Comparison of the Catalytic Roles Played by the KMSKS Motif in the Human and Bacillus stearothermophilus Tyrosyl-tRNA Synthetases*

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The Class I aminoacyl-tRNA synthetases are characterized by two signature sequence motifs, “HIGH” and “KMSKS.” In Bacillus stearothermophilus tyrosyl-tRNA synthetase, the KMSKS motif (220KFGGKT234) has been shown to stabilize the transition state for tyrosine activation through interactions with the pyrophosphate moiety of ATP. In most eukaryotic tyrosyl-tRNA synthetases, the second lysine in the KMSKS motif is replaced by a serine or an alanine residue. Recent kinetic studies indicate that potassium functionally compensates for the absence of the second lysine in the human tyrosyl-tRNA synthetase (222KKSSS226). In this paper, site-directed mutagenesis and pre-steady state kinetics are used to determine the roles that serines 224, 225, and 226 play in catalysis of the tyrosine activation reaction. In addition, the catalytic role played by a downstream lysine conserved in eukaryotic tyrosyl-tRNA synthetases, Lys-231, is investigated. Replacing Ser-224 and Ser-226 with alanine decreases the forward rate constant 7.5- and 60-fold, respectively. In contrast, replacing either Ser-225 or Lys-231 with alanine has no effect on the catalytic activity of the enzyme. These results are consistent with the hypothesis that the KMSSS sequence in human tyrosyl-tRNA synthetase stabilizes the transition state for the tyrosine activation reaction by interacting with the pyrophosphate moiety of ATP. In addition, although they play similar roles in catalysis, the overall contribution of the KMSKS motif to catalysis appears to be significantly less in human tyrosyl-tRNA synthetase than it is in the B. stearothermophilus enzyme.

Aminoacyl-tRNA synthetases (AARS) catalyze the attachment of amino acids (AA) to their cognate tRNA\textsuperscript{AA} by an ATP-dependent two-step reaction mechanism. In the first step (Equation 1), the amino acid is activated by MgATP to form an enzyme-bound aminoacyl-adenylate intermediate. The second step (Equation 2) consists of the transfer of the amino acid to the 3’ end of its cognate tRNA\textsuperscript{AA}.

\[
\text{AARS} + \text{AA} + \text{MgATP} \rightleftharpoons \text{AARS-\text{AA} - AMP + PPi} \quad \text{(Eq. 1)}
\]

\[
\text{AARS-\text{AA} - AMP + tRNA} \rightleftharpoons \text{AARS + AA - tRNA} + \text{AMP} \quad \text{(Eq. 2)}
\]

