# Mutational and Kinetic Analysis of a Mobile Loop in Tyrosyl-tRNA Synthetase†

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ABSTRACT: The role of a mobile loop in tyrosyl-tRNA synthetase has been investigated by mutating each amino acid in the loop and kinetically analyzing the effect that each mutation has on the formation of the enzyme-bound tyrosyl adenylate intermediate. Kinetic analyses of mutations at three of the nine positions in the loop, K230, K233, and T234, have been reported elsewhere (Fersht et al., 1988; First & Fersht, 1993a,b). In this paper, the kinetic analyses of mutants in the remaining six positions, as well as a mutant in which the entire loop is deleted, are reported. With the exception of E235, which stabilizes the E·[Tyr-ATP]* and E·Tyr·ATP complexes by 1.0 and 1.2 kcal/mol, respectively, none of the remaining amino acids appears to be directly involved in the catalytic mechanism of the enzyme. Instead, mutation of these residues results in small alterations in the stability of E·Tyr·ATP, E·[Tyr-ATP]* and E·Tyr·AMP·$PP_i$ complexes. The precise amino acid residues which stabilize each state vary, suggesting that the loop adopts different conformations in each of the complexes with the most highly constrained conformation being in the E·[Tyr-ATP]* complex. Deletion of the loop reveals that the net effect of the loop in catalysis is two-fold: (1) to destabilize the E·Tyr·ATP complex preceding formation of the E·[Tyr·ATP]* complex and (2) to stabilize the E·[Tyr-ATP]* complex, indicating that the involvement of the loop in catalysis occurs at the expense of ATP-binding energy. As destabilization of the E·Tyr·ATP complex cannot be accounted for by analysis of single mutations in the loop, it is likely that this effect arises from the energetic coupling of amino acid residues in the loop.

Surface loops are a common secondary motif in proteins. They are characterized by the following properties [for review, see Rose et al. (1985)]: (1) all of the residues are in a continuous polypeptide chain, (2) the segment length of the polypeptide chain is between six and 16 residues, (3) there is no regular secondary structure, and (4) the distance between the segment termini (from first $\alpha$-carbon to last $\alpha$-carbon) is less than 10 Å and does not exceed two-thirds the maximum distance between any two $\alpha$-carbons in the loop. While, in general, the amino acid sequences of surface loops are less conserved than the amino acids that make up the hydrophobic core of the protein, amino acid residues in loops that are involved in catalysis may be more highly conserved than amino acids in surface loops whose role is purely structural. This sequence conservation can involve amino acids that interact directly with the substrate of the reaction as well as amino acids that help stabilize various conformations the loop may adopt during catalysis. Nucleotide-binding proteins, in particular, generally possess a highly conserved mobile loop, which is essential for nucleotide binding (Joseph et al., 1990; Gernstein & Chothia, 1991, and references therein).

In tyrosyl-tRNA synthetase an analogous mobile loop plays a crucial role in catalyzing the formation of tyrosyl adenylate. In order to understand the energetics of the catalytic mechanism of tyrosyl-tRNA synthetase, it is necessary to determine which amino acid residues in the loop are involved in catalysis. Three residues in the loop, K230,[1] K233, and T234, have been shown to significantly stabilize the E·[Tyr-ATP]* complex (Fersht et al., 1988; First & Fersht, 1993a). Analysis of the sequence variability at each position in the loop suggests that other amino acids may also be involved in the catalytic mechanism. In this paper, site-directed mutagenesis is used to investigate the role of the remaining amino acid residues in the mobile loop in tyrosyl-tRNA synthetase from *Bacillus stearothermophilus*. In addition, the net effect of the loop in catalysis is investigated using a mutant enzyme in which the entire mobile loop has been deleted. The results of these investigations indicate that the mobile loop decreases the affinity of the enzyme for ATP but increases its affinity for the tyrosyl adenylate transition state. Thus, the involvement of this mobile loop in catalysis occurs at the expense of energy gained from the binding of ATP.

## EXPERIMENTAL PROCEDURES

### *Materials*

All enzymes and the phagemid vector, pTZ18u, were obtained from United States Biochemicals (Cleveland, OH), chemicals from Sigma Chemicals (London, UK), and radiochemicals from Amersham International. DNA Polymerase from *Thermus aquaticus* was obtained from Cetus Corporation (Emeryville, CA).

