Involvement of Threonine 234 in Catalysis of Tyrosyl Adenylate Formation by Tyrosyl-tRNA Synthetase†

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ABSTRACT: There is a mobile loop in the tyrosyl-tRNA synthetase that contains the KMSKS signature sequence of class I aminoacyl-tRNA synthetases. As it has not been possible to determine the role of the mobile loop in catalysis from X-ray crystallographic studies, we are investigating its importance by a series of site-directed mutagenic and kinetic studies. Here we examine the role of threonine 234 (T234) in the catalysis of tyrosyl adenylate formation by tyrosyl-tRNA synthetase from *Bacillus stearothermophilus*. This residue is the carboxy-terminal residue in the signature sequence and is either a serine or threonine in eight of the ten class I aminoacyl-tRNA synthetases isolated from *Escherichia coli*. Kinetic analyses of tyrosyl adenylate formation in the mutant enzymes indicate that k_3, the forward rate constant for the formation of tyrosyl adenylate, is reduced 500-fold on mutation of T234 to alanine. In contrast, mutation of T234 to serine results in only a 4-fold decrease in k_3, suggesting that the loss of the hydroxyl group in the T234A mutant is responsible for its decreased reaction rate. Deletion of the hydroxyl group destabilizes the transition state for the formation of tyrosyl adenylate by 2.7 kcal/mol. The transition state is also destabilized by 1.4 kcal/mol on the mutation of K230 to alanine. The effects of mutation of both T234 and K230 to alanine are less than additive; there is a coupling energy of −1.3 kcal/mol in the transition state. The effects of mutating K230 and T234 to alanine are also nonadditive in the E·Tyr-AMP complex (coupling energy = −1.9 kcal/mol). Finally, to investigate whether or not the stabilization of the transition state by T234 involves the metal ion bound to ATP, Mg^{2+} was replaced by the Cd^{2+} ion. Whereas the effect of Cd^{2+} on the stability of the wild-type transition state complex is negligible, the replacement of Mg^{2+} by Cd^{2+} in the transition state for the T234A mutant destabilizes it by 1.5 kcal/mol, indicating that there is an energetic coupling between the metal ion and T234.

Aminoacyl-tRNA synthetases catalyze the charging of tRNA molecules by their cognate amino acid. As such, they are a critical component in the translation apparatus of the cell. For most of the aminoacyl-tRNA synthetases (ATS), this process can be separated into two steps in vitro. The initial step results in the formation of a stable enzyme-bound aminoacyl adenylate intermediate (ATS·AA-AMP, eq 1). In the second step, this enzyme-bound aminoacyl adenylate intermediate transfers the amino acid (AA) to the 3′ end of the appropriate tRNA species (eq 2).

$$\text{ATS} + \text{AA} + \text{ATP} \rightarrow \text{ATS·AA-AMP} + \text{PP}_i \quad (1)$$

$$\text{ATS·AA-AMP} + \text{tRNA} \rightarrow \text{ATS} + \text{AMP} + \text{AA-tRNA} \quad (2)$$

Although all aminoacyl-tRNA synthetases perform this same function for the different amino acid substrates, there is very little sequence similarity among them. It has been shown that this family of proteins can be divided into two classes on the basis of primary as well as tertiary structure (Eriani et al., 1990; Cusack et al., 1990). In one of these classes, designated class I, all of the proteins possess a "Rossman fold" for binding ATP (Rossman et al., 1974). All of the class I aminoacyl-tRNA synthetases share both a common loop with the signature sequence K-M-S(T)-K-S(T) (Hountondji et al., 1986) and a second region of homology with the consensus sequence H-X-G-H (Webster et al., 1984). These sequences are absent in the class II aminoacyl-tRNA synthetases (Eriani et al., 1990). In addition, class I aminoacyl-tRNA synthetases aminoacylate on the 2-hydroxyl group of the terminal ribose, while class II aminoacyl-tRNA synthetases aminoacylate the 3-hydroxyl group (Moras, 1992). A sequence that is similar to the KMSKS signature sequence in class I aminoacyl-tRNA synthetases, the Walker consensus sequence, has been identified in a large number of other, unrelated proteins that bind nucleotides, including Ras P21 protein, elongation factor Tu, F_1 ATPase, and adenylate kinase (Walker et al., 1982, 1984).

The involvement of the KMSKS signature sequence in aminoacyl adenylate formation was first shown by mutation of K230[1] and K233 in tyrosyl-tRNA synthetase (Fersht et al., 1988). The sequence of this loop in tyrosyl-tRNA synthetase is (amino acid residues 227–235) DGTKFGKTE. In the X-ray crystal structure of tyrosyl-tRNA synthetase (Blow & Brick, 1985; Brick et al., 1989), these two residues are located in a loop that is at least 8 Å away from the active

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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 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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site of the enzyme. Thus, the involvement of this loop in catalysis could not have been predicted from the X-ray crystal structure. On the basis of the involvement of K230 and K233 in tyrosyl adenylate formation, Fersht et al. (1988) have postulated that this loop moves into the active site of the enzyme on formation of the transition state complex. That the loop is highly mobile is supported by the crystallographic temperature factors.