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The Class I aminoacyl-tRNA synthetase family, of which tyrosyl-tRNA synthetase is a member, is characterized by the presence of an amino-terminal Rossmann-fold catalytic domain and conserved HIGH and KMSKS signature sequences (1–9). The KMSKS signature sequence in the Bacillus stearothermophilus tyrosyl-tRNA synthetase (220KFGGKT234) participates in catalysis of the tyrosine activation reaction (10–15). Specifically, Lys-230, Lys-233, and Thr-234 stabilize the transition state by interacting with the pyrophosphate moiety of the ATP substrate (11–15). In the human tyrosyl-tRNA synthetase Gly-232, Lys-233, and Thr-234 are replaced with serine residues (222KKSSS226) (16). The absence of a second lysine in the KMSSS sequence in human tyrosyl-tRNA synthetase, which is the most highly conserved amino acid in the Class I aminoacyl-tRNA synthetase family (17), and the observation that the catalytic efficiency of human tyrosyl-tRNA synthetase is similar to that of the B. stearothermophilus enzyme (18) raises the question of how human tyrosyl-tRNA synthetase compensates for the absence of the second lysine in the KMSKS signature sequence. Recently, it was shown that in the human tyrosyl-tRNA synthetase, potassium stabilizes the transition state for tyrosine activation by interacting with the pyrophosphate moiety of ATP (19). Based on these observations, it was concluded that in human tyrosyl-tRNA synthetase, potassium functionally replaces the second lysine in the KMSKS signature sequence. In this paper, site-directed mutagenesis and pre-steady state kinetic methods are used to determine whether other amino acids in the KMSSS loop of human tyrosyl-tRNA synthetase help compensate for the absence of the second lysine. Specifically, the roles of the three serine residues in the KMSSS sequence, Ser-224, Ser-225, and Ser-226, play in the catalysis of tyrosine activation are investigated. In addition, the hypothesis that a downstream lysyl residue, Lys-231, which is conserved in eukaryotic tyrosyl-tRNA synthetases, also helps compensate for the absence of the second lysine in the KMSKS signature sequence is tested. The results presented in this paper indicate that of these four residues only Ser-224 and Ser-226 are involved in catalysis of the tyrosine activation reaction. Furthermore, the dissociation constant for potassium is not affected by replacing the serine residues in the KMSSS sequence of human tyrosyl-tRNA synthetase with alanine. These observations are consistent with the hypothesis that the KMSSS sequence in human tyrosyl-tRNA synthetase stabilizes the transition state for tyrosine activation through interactions with the pyrophosphate moiety of ATP. Quantitative analysis of tyrosyl-tRNA synthetase variants indicates that, although the KMSKS signature sequences play similar roles in the catalytic mechanisms of the B. stearothermophilus and human tyrosyl-tRNA synthetases, the extent to which the KMSSS sequence affects catalysis in the human enzyme is...
significantly less than that of the homologous sequence in the *B. stearothermophilus* tyrosyl-tRNA synthetase. This difference in the catalytic mechanisms of the *B. stearothermophilus* and human tyrosyl-tRNA synthetases potentially could be used to design inhibitors that selectively target the bacterial enzyme.

**Experimental Procedures**

**Materials**—Reagents were purchased from the following sources: nickel-nitrilotriacetic acid resin and 5 mL of polypropylene columns (Qiagen); l-[14C]tyrosine (Moravek Biochemicals); inorganic pyrophosphate and tetrasodium pyrophosphate (Sigma); nitrocellulose (Schleicher & Schuell); EMD-103 membranes and NAP-25 columns (Amersham Biosciences); Taq and Pfu DNA polymerases, T4 DNA ligase, Wizard PCR prep, and DNA Cleanup Systems (Promega); NuSieve low melting point agarose (ISC BioExpress); XL1 Blue competent cells (Stratagene); oligonucleotides (Operon and Invitrogen); and pET30a vector (Novagen). Automated DNA sequencing was performed by the DNA sequencing facility at Arizona State University. All other reagents were purchased from Fisher Scientific.