### *Methods*

*Production of Mutants.* All mutants, except E235A, were constructed by the method of Kunkel (1985) from pYTS5 as

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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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Table I: Sequences of Mutagenic Oligonucleotides[a]

| mutation | oligonucleotide sequence |
|---|---|
| D227A | 5′ TT CGT GCC GGC CGC TTT CG 3′ |
| D227N | 5′ TTT CGT GCC GTT CGC TTT CGT CAC A 3′ |
| G228A | 5′ AA TTT CGT GGC GTC CGC T 3′ |
| T229A | 5′ CC GAA TTT CGT GCC GGC CGC TT 3′ |
| T229S | 5′ CC GAA TTT CGA ACC GTC CGC TT 3′ |
| F231L | 5′ CGT TTT CCC GAG TTT CGT GCC 3′ |
| G232A | 5′ TC CGT TTT CGC GAA TTT C 3′ |
| E235A | 5′ CGT GCC GCT TGC CGT TTT CCC G 3′ |
| Δ(227–234) | 5′ GAG CCA AAT CGT GCC GCT TTC CGC TTT CGT CAC AAG CGG GAT CG 3′ |
| wild-type | 5′ CGT GCC GCT TTC CGT TTT CCC GAA TTT CGT GCC GTC CGC TTT CGT CAC A 3′ |

[a] All mutagenic oligonucleotides except Δ(227–234) are aligned with the nucleotide sequence for the wild-type active site loop. Mismatched bases are shown in bold typeface. All oligonucleotides were synthesized using an Applied Biosystems model 280B oligonucleotide synthesizer and purified using Pharmacia FPLC Mono Q anion exchange with a gradient elution from 20 mM NaOH to 20 mM NaOH and 1.5 M NaCl over 100 min.

described in an accompanying paper (First & Fersht, 1993a). Construction of the phagemid pYTS5, which contains the wild-type tyrosyl-tRNA synthetase gene from *B. stearothermophilus* preceded by a Trp-Lac promoter, is also described in an accompanying paper (First & Fersht, 1993a). The oligonucleotides used in the mutagenesis are shown in Table I. Mutation of E235A was performed in M13mp93 using procedures that have been described previously (Fersht et al., 1988). Following isolation of each mutant phagemid, the entire tyrosyl-tRNA synthetase gene was sequenced to ensure that no other mutations were present.

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

### *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 pH of this MgATP solution was adjusted to 6.5 by the addition of NaOH prior to addition to the assay mixtures.

*Activation.* The rate constants for the formation of enzyme-bound tyrosyl adenylate by all mutants other than the loop deletion mutant, Δ(227–234), were followed by stopped-flow fluorescence as described in the preceding papers. For the Δ(227–234) mutant, formation of enzyme-bound tyrosyl adenylate from [$^{14}$C]tyrosine and ATP was monitored by filtration through nitrocellulose filters (described in preceding papers). For all mutants except E235A, determination of $K_{Tyr}$was done using methodology described above and in the preceding papers by holding the ATP concentration constant (at 0.1 mM) and varying the concentration of tyrosine between 1 and 200 μM (Wells et al., 1991). The $K_{Tyr}$ for the E235A mutant was determined by equilibrium dialysis as previously described (Fersht, 1975). The $K_{Tyr}$ term for the Δ(227–234) mutant was determined by a procedure analogous to that used for the determination of $K'_{ATP}$: varying the concentration of tyrosine between 1 and 50 μM, while holding the concentration of ATP constant at 20 μM.

*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 nM E·[$^{14}$C]Tyr-AMP 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).

*Determination of Sequence Variability.* The sequence variability, $V_s$, for each position in the active site loop was calculated from 48 aligned sequences using the following equations (Shenkin et al., 1991):

$$S = -\sum_{i=1}^{k} p_i \log_2 p_i \quad (1)$$

$$V_s = 6 \times 2^S \quad (2)$$

where $S$ is the information-theoretical entropy, and $p_i$ is the observed probability of appearance of the $i$-th amino acid type at a given position in the active site loop. The 48 class I aminoacyl-tRNA active site loop sequences that were aligned (on the basis of the KMSKS signature sequence) are: arginyl-tRNA synthetase (*E. coli*), cysteinyl-tRNA synthetase (*E. coli*), glutamyl-tRNA synthetase (*Bacillus subtilis, B. stearothermophilus, Chlamydia psittaci, E. coli, Rhizobium meliloti, Thermus thermophilus*), glutaminyl-tRNA synthetase (*E. coli, Saccharomyces cerevisiae, Homo sapiens*), isoleucyl-tRNA synthetase (*E. coli, S. cerevisiae, Thermus thermophila*), leucyl-tRNA synthetase (*E. coli, S. cerevisiae,* mitochondrial *S. cerevisiae,* mitochondrial *Neurospora douglassi, Neurospora crassa,* mitochondrial *N. crassa*), methionyl-tRNA synthetase (*E. coli, S. cerevisiae,* mitochondrial *S. cerevisiae, T. thermophilus*), tryptophanyl-tRNA synthetase (*B. stearothermophilus, B. subtilis, H. sapiens,* mitochondrial *S. cerevisiae*), tyrosyl-tRNA synthetase (*B. stearothermophilus, B. subtilis, E. coli,* mitochondrial *N. crassa, S. cerevisiae*), and valyl-tRNA synthetase (*B. stearothermophilus, E. coli, S. cerevisiae,* mitochondrial *S. cerevisiae*). In addition, $V_s$ was also calculated using only the *E. coli* enzymes in order to allow each aminoacyl-tRNA synthetase to be equally represented.