As X-ray crystallography does not provide any insight into the role of this mobile loop in catalysis, we have combined site-directed mutagenesis with kinetic analysis of the mutant enzymes to determine the role of each of the amino acid residues in this loop. The role that a particular amino acid residue plays in the catalytic mechanism of an enzyme can be understood by determining its effect on the free energy of the enzyme·substrate complex at each step along the reaction coordinate. This can be done by removing the functional group of the amino acid of interest (i.e., by site-directed mutagenesis), analyzing the kinetics for the binding of substrate and product to this mutant enzyme, and using free energy equations and rate theory to determine the free energy of binding (relative to a specific standard state) for each step in the reaction coordinate (Fersht et al., 1992). The free energies for binding to the wild-type enzyme, determined in an analogous manner, can be subtracted from the free energies of binding to the mutant for each step in the reaction to determine the effect that the functional group has on the binding energy at each step in the reaction coordinate. In tyrosyl-tRNA synthetase the above technique has been previously used to analyze the effect of active site mutants at each step along the reaction coordinate [for review, see Fersht (1987)].

In this paper, we address the question of the role of T234 in tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* during the formation of the tyrosyl adenylate intermediate. This residue is of particular interest, as it is conserved both in the KMSKS signature sequence of other class I aminoacyl-tRNA synthetases, and in the Walker consensus sequence. Both serine and alanine mutations have been used to differentiate the role of the hydroxyl group in T234 from mutational effects that do not involve the hydroxyl group. The role of the coupling between K230 and T234 is investigated using double-mutant free energy cycles to analyze the effect of this coupling along the reaction coordinate. Finally, the question of whether this residue interacts with the Mg^{2+} ion is addressed by substituting Cd^{2+} for Mg^{2+} in the kinetic analysis of both wild-type and mutant enzymes.

MATERIALS AND METHODS

Materials

All enzymes except Taq polymerase were obtained from United States Biochemicals (Cleveland, OH), chemicals from Sigma Chemicals (London), and radiochemicals from Amersham International. *Thermus aquaticus* DNA polymerase was obtained from Perkin-Elmer Cetus (Norwalk, CT). The phagemid vector pTZ18u and M13K07 helper phage were obtained from Pharmacia (Uppsala, Sweden). The plasmid vector pMT416 was the generous donation of R. W. Hartley.

Methods

Construction of Phagemid Vector Containing Tyrosyl-tRNA Synthetase. The tyrosyl-tRNA synthetase gene (Winter et al., 1982) was removed from M13mp93 using the polymerase chain reaction with the following primers: 5′ AGA *GGA TCC* TTA TAA AGA GGT GAA GGA CAT GGA 3′ (forward primer, *Bam*H I site in italics), 5′ CAG *GTC GAC* GGA CGA TCT TTT TTC TTA GGC GTA 3′ (reverse primer, *Sal*I site in italics). This gene was digested with *Bam*HI and *Sal*I and subcloned into the polylinker region of the phagemid pTZ18u to create pYTS4. The Trp-Lac promoter was removed from the pMT416 plasmid using the polymerase chain reaction with the following primers: 5′ *GAA TTC* TTA CTC CCC ATC CCC CTG T 3′ (forward primer, *Eco*RI site in italics), 5′ *GGA TCC* TGT TTC CTG TGT GAA 3′ (reverse primer, *Bam*HI site in italics). This promoter was digested with *Eco*RI and *Bam*HI and subcloned into pYTS4 immediately 5′ to the tyrosyl-tRNA synthetase gene to create pYTS5 (Figure 1). The sequence of the tyrosyl-tRNA synthetase gene in this construct is identical to that of the M13mp93 vector with the exception of a silent mutation in the third codon of V342 (GTA to GTG).

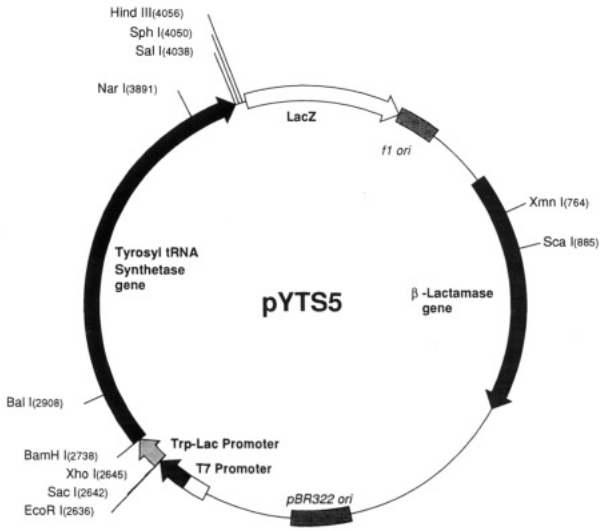

FIGURE 1: Construction of pYTS5. pYTS5 was constructed from pTZ18u, M13mp93, and pMT416. M13mp93 contains both the promoter and coding sequences for tyrosyl-tRNA synthetase (YTS, solid arrow) inserted into the multiple cloning site. pMT416 contains the coding sequence for the *B. amyloliquefaciens* ribonuclease (barnase) under control of a Trp-Lac promoter (lightly stippled arrow, R. W. Hartley, personal communication). The polymerase chain reaction (PCR) was used to excise the coding region of the tyrosyl-tRNA synthetase gene and the Trp-Lac promoter from M13mp93 and pMT416, respectively. The *lac Z* gene is indicated by the split arrow (not stippled) surrounding the promoter/tyrosyl-tRNA synthetase insert.

