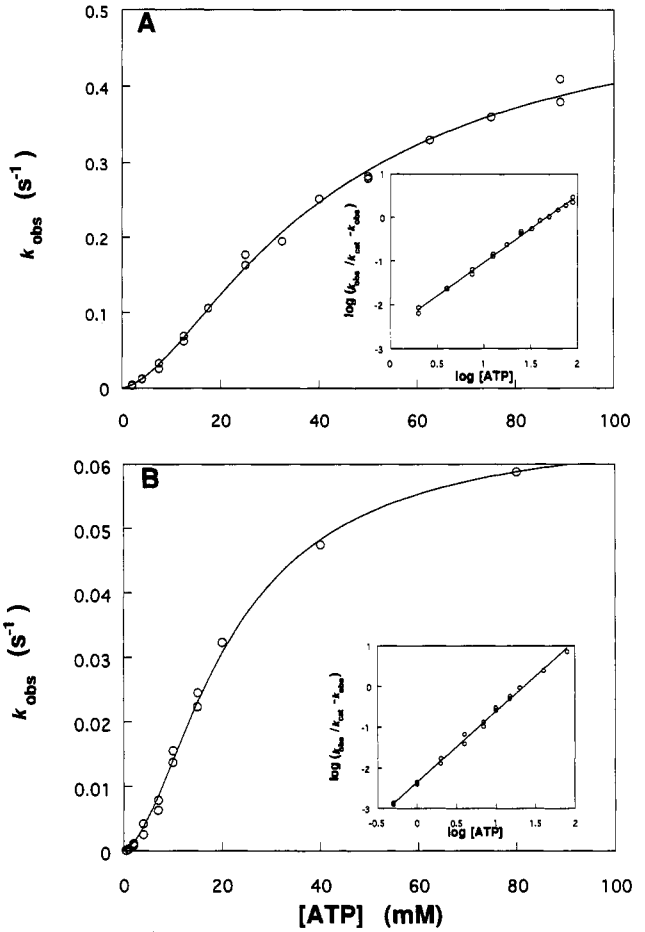

FIGURE 2: Sigmoidal ATP dependence of tyrosyl adenylate formation in the K233A mutant. The dependence of the initial reaction rate on ATP concentration in the presence of either (A) 200 μM tyrosine or (B) 1 μM tyrosine is shown. The model used to fit the data is described under Experimental Procedures. Hill plots are shown as insets.

The dependence of the initial rate for the formation of tyrosyl adenylate on the concentration of ATP was measured in the presence of 200 μM tyrosine (Figure 2A). As the value of K_{Tyr} for the K233A mutant is 10.4 μM, 95% of the enzyme in this assay is complexed to tyrosine prior to the addition of ATP. Analysis of the K233A mutant protein obtained from either K233A-1a or K233A-13a shows the same sigmoidal dependence on ATP concentration of the initial rate for the formation of tyrosyl adenylate. In addition, K233A purified from the M13mp93 clone used in previous studies (Fersht et al., 1988) also displays sigmoidal kinetics with respect to ATP binding (data not shown). Finally, reanalysis of the data from the previous studies also suggests that ATP binds with sigmoidal kinetics, although, due to the more restricted range of ATP concentrations used in these studies, the sigmoidal shape of the curve is not as obvious as it is in the present investigation.

The thermodynamic cycle for the binding of ATP is shown in Figure 1. Although this gives rise to four binding constants for ATP, only three of the binding constants are independent. As the relative activities of species B and D are not known (nor whether species B is active at all) it is not possible to obtain meaningful values for the dissociation constants. The data have therefore been fitted for the sole purpose of emphasizing the sigmoidal nature of the ATP dependence of activation (Figure 2). Although the curve fit itself is good (R = 0.998), due to the large errors in fitting the data to an Adair

equation with three independent variables, the α, β, and γ values obtained from these curve fittings should be considered only as very approximate values. For the ATP dependence of tyrosyl–adenylate formation at saturating (50 μM) tyrosine concentrations (Figure 2A), α = 400 mM, β = 40 mM, and γ = 1000 mM^2. The cooperative dependence of tyrosyl adenylate formation on ATP concentration is also apparent in the Hill plot (Figures 2A, inset; Hill, 1925) which has a slope of 1.54 (correlation coefficient = 0.998). The dissociation constant and k_{cat} values obtained from the Hill plot are 370 mM and 0.56 s^{-1}, respectively.