## RESULTS

*Effect on Binding and Rate Constants of Mutations in the Active Site Loop of Tyrosyl-tRNA Synthetase.* The binding and rate constants for mutants at each position in the active site loop of tyrosyl-tRNA synthetase are shown in Table II. The binding of tyrosine to the free enzyme is not significantly affected by mutations in the active site loop. In contrast, the binding of ATP to the E·Tyr complex is generally 2–4-fold tighter on mutation of the active site loop. The binding of pyrophosphate by the E·Tyr-AMP intermediate is decreased relative to wild-type enzyme in mutants at all positions except T229 and G232. This decreased affinity for pyrophosphate is most noticeable in the previously characterized K230A and

Table II: Binding and Rate Constants for each of the Tyrosyl-tRNA Synthetase Mutants[a]

| enzyme | $K_{Tyr}$ (μM) | $K'_{ATP}$ (mM) | $k_3$ ($s^{-1}$) | $k_3/K'_{ATP}$ ($s^{-1}$ $M^{-1}$) | $k_{-3}$ ($s^{-1}$) | $K_{PP_i}$ (mM) | $k_{-3}/K_{PP_i}$ ($s^{-1}$ $M^{-1}$) |
|---|---|---|---|---|---|---|---|
| wild-type[b] | 12 | 4.7 | 38 | 8080 | 16.6 | 0.61 | 27200 |
| D227A | 14 (±2) | 2.4 (±0.1) | 11.8 (±0.2) | 4900 | 11 (±1) | 1.5 (±0.4) | 7300 |
| D227N | 11 (±2) | 1.9 (±0.3) | 23 (±1) | 12100 | 15 (±2) | 0.5 (±0.2) | 30000 |
| G228A | 20 (±5) | 2.6 (±0.2) | 11.8 (0.6) | 4500 | 17 (±3) | 1.7 (±0.5) | 10000 |
| T229A | 16 (±1) | 3.1 (±0.2) | 43 (±2) | 13900 | 15 (±1) | 0.38 (±.08) | 40000 |
| T229S | 13 (±2) | 2.1 (±0.2) | 20 (±1) | 9500 | 12 (±1) | 0.4 (±0.1) | 30000 |
| K230A[b] | 23 | 4.9 | 0.39 | 80 | | | 130 |
| F231L | 12 (±4) | 2.3 (±0.2) | 6.6 (±0.3) | 2800 | 7.0 (±0.6) | 1.6 (±0.2) | 4700 |
| G232A | 24 (±1) | 3.3 (±0.5) | 15 (±1) | 4500 | 4 (±1) | 0.4 (±0.2) | 10000 |
| K233A[b,d] | 10.4 | | 0.55 | | | | |
| T234A[c] | 9 (±1) | 1.1 (±0.4) | 0.06 (±.01) | 60 | 0.31 (±.03) | 4.4 (±0.8) | 70 |
| T234S | 13 (±2) | 1.4 (±0.1) | 9.7 (±0.4) | 6900 | 18 (±1) | 1.4 (±0.2) | 12900 |
| E235A | 21 (±2) | 4.4 (±0.5) | 9.5 (±0.4) | 2200 | 19 (±2) | 2.9 (±0.1) | 6600 |
| Δ(227–234) | 6.1 (±0.4) | 0.15 (±0.04) | 0.0018 (±0.0003) | 12 | | | |

[a] Experimental errors are indicated in parentheses. [b] Data taken from Fersht et al. (1988). [c] Data taken from First and Fersht (1993a). $K'_{ATP}$ data were obtained in the presence of 50 mM tyrosine. [d] The $K'_{ATP}$ value for the K233A mutant cannot be accurately determined in the absence of 0.5 M NaCl (First & Fersht, 1993b).