Production of Mutants. Mutagenesis was performed using the method of Kunkel (1985) with a pTZ18u phagemid, which contains the wild-type tyrosyl-tRNA synthetase gene from *B. stearothermophilus* and is preceded by a Trp-Lac promoter (pYTS5). The following oligonucleotides were used to create the desired mutants (mismatches are shown in italics): 5′ T GCG GCT TTC *AC*T TTT CCC GAA 3′ (T234S), 5′ GCG GCT TTC *AGC* TTT CCC GAA 3′ (T234A), and 5′ GCG GCT TTC *AGC* TTT CCC GAA T*GC* CGT GCC GTC CG 3′ (K230A/T234A). Uracil-containing single-stranded template was obtained from wild-type phagemid harbored in *Escherichia coli* CJ236 following the procedure described by Pharmacia. Following in vitro mutagenesis, the phagemids were transformed into *E. coli* TG2 cells [recA form of TG1 (Gibson, 1984)], and mutants were screened by dideoxy sequencing of the phagemid templates. Clones found to contain the desired mutants were then retransformed into *E. coli* TG2 cells and phagemid template was purified from these cells. This template was used to sequence the entire tyrosyl-tRNA synthetase gene to ensure that no other mutations were

present. All transformations were by the method of Hanahan (1985).

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

Purification of Enzymes. Mutant enzymes were expressed in *E. coli* TG2 hosts and purified to electrophoretic homogeneity as described previously (Fersht et al., 1988 ; First & Fersht, 1993).

Kinetic Procedures. All experiments, except those in which Cd^{2+} replaced Mg^{2+}, were performed in a standard buffer containing 144 mM Tris-HCl (pH 7.78) and 10 mM $MgCl_2$, at 25 °C. ATP was added as the magnesium salt to maintain the free Mg^{2+} at 10 mM. The final pH of the reaction was adjusted to 7.78 with NaOH. In experiments where Cd^{2+} replaced Mg^{2+}, all buffers and enzymes were treated with Chelex resin to remove trace amounts of contaminating metals prior to the addition of Cd^{2+} (Garcia et al., 1990). ATP was added as the cadmium salt to maintain the free Cd^{2+} at 10 mM.

Activation. The rate of formation of tyrosyl adenylate in the wild-type and T234S mutant enzymes was followed using pre-steady-state stopped-flow kinetics (Fersht et al., 1975; Wells & Fersht, 1986). The formation of tyrosyl adenylate in the T234A and K230A/T234A mutants was sufficiently slow that it could be monitored using a conventional filter assay (Calender & Berg, 1966; Leatherbarrow & Fersht, 1987).

The equilibrium constants for the dissociation of tyrosine from the E·Tyr complex, K_{Tyr}, were determined for the T234S and T234A mutants by measuring the tyrosine dependence of the rate of formation of the tyrosyl adenylate in the presence of ATP concentrations (0.1 mM ATP for T234S, 0.02 mM ATP for T234A, and 0.02 mM ATP for K230A/T234A) which were less than one-tenth the value of the K_{ATP} for the mutant enzyme. At these concentrations of ATP, over 90% of the tyrosyl-tRNA synthetase is present as the free enzyme, and the K_m determined from the tyrosine dependence of the rate of tyrosyl adenylate formation is equal to the dissociation constant for the E·Tyr complex (Wells et al., 1991). For the T234S mutant, in which the formation of the enzyme-bound tyrosyl adenylate complex was monitored by stopped-flow fluorescence, one syringe contained increasing concentrations of tyrosine (1–200 μM) in standard buffer, while the other syringe contained enzyme in standard buffer. MgATP was present in both syringes at a concentration of 0.1 mM. The reaction was monitored using the change in fluorescence of the tyrosyl-tRNA synthetase to follow the time course of the reaction (excitation at 292 nm and emission at >320 nm). An approximately 5% decrease in the total fluorescence was observed on formation of the enzyme-bound tyrosyl adenylate complex. For the T234A mutant, the formation of the enzyme-bound tyrosyl adenylate complex from [^{14}C]tyrosine and ATP was monitored using nitrocellulose filter assays. Assay solutions (260 μl) were made up such that, after addition of enzyme (40 μL, 4 μM), the assay solutions contained 144 mM Tris-HCl, pH 7.78, 10 mM $MgCl_2$, 0.001 units/mL inorganic pyrophosphatase, 0.02 mM MgATP, and 1–50 μM [^{14}C]-tyrosine (15 Bq/mmol). Assays were incubated at 25 °C, and prewarmed enzyme was added to initiate the reaction. Nine to twelve aliquots were periodically taken over at least four half-lives of the reaction, filtered through nitrocellulose filters, washed with 5 mL of ice-cold 144 mM Tris-HCl, pH 7.78, and dried. Retained E·[^{14}C]Tyr-AMP was assayed using scintillation counting. Rate constants were calculated using nonlinear regression to a first-order theoretical curve (Leatherbarrow, 1987). As the rate of formation of the tyrosyl adenylate complex was too slow to determine accurately the K_{Tyr} values for the K230A/T234A mutant by kinetic analysis, the dissociation constant for this mutant was determined from equilibrium dialysis in the absence of ATP as previously described (Fersht, 1975).