The ATP dependence of the initial rate for the formation of tyrosyl adenylate was also measured at subsaturating (1 μM) concentrations of tyrosine (Figure 2B). Under these conditions, only 10% of the enzyme in the assay is complexed with tyrosine prior to the addition of ATP. Both K233A-1a and K233A-13a show a sigmoidal ATP dependence for the formation of tyrosyl adenylate. As tyrosine is at subsaturating levels in this experiment, the kinetics of ATP binding are more complex than those in Figure 2A. For the ATP dependence of tyrosyl adenylate formation at subsaturating (1 μM) tyrosine concentrations (Figure 2B), α = 180 mM, β = 60 mM, and γ = 400 mM^2. Again, these should only be considered as very approximate values. The increase in cooperativity at low tyrosine concentrations is reflected in the shape of the activation curve as well as in the Hill plot (Figure 2B, inset), which has a slope of 1.73 (correlation coefficient = 0.991). The dissociation constant and k_{cat} values obtained from the Hill plot are 220 mM and 0.071 s^{-1}, respectively.

Effect of 0.5 M NaCl on the Kinetics of the K233A Mutants. When the ATP dependence of tyrosyl adenylate formation is measured in the presence of 0.5 M NaCl, the K233A mutant displays classical Michaelis–Menten kinetics. This is true both when the concentration of tyrosine is well above its dissociation constant (200 μM tyrosine, Figure 3A) and when the concentration of tyrosine is well below its dissociation constant (1 μM tyrosine, Figure 3B). As the effect of increased ionic strength is to convert the kinetics for the K233A mutant to classical Michaelis–Menten kinetics, it eliminates the possibility that the sigmoidicity in the curve shown in Figure 2 results from an increase in ionic strength at higher ATP concentrations. This is confirmed by kinetic analyses for ATP concentrations below 10 mM; in the K233A mutant, the sigmoidal nature of the curve is evident, whereas, in both wild-type enzyme and other active site mutants of tyrosyl-tRNA synthetase, no evidence of sigmoidal behavior is observed (data not shown). The kinetic constants determined for both wild-type and the K233A mutant tyrosyl-tRNA synthetase are summarized in Table I. Attempts to determine the K_{ATP} value for wild-type tyrosyl-tRNA synthetase from pre-steady-state kinetic analysis using stopped-flow fluorescence were ambiguous, as the individual reaction rates for each concentration of ATP could not be fitted to a single exponential. As a result, it was necessary to measure this value using steady-state pyrophosphate exchange kinetics. As previously shown by Wells et al. (1991), when [tyrosine] $\ll$ K_d^{Tyr}, this method will yield the true value for K_{ATP}. The effect of 0.5 M NaCl on wild-type enzyme is to increase the affinity for ATP 2-fold when [Tyr] = 1 μM and to decrease it 2-fold when [Tyr] = 200 μM. The forward rate constant, k_3, decreases 2.5-fold in the presence of 0.5 M NaCl.

The binding of ATP to the K233A mutant is approximately 3-fold weaker than the binding of ATP to wild-type enzyme, while k_3 is 20-fold lower, resulting in a 50-fold decrease in the specificity constant (k_3/K'_{ATP}). Comparison with the ATP