K233A mutants where the affinity for pyrophosphate is too low to be accurately determined and, in the T234A mutant, where the affinity is 7-fold less than it is for wild-type enzyme.

The most noticeable effect of mutation of the active site loop is on the rate constants of the forward and reverse reaction ($k_3$ and $k_{-3}$). Mutation at most positions in the loop results in a 2–6-fold decrease in the $k_3$ values. This is substantially less than for the K230A, K233A, and T234A mutants, where the forward rate constant is decreased by 2–3 orders of magnitude. With the exception of the K230A, K233A, and T234A mutants, the reverse rate constant, $k_{-3}$, is generally unaffected by mutation of the active site loop, although F231L and G232A show 2- and 4-fold decreases in $k_{-3}$, respectively. The specificity constants, $k_3/K'_{ATP}$ and $k_{-3}/K'_{PP_i}$ for active site loop mutants are, with the exception of K230A, K233A, and T234A, within 4-fold of the values for wild-type enzyme.

*Effect of the Loop Mutations on the Stability of Each State in the Reaction Pathway.* The differences between the free energy of binding for each mutant enzyme and the wild-type enzyme ($\Delta\Delta G_{app}$) are shown in Figures 1 and 2. The binding of free tyrosine is not significantly altered in any of the mutants. This is consistent with previous analyses of mutations at K230, K233, and T234 and supports the hypothesis that the active site loop is not involved in the binding of free tyrosine to the enzyme.

Four mutants, D227A, D227N, T229S, and F231L, show a small (0.4 kcal/mol) increase in binding affinity for the Tyr·ATP complex. This is similar to the effect of the T234A mutation on binding of the Tyr·ATP complex (First & Fersht, 1993a), although the affect is slightly larger (1 kcal/mol) for the T234A mutant.

For binding of the tyrosyl adenylate transition state, [E·Tyr-ATP]‡, except for mutants of the three amino acids that are known to participate in catalysis (K230, K233, and T234), only the E235A mutant shows a $\Delta\Delta G_{app}$ above 0.7 kcal/mol (1.0 kcal/mol). This is not as large an affect as previously seen for mutants of K230, K233, and T234, which all decrease the stability of the tyrosyl adenylate transition state by 2.5–3.0 kcal/mol. Of the remaining mutants, three exhibit decreased binding to the transition state of more than 0.5 kcal/mol (G228A, F231L, and G232A).

The E235A and G228A mutants decrease the stability of the E·Tyr-AMP·$PP_i$ complex by 1.2 and 0.7 kcal/mol, respectively. Neither F231L nor G232A, which destabilize the transition state complex, were found to similarly destabilize the E·Tyr-AMP·$PP_i$ complex.

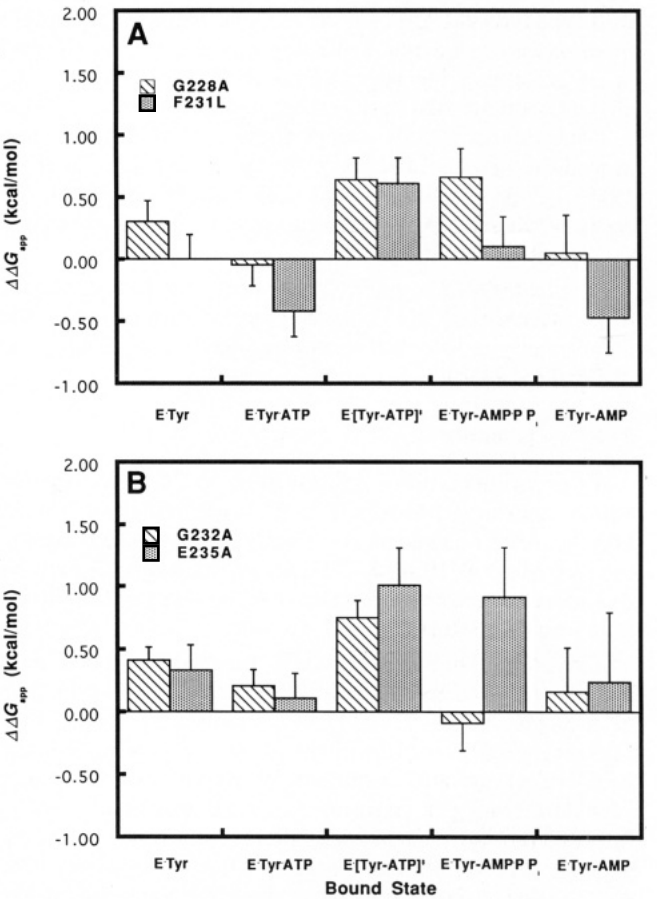


FIGURE 1: Difference free energy diagrams for G228A and F231L (A) and G232A and E235A (B). The difference free energy ($\Delta\Delta G_{app}$) for each state in the reaction pathway was calculated by subtracting the free energy of the wild-type enzyme from the free energy for the mutant enzyme, for each state. Positive values for $\Delta\Delta G_{app}$ indicate that the mutation destabilizes the bound state. Error bars are indicated by single hashed lines on top of the column bars.