**Site-directed Mutagenesis, Purification of the Wild-type and Variant Human Tyrosyl-tRNA Synthetases, and Standard Buffer**—All tyrosyl-tRNA synthetase variants were created by the PCR-mediated overlap-extension method (20). The T7SS3 wt plasmid, a derivative of the pET30a (+) cloning vector containing the wild-type human tyrosyl-tRNA synthetase gene (16), was used as the template for the initial PCR mutagenesis reactions. The following oligonucleotides were used to introduce the desired mutations and to remove a SacI restriction site (the mutated nucleotides are in italics): (S224A) 5'-GGA CTC CTC TTC TGA AGC TGC TGT TAA TCC-3'; (S225A) 5'-GGA CTC CTC TTC TGA GCC GCT CAT TTT GCC-3'; (K231A) 5'-GGA CTC CTC TTC TGA ACT CAT TTT GCC-3'; (K231A-fwd) 5'-GAA GAG GAG GAG GCC GCC GCT ATG CTC TAT GCC-3'; and (K231A-rvs) 5'-GGA CTC CTC TTC TGA AGC ATG CAT TTT GCC-3'. One additional primer, 5'-CAA GAG GAG GAG GCC GCC GCC TGC CAT ATG CTC-3', was used as an internal primer, and T7 promoter and T7 terminator primers were used as outside primers. The removal of the SacI site did not alter the amino acid sequences of the final products. All of the PCR products were purified with the Wizard PCR prep DNA Purification and DNA Cleanup Systems from Promega. The final PCR products were digested with the KpnI and HindIII restriction enzymes and subcloned into the pET30a (+) vector that had been digested with the same two restriction enzymes. Positive clones were selected by PCR amplification of the human tyrosyl-tRNA synthetase coding sequence followed by digestion with the SacI restriction enzyme to ensure that the SacI site was removed. The tyrosyl-tRNA synthetase coding sequence of each variant was then sequenced by automated DNA sequencing to ensure that no secondary mutations had occurred. Purification of the wild-type and variant human tyrosyl-tRNA synthetases has been described previously (16, 18). Briefly, the wild-type and tyrosyl-tRNA synthetase variants were expressed with a removable amino-terminal His tag/S tag. The cells were lysed by sonication in buffer A (50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole, and 1 mM phenylmethanesulfonyl fluoride, pH 8.0), and the cellular debris was removed by centrifugation. The cleared lysate was then loaded onto a nickel-nitrilotriacetic acid gravity flow column equilibrated with buffer A, and the human tyrosyl-tRNA synthetase was eluted with buffer A containing 250 mM imidazole. The peak fractions were pooled and dialyzed against the standard buffer (minus KCl) and 2 units/ml inorganic pyrophosphatase. The peak eluting at 140 mM NaCl was collected and dialyzed against two changes of buffer C (50 mM Tris, pH 7.5, 2 mM NaCl, 10 mM MgCl₂, and finally against buffer C plus 10% glycerol (v/v)). SDS-PAGE analysis was used to assess the purity of the purified enzymes (21). The concentration of the enzyme was determined using a filter-based active site titration assay as described in Ref. 22 and measurements in the presence of 6 M guanidine hydrochloride (e = 89040 m⁻¹ cm⁻¹ as determined by the ExPASy ProtParam tool (23)). The enzyme was stored at −70 °C. Unless stated otherwise, all kinetic experiments were performed in a standard buffer solution containing 150 mM Tris–Cl, pH 7.5, 20 mM 2-mercaptoethanol, 10 mM MgCl₂, and 150 mM KCl.

**Equilibrium Dialysis**—Equilibrium dialysis was performed using a modification of the method previously described in Ref. 22. Briefly, 35–40 μM tyrosyl-tRNA synthetase, in the standard buffer, was placed in one chamber (chamber A) of each cell in an eight-cell equilibrium dialyzer (Hoeffer). The other chamber (chamber B) of each cell contained concentrations of l-[14C]tyrosine ranging from 2 to 200 μM in the same buffer. After overnight dialysis at 4 °C, the l-[14C]tyrosine concentrations in each chamber were determined by removing 40-μl aliquots, adding each aliquot to 5 ml of cytoscint (Fish er), and counting in a Beckman LS 6500 scintillation counter. The concentration of tyrosine in each chamber was calculated from the specific activity of the stock l-[14C]tyrosine (3 μl of the 300 μM stock l-[14C]tyrosine was counted to determine the specific activity). The concentrations of enzyme-bound and free tyrosine were calculated by subtracting the tyrosine concentration in chamber B (Ffree) from that in chamber A (Fbound + Ffree). The data were analyzed by both nonlinear and linear curve fitting using Equations 3 and 4:

\[
\frac{[Tyrs]\text{bound}}{[Tyrs]} = \frac{1}{K_{Tyr}} + \frac{n[E]}{K_{Tyr}} \quad (\text{Eq. 3})
\]

\[
\frac{[Tyrs]\text{bound}}{[Tyrs]} = 1 - \frac{1}{K_{Tyr}} \frac{n[E]}{K_{Tyr}} \quad (\text{Eq. 4})
\]

where \( a = [Tyrs]\text{bound}/[E] \), \( K_{Tyr} \) is the dissociation constant for tyrosine, \( [Tyrs]\text{bound} \) is the concentration of tyrosine bound to the enzyme, \( [Tyrs] \) is the concentration of unbound tyrosine at equilibrium, \( n \) is the number of active sites, and \( [E] \) is the total enzyme concentration as determined by A_{280} (24, 25).