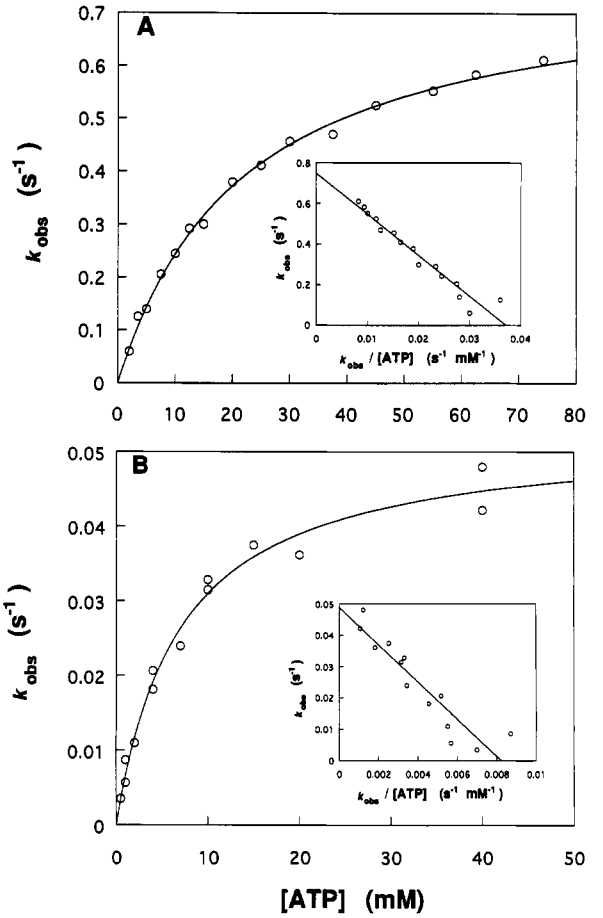

FIGURE 3: Enhanced binding of ATP to the active subunit and loss of cooperativity with respect to ATP binding in the K233A mutant on addition of NaCl. The effect of ATP concentration on the initial reaction rate in the presence of 0.5 M NaCl is shown. The concentration of tyrosine in the reaction solution is either (A) 200 μM or (B) 1 μM. Eadie–Hofstee plots are shown as insets.

binding constants determined in the presence and absence of NaCl indicates that, in the presence of NaCl, ATP binds with an affinity similar to that calculated for the binding of the second molecule of ATP in the absence of NaCl.

Second Site Mutation of K230 and T234. The effect of introducing a second alanine mutation at either K230 or T234 is shown in Figure 4. The binding of ATP in these double mutants follows classical Michaelis–Menten kinetics at either saturating (Figure 4) or subsaturating (data not shown) concentrations of tyrosine. Kinetic analyses of these double mutants were carried out in the absence of NaCl. Binding and rate constants for the double mutants are summarized in Table I.

Free Energy Profile for the Tyrosyl tRNA Synthetase K233A Mutant. The free energy profile for the K233A mutant is shown in Figure 5. Free energies for the binding of ATP in the E·Tyr·ATP and the E·[Tyr-ATP]* complexes were calculated from dissociation constants for ATP determined in the presence of 0.5 M NaCl. The $\Delta\Delta G_{app}$ for ATP binding to K233A was calculated by subtracting the ΔG_{app} for wild-type enzyme (in the presence of 0.5 M NaCl) from the ΔG_{app} for the K233A mutant enzyme (in the presence of 0.5 M NaCl). The value for the E·Tyr state in the reaction profile was taken from Fersht et al. (1988).

DISCUSSION

The value of K_m for the binding of ATP to the active subunit in wild-type tyrosyl-tRNA synthetase is 4.7 mM in the presence

Table I: Kinetic Constants for Wild-Type, K233A, K230A/K233A, and K233A/T234A Tyrosyl-tRNA Synthetase[a]

enzyme	K_{ATP} (mM)	K'_{ATP} (mM)	k_3 (s^{-1})	k_3/K'_{ATP} (s^{-1} M^{-1})
wild type (no NaCl)	3.5	4.7	38	8080
wild type (0.5 M NaCl)	1.8 (±0.2)	8 (±1)	15 (±2)	1875
K233A (no NaCl)			0.56 (±0.05)	
K233A (0.5 M NaCl)	7.0 (±0.8)	22 (±2)	0.78 (±0.06)	35.5
K230A/K233A (no NaCl)	3.2 (±0.8)	0.16 (±0.01)	0.001 (±0.0003)	6.3
K233A/T234A (no NaCl)	1.7 (±0.9)	0.14 (±0.03)	0.0008 (±0.0002)	5.7