Finally, none of the mutations investigated in this paper appears to alter the stability of the E·Tyr-AMP intermediate.

*Effect of Altering Side Chains at Positions 227 and 229.* With the exception of the two glycine residues, which are mutated to alanine, mutations were designed such that (1) the functional group was removed from the wild-type side chain, (2) no new functional group was introduced, and (3) the remainder of the side chain was left as intact as possible. At two positions, D227 and T229, additional mutations were constructed, converting D227 to an asparagine and T229 to

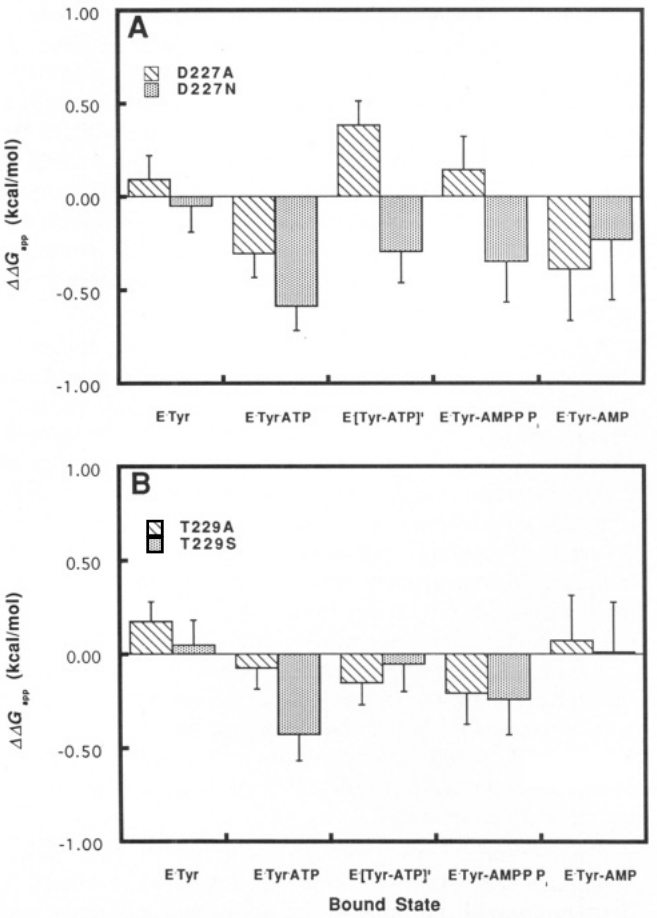


FIGURE 2: Difference free energy diagrams for position 227 (A) and 229 (B) mutants. The difference free energy ($\Delta\Delta G_{app}$) for each state in the reaction pathway was calculated as indicated in the legend to Figure 1. Error bars are indicated by single hashed lines on top of the column bars.

a serine. This was done to provide further insight into the roles of the functional groups at these two positions. As discussed above, mutation of D227 to alanine alters the binding of both the Tyr·ATP complex preceding the formation of the tyrosyl adenylate transition state as well as the binding of the tyrosyl adenylate transition state itself. Mutation of D227 to either alanine or aspartic acid increases the binding of the Tyr·ATP complex, although the magnitude of this affect differs for the two mutants (Figure 2). In contrast, while the D227A mutation destabilizes the binding of the tyrosyl adenylate transition state, the D227N mutation actually stabilizes this state by 0.5–0.6 kcal/mol. A similar effect is seen in the binding of the Tyr·ATP complex to position 229 mutants (Figure 2B). In this case, the T229A mutant fails to alter the binding affinity of the enzyme for this complex, whereas the T229S mutant shows an increased binding affinity of 0.4–0.5 kcal/mol. It is apparent from these results that either the introduction of new functional groups (D227N) or the alteration of the steric constraints placed on a functional group (T229S) can affect the stability of various steps in the reaction pathway. This effect has previously been observed in tyrosyl-tRNA synthetase, most notably in the K82N mutation (Fersht et al., 1988).