**Kinetic Analyses**—All kinetic analyses were performed in the standard buffer + KCl at 25 °C.Stopped-flow fluorescence studies were used to monitor the ATP dependence of tyrosyl-adenylate formation (i.e., the forward reaction) and the pyrophosphate dependence of the reverse reaction (i.e., the conversion of E + Tyr-AMP + PP to E + Tyr + ATP) in the pre-steady-state (26). In these studies an SX 18.MV stopped-flow spectrophotometer (Applied Photophysics) was used to monitor the increase in fluorescence associated with the reverse reaction (λₘₐₓ = 295 nm, λₐₜₜ > 320 nm) (18). In the ATP dependence experiments, one of the syringes of the stopped-flow instrument contained the enzyme (0.3–0.5 μM), while the other syringe contained varying concentrations of MgATP (0.8–25 μM). Both syringes contained pyrophosphatase, 200 μM tyrosine, and the standard buffer. Upon mixing equal volumes from each syringe the decrease in the intrinsic fluorescence of the protein was monitored over time. For analysis of the reverse reaction, the TyrRS + Tyr-AMP complex was formed by incubating the enzyme in the standard buffer (minus KCl) and 2 units/ml inorganic pyrophosphatase with 100 μM tyrosine and 10 mM MgATP at 25 °C for 1 h (27). The Tyr-AMP produced from free ATP by gel filtration on a NAP-25 column (Amersham Biosciences) (27). The reverse reaction was monitored by mixing varying concentrations of tetrasodium-pyrophosphate (0.1–0.8 μM) with the TyrRS + Tyr-AMP complex (0.3 μM) in the standard buffer containing 75 mM KCl at 25 °C and measuring the increase in the intrinsic fluorescence of the protein (18, 26). Due to the low solubility of MgPP i, it was not possible to achieve saturating pyrophosphate concentrations for the reverse reaction. As a result, it was not possible to determine kₖₐₜ, independently of Kₕₚ. Instead, the reverse reaction was monitored under conditions where Kₕₚ >> [PP] and kₖₐₜ/Kₕₚ were determined by fitting the data to the resulting linear approximation of Equation 5. In the above calculations, a rapid equilibrium assumption is used for the binding of the substrates (18). All kinetic data were fit to a single exponential fitting end point equation using the Kaleidagraph and Applied Photophysics stopped-flow software packages to determine the observed rate constants. The Kaleidagraph software was used to plot the observed rate constants versus the substrate concentrations and to fit these plots to the following hyperbolic function (Equation 5)

\[
k_{obs} = \frac{k_{cat}}{K_{M} + [S]} \quad (\text{Eq. 5})
\]

where \( k_{obs} \) is the observed rate constant, \( k_{cat} \) is the either the forward rate constant (kₖₐₜ) or the reverse rate constant (kₙₜ) for the tyrosine activation reaction, [S]ₘₜ is the total substrate concentration, and Kₕₚ is the dissociation constant for tyrosine. 1

\[1\] The abbreviations used are: TyrRS, tyrosyl-tRNA synthetase; \( ^* \), non-covalent bond; \( ^*^* \), covalent bond.
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Determination of the Dissociation Constant for Tyrosine (K_{Tyr})—The reaction mechanism for tyrosine activation is shown in Scheme I. To determine whether serines 224, 225, 226, and lysine 231 interact with the tyrosine substrate, alanine variants at each of these positions were generated and the dissociation constant for tyrosine in the absence of ATP (K_{Tyr}) was determined for each variant using equilibrium dialysis. As shown in Table I, each of the variants bound tyrosine with an affinity similar to that of the wild-type enzyme (34 \mu M).