[a] All values were measured at 25 °C. Wild-type and K233A enzyme values were determined in the presence of 0.5 M NaCl. Kinetic constants for wild-type in the absence of 0.5 M NaCl are taken from Fersht et al. (1988). Kinetic constants for the K230A/K233A and K233A/T234A double mutants were determined in the absence of 0.5 M NaCl. K_{ATP} is the dissociation constant for ATP from the E·ATP complex, K'_{ATP} is the dissociation constant for ATP from the E·Tyr·ATP complex, and k_3 is the forward rate constant for the formation of tyrosyl adenylate. Values represent means ± SEM (in parentheses) for at least three separate determinations. k_3 for K233A (no NaCl) was determined from the Hill plot shown in Figure 2A. All values were obtained by fitting plots of initial reaction rates versus ATP concentration to a Michaelis–Menten equation using Kaleidograph 2.1.1 (Abelbeck Software).

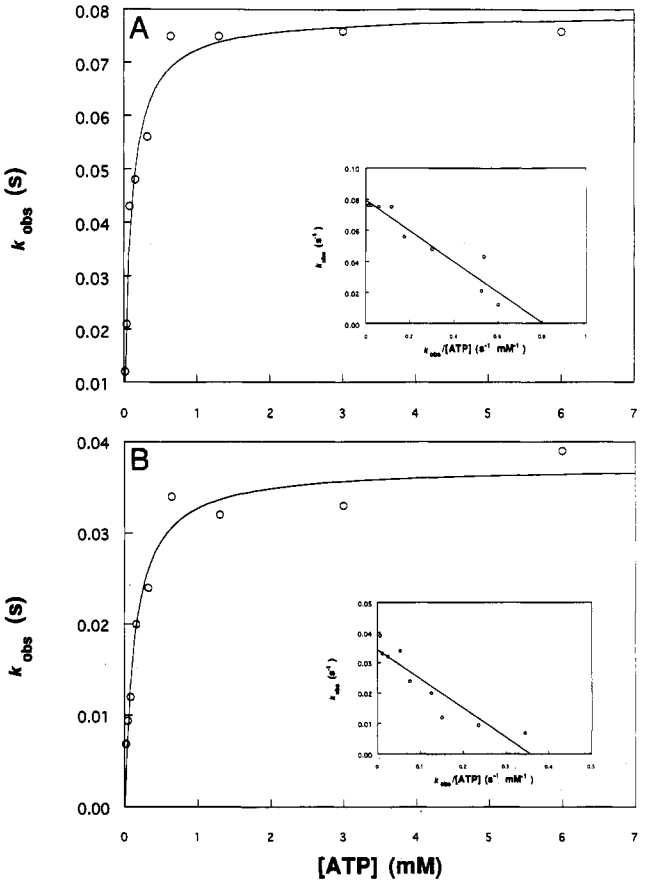

FIGURE 4: Enhanced binding of ATP to the active subunit and loss of cooperativity with respect to ATP binding in the K233A mutant on introduction of a second mutation in the active site loop. The effect that introducing a second alanine mutation at either residue K230 (panel A) or T234 (panel B) in the active site loop has on the ATP-dependent tyrosyl adenylate formation is shown. The data are fit to a model which assumes classical Michaelis–Menten kinetics. Eadie–Hofstee plots are shown as insets.

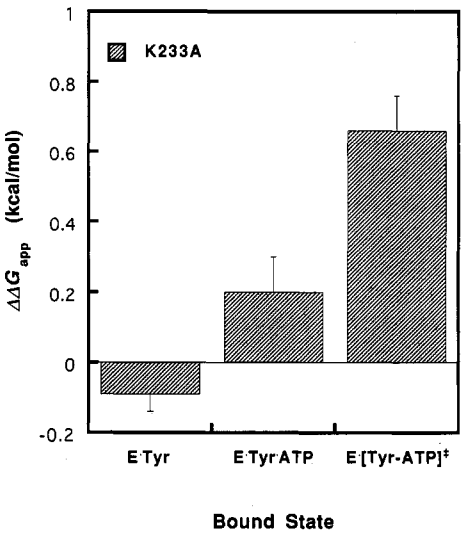

FIGURE 5: Difference free energy diagrams for the K233A mutant in the presence of 0.5 M NaCl. Difference free energies for the K233A mutant of tyrosyl-tRNA synthetase (K233A) were calculated by subtracting the free energy of the wild-type enzyme complex from the free energy of the mutant enzyme complex at each step in the reaction profile. Noncovalent bonds are indicated by a period and covalent bonds are indicated by a hyphen. The transition state is denoted by ‡. The value for the K233A·Tyr state was taken from Fersht et al. (1988). K'_{ATP} and k_3 values used in the calculation of $\Delta\Delta G_{app}$ values were obtained in the presence of 0.5 M NaCl for both mutant and wild-type tyrosyl tRNA synthetase.