*Comparison of Reaction Profiles for Mutants at Each Position in the Loop.* To evaluate the relative contribution that each residue has on the reaction pathway, mutants at each position have been compared (not shown). The net effect of the amino acid side chains appears to be one of stabilizing the transition state and the E·Tyr-AMP·$PP_i$ complex. No clear net effect on the stability of the other states in the reaction

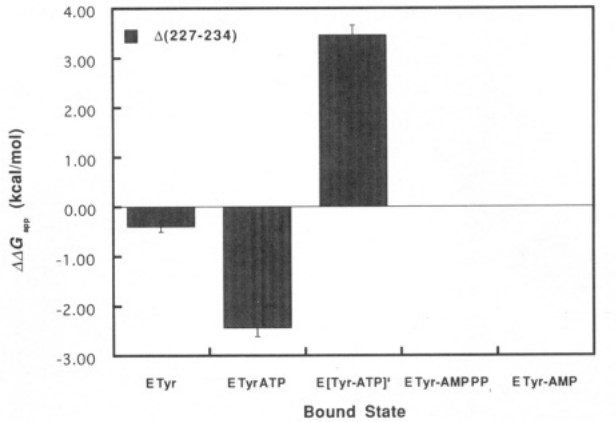


FIGURE 3: Free energy difference diagram for the Δ(227–234) mutant. Free energy differences were calculated as described in the legend to Figure 1. Blank columns for the E·Tyr-AMP·$PP_i$ and E·Tyr-AMP complexes indicate the inability to calculate these values due to the inaccessibility of $k_{-3}$ and $K_{PP_i}$ for this mutant.

pathway was observed. It should be noted that this comparison indicates the contribution of each amino acid residue in the loop in isolation from other amino acid residues and consequently does not reflect the affect of energetic coupling between the amino acid residues.

*Effect of Deleting the Active Site Loop.* Deletion of residues 227–234 has no significant affect on the binding of free tyrosine to tyrosyl-tRNA synthetase (Figure 3), suggesting that deletion of these residues leaves the overall structure of the enzyme intact. Surprisingly, in view of the results discussed in the previous section, deletion of the loop results in a large (2.5 kcal/mol) increase in the stability of the E·Tyr·ATP complex. The affinity of the enzyme for the tyrosyl adenylate transition state is reduced by 3.5 kcal/mol when the loop is deleted. Binding to the final two states in the reaction pathway could not be calculated for the Δ(227–234) mutant as the rate of pyrophosphorolysis by this mutant is indistinguishable from the background hydrolysis rate.

*Analysis of the Sequence Variability in the Active Site Loop.* Shenkin et al. (1991) have suggested a measure of variability, $V_s$, based on the information-theoretical entropy. This measure of variability is more mathematically robust and continuous than the method of Wu and Kabat (1970). Figure 4 shows the application of this method for measuring sequence variability to the active site loop in tyrosyl-tRNA synthetase. Comparison of Figure 4 with the effect of mutation at each position indicates that, while the three residues that are most essential in catalyzing the formation of tyrosyl adenylate (K230, K233, and T234) all have low values for $V_s$, there are two positions (F231 and G232) that exhibit low values for $V_s$ but do not appear to be substantially involved in the formation of tyrosyl adenylate, and one position (E235) which exhibits a high value for $V_s$, but stabilizes two of the steps in the reaction pathway by 1 kcal/mol.

## DISCUSSION

*Role of the Mobile Loop in the Catalytic Mechanism of Tyrosyl Adenylate Formation in Tyrosyl-tRNA Synthetase.* The mobile loop plays a significant role in at least three of the five steps thus far elucidated for the formation of the E·Tyr-AMP intermediate. Specifically, the loop (1) is not involved in the binding of free tyrosine to the enzyme, (2) destabilizes the E·Tyr·ATP complex prior to formation of the transition state complex, (3) stabilizes the [E·Tyr-ATP]‡ complex, (4) stabilizes the E·Tyr·AMP·$PP_i$ complex, and (5) appears to

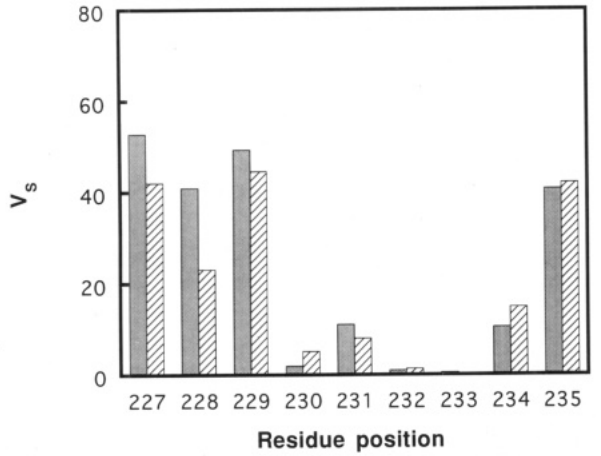


FIGURE 4: Sequence variability in the active site loop of the aminoacyl-tRNA synthetases. The sequence variability, $V_s$, was calculated according to Shenkin et al. (1991) for 48 class I aminoacyl-tRNA synthetases from various sources (stippled bars) and for each of the class I aminoacyl-tRNA synthetases from *E. coli* (hatched bars). At position 233, all of the *E. coli* class I aminoacyl-tRNA synthetases contain a lysine residue.