The equilibrium constants for the dissociation of ATP from the E·Tyr·ATP complex were determined by measuring the ATP dependence on the rate of formation of the tyrosyl adenylate in the presence of saturating concentrations of tyrosine (50 μM tyrosine for the T234A and K230A/T234A mutants and 200 μM tyrosine for the T234S mutant). At these concentrations of tyrosine, over 80% of the enzyme is present as the E·Tyr complex, and the K_m determined from the ATP dependence of the rate of tyrosyl adenylate formation is equal to the equilibrium constant for the dissociation of ATP from the E·Tyr·ATP complex. The K'_{ATP} values for the wild-type and T234S mutant enzymes were determined using the method described above for the determination of the K_{Tyr}, except that the concentration of tyrosine in both syringes was constant (200 μM) and ATP (0.2–20 mM) was added to the syringe that lacked enzyme. The K'_{ATP} values for the T234A and K230A/T234A mutant enzymes were determined using the method described above for the determination of the K_{Tyr} value for the T234A mutant. The concentration of [^{14}C]-tyrosine was held constant (50 μM) in these assays, while the concentration of ATP varied between 0.02 and 10 mM.

Pyrophosphorolysis. Enzyme-bound tyrosyl adenylate was prepared for each mutant enzyme and stored at –70 °C as described by Fersht et al. (1988). Pyrophosphorolysis was initiated by the addition of tetrasodium pyrophosphate to a solution of 100–400 mM enzyme·[^{14}C]tyrosine-AMP intermediate in 144 mM Tris-HCl (pH 7.78) and 10 mM $MgCl_2$. Both solutions were preincubated at 25 °C prior to the start of the reaction.

Analysis of Kinetics. Kinetic analysis has been previously described (Fersht et al., 1988; Wells et al., 1991). Analysis of coupling energies using double mutant cycles has also been described (Carter et al., 1984; Horovitz, 1987).

RESULTS

Expression of Mutants. The expression of tyrosyl-tRNA synthetase from the phagemid pYTS5 consistently yields 30–100 mg of enzyme per liter of cell culture. This is in contrast to the expression of tyrosyl-tRNA synthetase from the M13mp93 vector, which produces highly variable yields, ranging from less than 5 mg of enzyme per liter of cell culture to 50 mg of enzyme per liter of cell culture, and gives especially low yields for mutants that are kinetically slow. In contrast, the yield from pYTS5 is not significantly decreased in tyrosyl-tRNA synthetase mutants. This is presumably a consequence of both the strong, IPTG-inducible Trp-Lac promoter and the presence on the plasmid of a gene encoding ampicillin resistance. This latter property allows the selection of cells that contain the plasmid encoding the mutant tyrosyl-tRNA synthetase gene.

Effect of Mutation of T234 on the Free Energy of Each Step Along the Reaction Coordinate. To analyze the role of the functional groups in T234 in the catalytic mechanism of tyrosyl-tRNA synthetase, two different mutations were made in the wild-type enzyme. First, T234 was mutated to an alanine residue. This mutation removes the hydroxyl group while preserving the methyl group of the threonine side chain. Second, T234 was mutated to a serine residue. This mutation

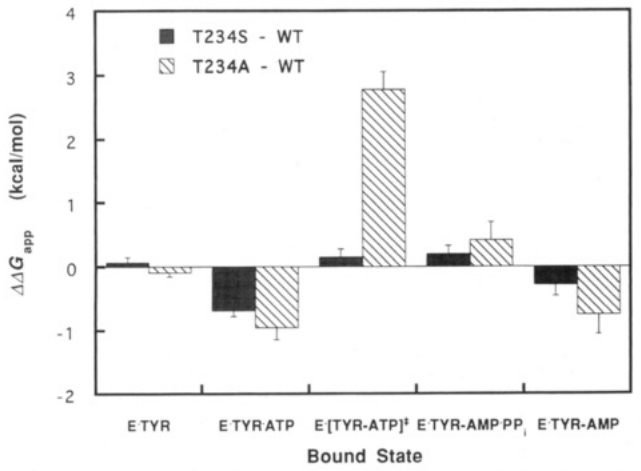

FIGURE 2: $\Delta\Delta G_{app}$ for Mutants of T234. Differences in free energies between the complexes of the mutants and wild-type are shown for each step leading to the formation of the tyrosyl adenylate intermediate. Tyrosyl-tRNA synthetase is represented by E, "·" represents noncovalent association, and "-" represents covalent bonds between species. The transition state is indicated by "‡". Errors (bars) are calculated as $e_t = (\sum \chi_i^2)^{1/2}$, where e_t is the total error in $\Delta\Delta G_{app}$, and χ_i is the error for each rate or binding constant used to calculate the free energies of binding.