of saturating tyrosine (Fersht et al., 1988). From Figure 2, it appears that this is at least 5-fold tighter than the dissociation constant for the binding of ATP to the K233A mutant (in the presence of saturating tyrosine). Thus, one effect of this mutation is to decrease the affinity of the active subunit for ATP. One consequence of this decreased affinity is that it allows two molecules of ATP to bind to the K233A mutant prior to the formation of the tyrosyl adenylate intermediate. This is in contrast to the wild-type enzyme, which initially binds ATP at the active subunit and binds ATP to the inactive subunit only after the tyrosyl adenylate intermediate has formed (Fersht, 1975). In other words, in the wild-type enzyme, the binding of only one ATP per tyrosyl-tRNA synthetase dimer can be followed by monitoring the formation of the tyrosyl adenylate intermediate, whereas in the K233A mutant the binding of two molecules of ATP can be followed. The ability to monitor the binding of ATP to both subunits allows the positive cooperativity displayed by the two binding sites to be observed.

It appears likely that, by altering the number of ATP molecules binding to the enzyme prior to tyrosyl adenylate formation, the K233A mutation has uncovered an already existent cooperative mechanism. In agreement with this hypothesis is the observation of Jones et al. (1985) that disruption of the subunit interface in tyrosyl-tRNA synthetase alters the K_m for the binding of ATP to the active subunit, indicating that there is communication between the dimer interface and the ATP binding sites. This communication occurs despite the absence of direct contact between the dimer interface and the ATP binding sites (Bhat et al., 1982).

Although the ATP-binding affinity is reduced, the binding of tyrosine is not significantly altered in the K233A mutant (Fersht et al., 1988). This suggests that the positive cooperativity associated with the binding of ATP is not correlated with the negative cooperativity associated with the binding of

tyrosine. In mechanistic terms, the physical bases of the two types of cooperativity are dissimilar.

The effect of either adding NaCl or mutating a second site in the active site loop is to increase the affinity of the K233A mutant for ATP. This results in only one molecule of ATP binding before the formation on the tyrosyl adenylate intermediate and causes a reversion to Michaelis–Menten kinetics when the formation of tyrosyl adenylate is used to follow ATP binding. As the binding of ATP to the second site is never directly monitored, any cooperativity in ATP binding will not be observed.

How do NaCl and second site mutations increase the binding of ATP to the active subunit? While global effects cannot be excluded, the ability to restore high-affinity binding by mutating a second residue in the active site loop suggests that local interactions are responsible for the decreased affinity of ATP in the K233A mutant. The ability of NaCl to counteract the effect of the K233A mutation suggests that electrostatic interactions can alter these local interactions. The effect of increased ionic strength on K'_{ATP} and k_{cat} for the wild-type enzyme supports the hypothesis that electrostatic interactions affect the active site of the enzyme.

An additional piece of evidence suggesting that local changes are responsible for the decreased affinity of the K233A mutant for ATP (and the subsequent increase in binding affinity in the presence of NaCl or when a second site is mutated) comes from monitoring the binding of tyrosine to the mutant enzyme. Neither the mutation of K233 to alanine nor the subsequent treatment with NaCl or second site mutagenesis significantly alters either the binding affinity of tyrosine or the half-of-sites reactivity displayed by the enzyme with respect to tyrosine binding. If global effects were involved in the changes in ATP binding, one would anticipate a disruption at the dimer interface which should alter not only ATP binding but tyrosine binding and half-of-sites reactivity as well. That this is not observed argues against such global effects occurring in the K233A mutant.