affect the stability of the E·Tyr-AMP complex. The energetics on which these conclusions are based are summarized below.

That the loop does not stabilize the E·Tyr complex is demonstrated by the $K_{Tyr}$ values for tyrosyl-tRNA synthetase containing mutations in the loop. In no cases are the $K_{Tyr}$ values for the mutant enzymes substantially different from that of the wild-type enzyme. In addition, in the Δ(227–234) mutant, the $K_{Tyr}$ value is also similar to that of the wild-type enzyme, suggesting that deletion of the mobile loop leaves the remainder of the protein structurally intact.

In the case of the E·Tyr·ATP complex, with the exception of the T234 side chain, the mutation of individual residues suggests that the mobile loop is not involved in stabilizing this complex either. That this is not the case is demonstrated by the Δ(227–234) mutant, in which the E·Tyr·ATP complex is stabilized by 2.5 kcal/mol when compared with wild-type enzyme. The presence of the loop prevents the reaction from falling into a thermodynamic "pit" which would increase the activation barrier that must be overcome to form the transition state complex. In terms of energetics, the involvement of the loop in catalysis occurs at the expense of ATP-binding energy. While the magnitude of this effect is fairly substantial (2.5 kcal/mol), the observation that it is not entirely due to the individual amino acid residues suggests that the energetic coupling between amino acid residues in the mobile loop is involved in the destabilization of the E·Tyr·ATP complex.

Three residues in the mobile loop, K230, K233, and T234, are responsible for the majority of the stabilization of the transition state complex by the loop. While individually each of these residues contributes 2.5–3 kcal/mol to the stability of the E·[Tyr-ATP]‡ complex relative to alanine, the net effect of the loop on the stability of this complex is only 3.5 kcal/mol, indicating that a large amount of energetic coupling occurs between these three residues. In addition to these three residues, nearly every other amino acid in the loop stabilizes the E·[Tyr-ATP]‡ complex by 0.4–0.7 kcal/mol. Thus, even amino acid side chains that are not essential for the activity of the loop contribute to the stability of the E·[Tyr-ATP]‡ complex. Presumably, this is a consequence of van der Waals effects, suggesting that the loop is more conformationally constrained in the E·[Tyr-ATP]‡ complex than it is in either the E·Tyr·ATP complex preceding the transition state or the E·Tyr-AMP·$PP_i$ complex following it.

Owing to the inability to measure pyrophosphorolysis in the Δ(227–234) mutant, it is impossible to quantitate the net effect of the loop on the stability of the E·Tyr-AMP·$PP_i$ complex. That the loop is involved in stabilizing this complex is confirmed by the mutation of E235, which destablizes the E·Tyr-AMP·$PP_i$ complex by 1 kcal/mol, and mutation of K230 and K233, which effectively eliminates pyrophosphorolysis (the reverse reaction) implying that mutation of these amino acid residues destabilizes the E·Tyr-AMP·$PP_i$ complex. Deletion of the entire loop also eliminates pyrophosphorolysis, supporting this conclusion.

Analysis of the K230A/T234A double mutant indicates that energetic coupling between the K230 and T234 side chains stabilizes the E·Tyr-AMP complex by 1.9 kcal/mol (First & Fersht, 1993a). The X-ray crystal structure for the tyrosyl-tRNA synthetase E·Tyr-AMP complex has been solved to 2.3-Å resolution (Brick et al., 1989). In this structure there is no side chain density for either K230 or K233 beyond the β-carbon atoms and no indication that the amino acid residues in the mobile loop directly interact with the tyrosyl adenylate intermediate. Nevertheless, the energetics of the K230/T234 coupling indicate that the mobile loop affects the stability of this complex, suggesting that this effect may be mediated through other amino acid residues in the enzyme. Since this is only one of the six possible couplings between the K230, K233, and T234 side chains, it is not possible to draw general conclusions concerning the net effect of the mobile loop on the stability of this complex.