removes the methyl group of the threonine side chain, while preserving the hydroxyl group. The effect of these mutations on each step in the reaction coordinate can be seen in Figure 2. It should be noted that in the frame of reference used a positive free energy ($\Delta\Delta G_{app}$) indicates that the bound state is stabilized by the presence of the wild-type residue. This is in accord with the $\Delta\Delta G_{app}$ as defined in earlier papers (Carter et al., 1984; Fersht et al., 1988). Neither mutation has any detectable effect on the binding of tyrosine to the free enzyme. In contrast, the initial binding of ATP to the E·Tyr complex is stabilized by approximately 0.5 kcal/mol in both of the mutants. Examination of the binding constants (Table I) indicates that the binding of ATP is 4-fold tighter in the mutants than in the wild-type enzyme. This is markedly different from the binding of the transition state to the mutants. While the T234S mutant is indistinguishable from the wild-type enzyme in its binding of the transition state, the T234A mutant binds the transition state 2.7 kcal/mol less tightly than wild-type enzyme (Figure 2), indicating that the hydroxyl group of T234 interacts strongly with the tyrosyl adenylate transition state. Comparison of the rate constants indicates that the weaker binding of the transition state in the T234A mutant is accompanied by a 60-fold decrease in the rate of catalysis. Finally, analysis of the binding of tyrosine-AMP·PP_i and tyrosine-AMP to the enzymes indicates that there is no significant difference between the stabilization of these states by the wild-type enzyme and either of the mutant enzymes.

Coupling of the Effects of K230 and T234. The effect of the coupling between two amino acid residues on the stabilization of each step in the reaction coordinate can be analyzed thermodynamically using double mutant cycles (Carter et al., 1984; Horovitz, 1987). If the effects of two residues are independent, then the free energy change observed on mutation of one of the residues will not be affected by mutation of the other residue in the same enzyme. In contrast, if the interactions of two residues with the substrates are coupled, then the free energy change resulting from the mutation of one of the residues is dependent on whether the other amino acid residue has also been mutated. Graphically, if the free energy changes on opposite sides of a thermodynamic cycle are not identical, then the two amino acids being analyzed interact in a manner that either stabilizes or destabilizes the bound state being examined.

Figure 3 shows thermodynamic cycles for coupling between K230 and T234 on four of the steps in the reaction coordinate for the formation of tyrosyl adenylate. Analysis of the E·Tyr-AMP·PP_i complex has been omitted due to the inability to separate the reverse rate constant from the dissociation constant for PP_i in the K230A mutant (Fersht et al., 1988). In these cycles, the starting point for each cycle is the wild-type enzyme. The cycles are constructed by the sequential conversion of wild-type residues into mutant residues. Interpretation of the free energy cycles is facilitated by comparing the values obtained for $\Delta\Delta G_1$ and $\Delta\Delta G_4$. In the case of $\Delta\Delta G_1$, two terms contribute to the free energy change: (1) the free energy change resulting from removal of the K230 side chain (ΔG_{mut}) and (2) the free energy change resulting from removal of the coupling between K230 and T234 ($\Delta\Delta\Delta G_{int}$). In the case of $\Delta\Delta G_4$, however, the T234 side chain has been replaced by an alanine residue. Consequently, $\Delta\Delta G_4$ consists only of the ΔG_{mut} term. Thus, one can determine the effect that removal of the energetic coupling between K230 and T234 has on the stability of the wild-type enzyme (relative to the double alanine mutant) by comparing $\Delta\Delta G_1$ with $\Delta\Delta G_4$.

As shown in Figure 3, coupling between K230 and T234 is observed in three of the four steps leading to tyrosyl adenylate formation (e.g., $\Delta G_1 \neq \Delta G_4$). Only in the initial binding of tyrosine (Figure 3, panel A) is no coupling between K230 and T234 observed. As mutation of either K230 or T234 does not significantly affect the initial binding of tyrosine (Table I), it is likely that the additivity of this cycle is a consequence of the lack of involvement in the binding of tyrosine by the mobile loop. The subsequent binding of ATP is accompanied by a small coupling between K230 and T234 (Figure 3, panel B), which destabilizes the formation of this state by 0.4 kcal/mol. During the formation of the tyrosyl adenylate transition state these two residues couple to stabilize this complex by 1.3 kcal/mol (Figure 3, panel C). Finally, the coupling between K230 and T234 stabilizes the E·Tyr-AMP intermediate by almost 2 kcal/mol (Figure 3, panel D).

Interaction between T234 and Mg^{2+}. While Mg^{2+} is the metal that is used physiologically by tyrosyl-tRNA synthetase, other divalent cations can be substituted in place of the Mg^{2+} ion in vitro (Garcia et al., 1990). Several divalent metal ions were investigated in an effort to find one which clearly decreased the rate of formation of the tyrosyl adenylate species. Whereas Mn^{2+} does not substantially alter the rate of the reaction (data not shown), Cd^{2+} was found both to increase the binding affinity of the enzyme for ATP and to decrease the rate of the reaction (Table II). In order to elucidate further the nature of the transition state stabilization by T234, the effect of substituting Cd^{2+} for Mg^{2+} in both the wild-type and T234A mutant enzymes was investigated. If T234 does not interact with the Cd^{2+} ion during the reaction, then when Cd^{2+} is substituted for Mg^{2+}, both the wild-type and T234A mutants should exhibit identical changes in free energy stabilization for each step in the reaction coordinate. The $\Delta\Delta G_{app}$ values for the E·Tyr·ATP and E·[Tyr-ATP]‡ complexes are shown in Figure 4. In the wild-type enzyme, substitution of Cd^{2+} for Mg^{2+} destabilizes the E·Tyr·ATP complex by 0.7 kcal/mol. In contrast, in the T234A mutant, the substitution of Cd^{2+} for Mg^{2+} increases the stability of this complex by 0.4 kcal/mol. The effect of substitution of Cd^{2+} for Mg^{2+} is much more dramatic in the E·[Tyr-ATP]‡ complex. In the wild-type enzyme, substituting Cd^{2+} for Mg^{2+} has no effect on the stability of the E·[Tyr-ATP]‡ complex, but in the