Alteration of Cooperativity and Allostery in Other Enzymes. As discussed above, the most plausible explanation for the observation of cooperative ATP binding in the K233A mutant is that this cooperativity already exists in the wild-type tRNA synthetase and the K233A mutant merely uncovers this preexisting mechanism. Are there other cases where cooperativity has been concealed in oligomeric proteins? Recently, two groups have used site-directed mutagenesis to introduce cooperativity into normally noncooperative enzymes (Kuo et al., 1989; Stebbins & Kantrowitz, 1992). In both cases, the enzymes involved, ornithine transcarbamoylase from *E. coli* and aspartate transcarbamoylase from *B. subtillus*, are closely related to the aspartate transcarbamoylase enzyme from *E. coli*, in which cooperative behavior with regard to substrate binding in the holoenzyme has been well documented [reviewed by Kantrowitz and Lipscomb (1988), Schachman (1988), and Allewell (1989)]. In addition, the mutation R105A in the isolated catalytic trimer of *E. coli* aspartate transcarbamoylase, which is homologous to mutations that convert the first two noncooperative enzymes into cooperative enzymes (R106A and R99A, respectively), also converts the noncooperative isolated catalytic trimer into a cooperative enzyme (Stebbins et al., 1989). Like K233 in tyrosyl-tRNA synthetase, each of these residues is located at the active site of the enzyme and decreases the affinity of the active site for substrates. Thus it seems likely that in these enzymes a mechanism for cooperativity already exists and mutagenesis simply unmasks this mechanism. That it is necessary to decrease the affinity of the active sites for cooperative binding to occur is not surprising. In cooperative systems, the ability to undergo a sharp transition from the unbound state to the bound state is gained at the expense of tight binding at low substrate concentrations. In the case of both tyrosyl-tRNA synthetase and the transcarbamoylases discussed above, the enzymes have apparently evolved to maximize substrate binding over a broad range of substrate concentrations.

The balance between cooperative and noncooperative substrate binding is reminiscent of the balance that exists between positive and negative allostery in phosphofructokinase (Lau & Fersht, 1987, 1989). This enzyme is allosterically regulated by phosphoenolpyruvate (an inhibitor) and GDP (an activator). However, on mutation of a single amino acid residue (E187A), the role of these two regulators is reversed, with phosphoenolpyruvate activating the enzyme and GDP inhibiting it. It appears that, at least in the enzymes discussed above, a single point mutation is sufficient to shift the equilibrium between different enzyme conformations, causing a change in the allosteric or cooperative properties of the enzyme.

Comparison with Other Aminoacyl-tRNA Synthetases. With regard to the aminoacyl-tRNA synthetases, two distinct classes exist. Class I aminoacyl-tRNA synthetases, composed of the aminoacyl-tRNA synthetases for methionine, leucine, valine, isoleucine, arginine, glutamine, glutamic acid, tyrosine, tryptophan (Eriani et al., 1990), and cysteine (Hou et al., 1991), possess an active site loop containing a lysine that is homologous to K233 in tyrosyl-tRNA synthetase. Consequently, it is of interest to determine whether or not the cooperativity uncovered in the tyrosyl-tRNA synthetase is a general characteristic of the class I aminoacyl-tRNA synthetases. Two class I aminoacyl-tRNA synthetases, tyrosyl-tRNA synthetase from *Saccharomyces cerevisiae* (C. M. Chow and U. L. RajBhandary, unpublished results) and tryptophanyl-tRNA synthetase from *Homo sapiens* (Frolova et al., 1991; Rubin et al., 1991), contain an alanine residue at the position corresponding to K233 in tyrosyl-tRNA synthetase from *B. stearothermophilus*. Whether either of these enzymes displays cooperative behavior with respect to ATP binding is not known. In the other aminoacyl-tRNA synthetases (all of which contain a lysine at the position corresponding to K233 in tyrosyl-tRNA synthetase from *B. stearothermophilus*), only the homologous lysine in the methionyl-tRNA synthetase has been investigated (Mechulam et al., 1991). Mutation of the homologous lysine, K235, in this enzyme produces results that are qualitatively similar to those produced by mutating K233 in tyrosyl-tRNA synthetase, although the presence of cooperativity has not been observed. It remains to be determined whether mutation of the analogous lysine in other aminoacyl-tRNA synthetases results in the cooperative binding of ATP.

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