*Comparison of Tyrosyl-tRNA Synthetase with Other Aminoacyl-tRNA Synthetases.* As indicated in Figure 4, five amino acid positions in the active site loop have less variability than the remainder of the loop. These five residues correspond to the KMSKS signature sequence (Mechulam et al., 1991). Of these residues, three (K230, K233, and T234) have substantial roles in the catalytic mechanism of tyrosyl adenylate formation in tyrosyl-tRNA synthetase. The remaining two residues, F231 and G232, do not appear to contribute more than 0.7 kcal/mol to the stability of any of the complexes in the reaction pathway. It should be noted that F231 has been found in only three other aminoacyl-tRNA synthetases (two tyrosyl-tRNA synthetases and one methionyl-tRNA synthetase) and that G232 is found in only two other aminoacyl-tRNA synthetases (both tyrosyl-tRNA synthetases). Consequently, the role of these residues in tyrosyl-tRNA synthetase from *B. stearothermophilus* may differ from the roles of the corresponding methionine and serine residues found in most in other aminoacyl-tRNA synthetases. There is also one amino acid, E235, which displays a high sequence variability but is found to stabilize both the [E·Tyr-ATP]‡ transition state complex and the E·Tyr-AMP·$PP_i$ complex by 1 kcal/mol. Glutamic acid is only found at this position in tyrosyl-tRNA synthetase from *B. stearothermophilus* and *E. coli*, although several aminoacyl-tRNA synthetases have an aspartic acid in the corresponding position. It must therefore be concluded that, while there is a rough correlation between positions of low sequence variability in the active site loop of class I aminoacyl-tRNA synthetases and their relative importance in stabilizing complexes along the reaction pathway, examples of both amino acids in positions with low sequence variability that do not appear to be catalytically important, and amino acids in positions with high sequence variability that are catalytically important, have been observed. There are several possible reasons why the correlation between the calculated sequence variability and importance of the amino acid in the catalytic mechanism of

tyrosyl-tRNA synthetase is not better. First, at positions 231 and 232, there may be a restricted set of amino acids that are allowed (and consequently a low sequence variability). As only one mutant at each position was tested, restrictions in the set of allowable amino acids cannot be determined. Second, specific positions may exhibit a low sequence variability due to their importance in the second step of the catalytic mechanism, the aminoacylation of tRNA. Third, the functional or evolutionary differences between the aminoacyl-tRNA synthetases may necessitate variations on the catalytic mechanism for aminoacyl-adenylate formation. This last point may explain the high sequence variability observed at position 235. Perhaps in tyrosyl-tRNA synthetase, E235 has evolved as a way of fine-tuning the basic catalytic mechanism involving K230, K233, and T234.

*Summary of the Involvement of the Mobile Loop in Catalysis of Tyrosyl Adenylate Formation.* At this point, it is useful to summarize what has been learned about the mobile loop in these, and previous, investigations. First, the loop interacts with four of the complexes in the formation of tyrosyl adenylate. Specifically, it is not involved in stabilizing the E·Tyr complex, it destabilizes the E·Tyr·ATP complex, it stabilizes the E·[Tyr-ATP]$^*$ complex, it stabilizes the E·Tyr-AMP·$PP_i$ complex, and it appears to affect the stability of the E·Tyr-AMP complex. Although the precise nature of the interaction between the loop and the tyrosyl adenylate intermediate remains to be elucidated, the involvement of the loop at this stage in the reaction suggests that it may also play a role in the transfer of tyrosine to tRNA. Second, in terms of energetics, the stabilization of the E·[Tyr-ATP]$^*$ complex is achieved at the expense of ATP-binding energy. Third, it has been shown that three residues, K230, K233, and T234, are primarily responsible for the interaction between the loop and the tyrosyl adenylate transition state. The remaining residues in the loop stabilize the E·[Tyr-ATP]$^*$ complex by between 0.4 and 1 kcal/mol each. Fourth, T234 interacts with the $Mg^{2+}$ ion in the E·[Tyr-ATP]$^*$ complex. The precise nature of this interaction is not known. Fifth, the sum of the transition state stabilization due to K230, K233, and T234 does not equal the net effect of the loop on the stability of the transition state, implying that significant energetic coupling occurs between these three residues during formation of the transition state. This is consistent with a model in which the mobile loop becomes conformationally constrained in the E·[Tyr-ATP]$^*$ complex. Finally, alteration of the affinity of the active-site for ATP results in a shift from noncooperative to cooperative ATP-binding. Thus, the mobile loop in one subunit is tightly coupled to the active site in the other subunit.

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