Table I: Rate and Dissociation Constants for Tyrosyl-tRNA Synthetase Mutants[a]

enzyme	K_{Tyr} (μM)	K'_{ATP} (mM)	k_3 (s^{-1})	k_3/K'_{ATP} (s^{-1} M^{-1})	K_{PP_i} (mM)	k_{-3} (s^{-1})	k_{-3}/K_{PP_i} (s^{-1} M^{-1})
wild type	11.6	4.7	38	8080	0.61	16.6	27200
K230A	23	4.9	0.39	80			130
T234S	13 (±2)	1.4 (±0.1)	9.7 (±0.4)	6900 (±700)	1.4 (±0.2)	18 (±1)	13000 (±1000)
T234A	10 (±1)	1.1 (±0.4)	0.07 (±0.01)	60 (±20)	4.4 (±0.8)	0.310 (±0.03)	70 (±10)
K230A/T234A	14 (±1)	3.0 (±0.1)	0.012 (±0.002)	4.0 (±0.8)	2.8 (±0.5)	0.37 (±0.03)	0.13 (±0.01)

[a] All experiments were conducted in standard buffer at 25 °C and pH 7.78. K_{Tyr} is the dissociation constant of the E·Tyr complex, K'_{ATP} is the dissociation constant for ATP from the E·Tyr·ATP complex, and K_{PP_i} is the dissociation constant of PP_i from the E·Tyr·AMP·PP_i complex. k_3 and k_{-3} are the rate constants for the forward and reverse reactions. With the exception of the K_{Tyr} values for wild-type, K230A mutant, and K230A/T234A mutant enzymes, all values were determined by pre-steady-state kinetics. The K_{Tyr} values for wild-type, K230A mutant, and K230A/T234A mutant enzymes were determined by equilibrium dialysis. Values for the wild-type and K230A mutant enzymes are taken from Fersht et al. (1988). Errors (in parentheses) are the standard deviation from a mean of 3–5 samples.

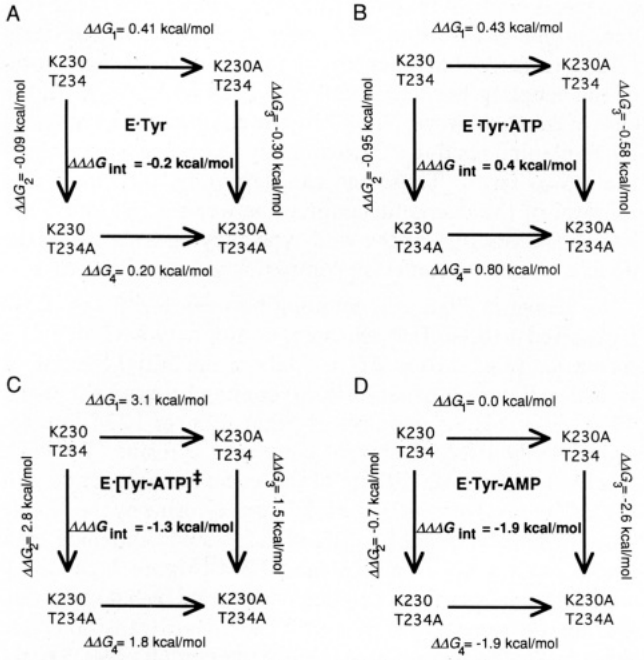

FIGURE 3: Coupling energies between K230 and T234. Double mutant cycles for the formation of enzyme-bound tyrosyl adenylate are shown for the E·Tyr complex (panel A), the E·Tyr·ATP complex (panel B), the E·[Tyr-ATP]‡ complex (panel C), and the E·Tyr-AMP complex (panel D). Abbreviations are as indicated in the legend to Figure 2. $\Delta\Delta G_1 = \Delta G_{app}$(K230A/T234) – ΔG_{app}(K230/T234), $\Delta\Delta G_2 = \Delta G_{app}$(K230/T234A) – ΔG_{app}(K230/T234), $\Delta\Delta G_3 = \Delta G_{app}$(K230A/T234A) – ΔG_{app}(K230A/T234), and $\Delta\Delta G_4 = \Delta G_{app}$(K230A/T234A) – ΔG_{app}(K230/T234A). $\Delta\Delta\Delta G_{int}$ values, the effect that the coupling between T234 and K230 has on the stability of each state, are determined by subtracting $\Delta\Delta G_1$ from $\Delta\Delta G_4$.