Determination of the Dissociation Constant of ATP (K_{ATP}) and the Forward Rate Constant (k_3)—To determine whether serines 224, 225, 226, and lysine 231 interact with the ATP substrate and/or are involved in catalysis of the tyrosine activation reaction, alanine variants at each of these positions were analyzed using pre-steady state kinetic methods. The dissociation constant for ATP in the presence of saturating tyrosine (K_{ATP}) and the forward rate constant (k_3) were determined from the ATP dependence of the tyrosine activation reaction in the presence of saturating tyrosine (200 \mu M). As shown in Fig. 1 and summarized in Table I, K_{ATP} for these variants is similar to that observed for the wild-type enzyme. In contrast, the forward rate constant for tyrosine activation (k_3) is reduced 8- and 60-fold for the S224A and S226A variants, respectively. The S225A and K231A substitutions display forward rate constant values similar to that of the wild-type enzyme.

Determination of the Specificity Constant for the Reverse Reaction (k_4/K_{PP})—Pre-steady state kinetic analyses of the reverse reaction for the S224A, S225A, and S226A variants were performed. Due to the low solubility of MgPP_i, it is not possible to determine the reverse rate constant (k_4) and the dissociation constant for pyrophosphate (K_{PP}) independently of each other. Under conditions where [PP_i] \ll K_{PP}, however, Equation 5 can be approximated by a linear equation whose slope is given by the specificity constant (k_4/K_{PP}). As a result, even though k_4 and K_{PP} cannot be determined independently of each other, the specificity constant can be determined by fitting a plot of k_{obs} versus [PP_i] to a linear equation. As shown in Fig. 2 and summarized in Table I, k_4/K_{PP} was reduced -12- and 100-fold for the S224A and S226A variants, respectively, whereas the S225A substitution has only a small effect on the value of k_4/K_{PP}. Due to its lack of effect on K_{PP}, the specificity constant for the reverse reaction was not determined for the K231A variant.

Potassium Does Not Interact with the Serine Residues of the K MSSS Sequence—Unlike bacterial tyrosyl-tRNA synthetases, tyrosine activation by the human tyrosyl-tRNA synthetase is potassium-dependent. To determine whether potassium interacts with the serine residues in the K MSSS sequence, pre-steady state kinetic methods were used to determine whether the alanine variants display altered affinities for potassium relative to that of the wild-type enzyme. The dissociation constant for potassium was determined from the potassium dependence of the tyrosine activation reaction in the presence of saturating tyrosine (200 \mu M) and ATP (10 mM). All three of the variants bind potassium with affinities similar to that observed for the wild-type tyrosyl-tRNA synthetase, indicating that the K MSSS sequence is not involved in binding the potassium ion (Fig. 3, Table I).

Free Energy Calculations for Each Step Along the Reaction Pathway—The binding and rate constants summarized in Table I were used to calculate the standard free energy values for each step along the reaction pathway for the S224A, S225A, S226A, and K231A variants. For the S225A and K231A variants, the standard free energies of each complex are similar to those observed for the wild-type enzyme. For this reason, only the free energy profile for the S225A variant is shown (Fig. 4, B). In contrast, the standard free energy values of the transition state complexes for the S224A and S226A variants are 1.5 and 2.6 kcal/mol higher, respectively, than the value for the wild-type enzyme (Fig. 4, A and C). In addition, the activation energy is increased 1.3 and 2.4 kcal/mol by the S224A and S226A substitutions, respectively. The results of the S226A substitution are similar to those observed for the T234A substitution in the B. stearothermophilus enzyme, which increases the free energy for the transition state by 2.8 kcal/mol and increases the activation energy by 3.8 kcal/mol (14).

DISCUSSION

The K MSSS Sequence Catalyzes the Formation of Tyrosyl-Adenylate by Interacting with the Pyrophosphate Moiety of ATP—In the B. stearothermophilus tyrosyl-tRNA synthetase sequence, three residues, Lys-230, Lys-233, and Thr-234, stabilize the transition state for tyrosine activation by interacting with the pyrophosphate moiety of the ATP substrate (11–15). In the human tyrosyl-tRNA synthetase, Lys-230 is conserved, whereas Gly-232, Lys-233, and Thr-234 are replaced with Ser-224, Ser-225, and Ser-226, respectively (16). Previous studies indicate that Gly-232 in the B. stearothermophilus tyrosyl-tRNA synthetase can be replaced by alanine without significantly destabilizing the transition state complex (12). The results presented in this study indicate that replacement of the corresponding residue in the human tyrosyl-tRNA synthetase, Ser-224, by alanine destabilizes the transition state for the tyrosine activation reaction by 1.5 kcal/mol. The observation that the S224A substitution does not affect the stabilities of the TyrRS•Tyr, TyrRS•Tyr•ATP, or TyrRS•Tyr•AMP complexes suggests that Ser-224 interacts with the pyrophosphate moiety of the ATP substrate and that this interac-
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TABLE I
Dissociation and rate constants for each of the tyrosyl-tRNA synthetase variants