T234A mutant enzyme, this substitution destabilizes the complex by 1.7 kcal/mol. Examination of the binding and rate constants indicates that, in the wild-type enzyme, the decreased rate observed on substituting Cd^{2+} for Mg^{2+} is compensated by the increased affinity of the enzyme for ATP. In the T234A mutant, the rate decrease is more extreme and is not compensated for by an increased affinity for ATP. One can consider this as a thermodynamic cycle, analogous to the double mutant cycles described above, in which one mutation is T234 to alanine and the other mutation is Mg^{2+} to Cd^{2+}.

Determination of the $\Delta\Delta\Delta G_{int}$ terms for such a thermodynamic cycle indicates that the energetic coupling between the T234 side chain and the Mg^{2+} ion destabilizes both the E·Tyr·ATP complex (by 1.1 kcal/mol) and the E·[Tyr-ATP]‡ complex (by 1.5 kcal/mol).

DISCUSSION

The Role of Threonine 234 in Catalysis. The combination of site-directed mutagenesis and pre-steady-state kinetic analysis has made it possible to analyze the role of T234 at every step leading to tyrosyl adenylate formation in tyrosyl-tRNA synthetase. Mutation of T234 to alanine allows the role of the hydroxyl group on threonine to be analyzed, while maintaining the contacts that the methyl group on the threonine side chain makes with the protein·substrate complex. Conversely, mutation of T234 to serine allows the role of the methyl group on the side chain to be analyzed, while maintaining the contacts that the hydroxyl group makes with the protein·substrate complex. That neither mutation alters the ability of the enzyme to bind tyrosine is consistent with

Table II: Rate Constants, Dissociation Constants, and Free Energies of Binding for Wild-Type and T234A Mutant Tyrosyl-tRNA Synthetase[a]

enzyme		wild type	T234A
$K'_{ATP}{}^{Cd}$	(mM)	1.33 (±0.3)	2.1 (±0.5)
$k_3{}^{Cd}$	(s^{-1})	8.2 (±0.5)	0.008 (±0.002)
$\Delta G_{E\cdot Tyr\cdot ATP^{Cd}}$	(kcal/mol)	–10.6 (±0.1)	–10.5 (±0.1)
$\Delta G_{E\cdot[Tyr\text{-}ATP]^{*Cd}}$	(kcal/mol)	5.5 (±0.1)	9.8 (±0.2)
$\Delta G_{E\cdot Tyr\cdot ATP^{Mg}}$	(kcal/mol)	–9.9	–10.9 (±0.2)
$\Delta G_{E\cdot[Tyr\text{-}ATP]^{*Mg}}$	(kcal/mol)	5.4	8.2 (±0.3)

[a] All experiments were conducted at pH 7.78 and 25 °C. $K'_{ATP}{}^{Cd}$ is the dissociation constant of ATP from the E·Tyr·ATP complex and $k_3{}^{Cd}$ is the rate constant for the forward reaction when Cd^{2+} is the metal cofactor. Calculations of free energies of binding when either Mg^{2+} or Cd^{2+} is present (indicated by superscript) assume that the initial binding of tyrosine is independent of the metal cofactor. $\Delta G_{E\cdot Tyr\cdot ATP^{Mg}}$ and $\Delta G_{E\cdot[Tyr\text{-}ATP]^{*Mg}}$ values for wild-type enzyme are calculated from previously published data (Fersht et al., 1988). Tyrosine dissociation constants used in the calculation of $\Delta\Delta G_{E\cdot Tyr\cdot ATP}$ and $\Delta\Delta G_{[E\cdot Tyr\text{-}ATP]^*}$ were taken from Table I (T234A mutant) and Fersht et al. (wild-type enzyme, 1988).

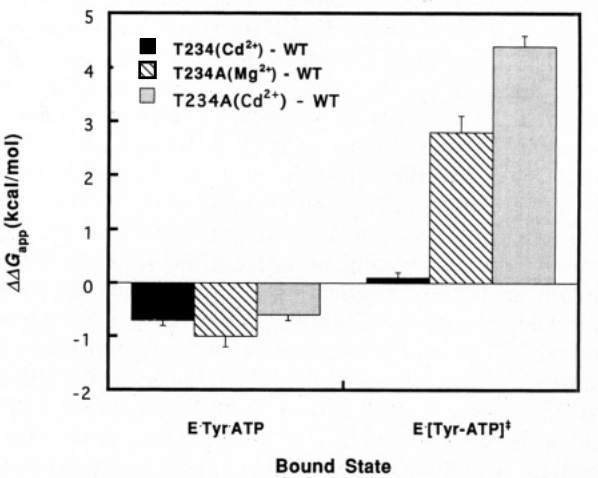

FIGURE 4: $\Delta\Delta G_{app}$ for T234 complexed with Cd^{2+} and T234A complexed with either Mg^{2+} or Cd^{2+}. The effect that substituting Cd^{2+} for Mg^{2+} has on the $\Delta\Delta G_{app}$ values for the E·Tyr·ATP and E·[Tyr-ATP]‡ complexes of both wild-type (T234) and mutant (T234A) enzyme is shown. Metal ions are shown in parentheses. WT indicates wild-type enzyme (T234) in the presence of Mg^{2+}.

previous investigations, which indicate that the mobile loop is not involved in the binding of the tyrosine substrate (Fersht et al., 1988). The small (0.5 kcal/mol) stabilization of the E·Tyr·ATP complex on mutating T234 to either alanine or serine suggests that one function of the threonine residue is to destabilize this complex. This prevents the complex from being trapped in a local energy minimum prior to the formation of the transition state. This is consistent with comparison of the binding and rate constants of the wild-type and T234S mutant enzymes which indicates that the wild-type enzyme has sacrificed tighter binding of the ATP substrate for a 4-fold increase in reaction rate. As the cellular concentration of ATP is approximately 5 mM (Lehninger, 1975), this decrease in binding affinity for ATP has little physiological effect on the catalytic activity of the enzyme. Since both T234S and T234A show similar increases in the stabilization of the E·Tyr·ATP complex, this effect is presumably due to steric interactions between the mobile loop and other regions of the ATP-binding site.

The hydroxyl group of T234 stabilizes the tyrosyl adenylate transition state by 2.7 kcal/mol relative to T234A. This is equivalent to the energy of disrupting a hydrogen bond involving a charged species (Fersht et al., 1985), suggesting that T234 may form a hydrogen bond with one of the phosphate groups on ATP, as has been proposed for K230 and K233 (Fersht et al., 1988). An alternative possibility, based on the X-ray data for the homologous serine, S17, in the Ras P21 protein (Milburn et al., 1990), is that T234 is coordinated to the Mg^{2+} ion. To determine whether or not Mg^{2+} and T234 interact, the coupling energy between Mg^{2+} and T234 was analyzed in both the E·Tyr·ATP complex and the E·[Tyr·ATP]‡ transition state complex and compared with the coupling energy between T234 and Cd^{2+}. Prior to binding to tyrosyl-tRNA synthetase, Mg^{2+} and Cd^{2+} are coordinated to both of the phosphate groups of ATP and to H_2O. Upon binding to the enzyme, the H_2O coordinated to the metal ion must be replaced by amino acid side chains on the enzymes. Until this occurs, the metal–ATP complex is not bound in a functional conformation. In the wild-type enzyme, the stability of the E·[Tyr-ATP]‡ complex is not altered when Mg^{2+} is replaced by Cd^{2+}, suggesting that Cd^{2+}ATP binds to the wild-type enzyme in a conformation which is functionally equivalent to that of Mg^{2+}ATP. In contrast, in the T234A mutant, the E·[Tyr-ATP]‡ complex is less stable when Cd^{2+} is bound than when Mg^{2+} is bound. Although it is clear from this that there is some type of coupling between the metal ion and T234, whether this is a direct or indirect effect cannot be determined from the analysis of thermodynamic cycles.

The Coupling of the Effects of K230 and T234 on the Catalytic Mechanism. In addition to having a direct role in stabilizing various steps in a reaction coordinate, amino acid residues can influence catalysis through their interactions with other amino acid residues in the protein. These interactions can be analyzed by the use of double mutant cycles (Carter et al., 1984; Horovitz, 1987). Analysis of the energetic coupling between K230 and T234 for each step in the reaction coordinate shows that the K230–T234 coupling has no effect on tyrosine binding, destabilizes the E·Tyr·ATP complex slightly, and significantly stabilizes both the E·[Tyr-ATP]‡ and the E·Tyr-AMP complexes. The destabilization of the E·Tyr-AMP complex by the T234A mutation and its stabilization by the K230–T234 coupling is the first evidence that the mobile loop affects the stability of the E·Tyr-AMP complex.

Mechanistic Relevance to Other Aminoacyl-tRNA Synthetases. The aminoacyl-tRNA synthetases can be divided into two distinct classes (Eriani et al., 1990). Class I aminoacyl-tRNA synthetases all contain a loop that is similar in sequence to that found in tyrosyl-tRNA synthetase. More specifically, in members of this class, a residue that is homologous to K233 in tyrosyl-tRNA synthetase is nearly always conserved and residues homologous to K230 and T234 are also generally conserved (Hountondji et al., 1986; Nagel & Doolittle, 1991). In class II aminoacyl-tRNA synthetases, this loop is absent, and the transition state is stabilized by an alternate mechanism (Moras, 1992). Of the aminoacyl-tRNA synthetases with a mobile loop, only arginyl- and glutaminyl-tRNA synthetases do not have a threonine or serine residue that is homologous to T234 in tyrosyl-tRNA synthetase (Avalos et al., 1991). This is surprising, as this residue is conserved in other types of nucleotide-binding proteins (Walker et al., 1982, 1984). Analysis of the glutaminyl-tRNA synthetase by X-ray crystallography indicates that the analogous loop in this enzyme has a different packing structure than the mobile loop in tyrosyl-tRNA synthetase (Rould et al., 1991). Consequently, it is possible that a serine or threonine homologous to T234 would not be properly positioned in the glutaminyl-tRNA synthetase.

Conclusion. Threonine 234 has been found to stabilize the transition state during the formation of tyrosyl adenylate in tyrosyl-tRNA synthetase. Coupling between K230 and T234 has the effect of stabilizing the E·[Tyr-ATP]‡ complex. In addition, both destabilization by the T234A mutant and coupling between K230 and T234 indicate that the mobile loop affects the stability of the E·Tyr-AMP complex. Finally, there is energetic coupling between T234 and the divalent metal ion, although the precise nature of this coupling is undetermined.

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