| Enzyme       | $K_{\text{TyT}}$ (µM) | $K'_{\text{ATP}}$ (µM) | $k_3$ (s⁻¹) | $k_{\text{acy}}/K_{\text{TyT}}$ (s⁻¹ µM⁻¹) | $K_{\text{ac}}$ (µM) |
|--------------|------------------------|-------------------------|-------------|------------------------------------------|---------------------|
| Wild-type    | 34 (±8)                | 4.0 (±0.1)              | 45 (±4)     | 58000 (±7000)                            | 32 (±2)             |
| S224A        | 50 (±5)                | 4.0 (±1.0)              | 6 (±2)      | 5000 (±100)                              | 24 (±1)             |
| S225A        | 21 (±5)                | 3.0 (±0.3)              | 31 (±1)     | 30000 (±4000)                            | 30 (±5)             |
| S226A        | 42 (±2)                | 4.1 (±0.1)              | 0.78 (±0.01)| 600 (±40)                                | 22 (±1)             |
| K231A        | 30 (±5)                | 3.0 (±0.2)              | 30 (±1)     | ND                                       | ND                  |

* The values for the wild-type enzyme are taken from Ref. 18.

# Standard deviations for three independent repetitions of each experiment are indicated in parentheses.

FIG. 1. Pre-steady state kinetic analysis of the tyrosine activation reaction. Typical plots of $k_{\text{obs}}$ versus substrate concentration for the MgATP dependence of tyrosine activation by the S224A (filled circle), S225A (filled square), and S226A (filled diamond) variants of the human tyrosyl-tRNA synthetase are shown (0.5 µM enzyme, 200 µM tyrosine, and 0.8–25 mM MgATP). The plots are fit to Equation 5.

FIG. 2. Pre-steady state kinetic analysis of the reverse reaction. Typical plots of $k_{\text{obs}}$ versus substrate concentration for the pyrophosphate dependence of the reverse reaction for the S224A (filled circle), S225A (filled square), and S226A (filled diamond) variants of the human tyrosyl-tRNA synthetase are shown (0.25 µM TyrRS•Tyr•AMP, and 0.08–0.4 mM tetrasiomium pyrophosphate). The plots are fit to the linear approximation of Equation 5 under conditions where [PPi]/[E] ≪ $K_{\text{PPi}}$.

FIG. 3. Potassium dependence of tyrosine activation reaction. The effects that the S224A (filled circle), S225A (filled square), and S226A (filled triangle) variants of the human tyrosyl-tRNA synthetase have on the potassium dependence of the tyrosine activation reaction are shown. Typical $k_{\text{obs}}$ versus substrate concentration plots for the potassium dependence of tyrosine activation in the presence of 200 µM tyrosine and 10 mM MgATP are shown (0.5 µM enzyme and 5–150 mM KCl). The plots are fit to Equation 5.

introduces positive cooperativity into the enzyme with respect to the binding of ATP (13). It is postulated that this cooperativity is always present in B. stearothermophilus tyrosyl-tRNA synthetase but is only uncovered in the K233A variant due to the lower affinity of this variant for ATP. The addition of 0.5 M NaCl to the reaction mixture restores the high affinity binding of ATP to the active site and abolishes the cooperative kinetics (13). In the presence of NaCl, the primary effect of replacing Lys-233 with alanine is a 50-fold decrease in the forward rate constant. These results are consistent with the hypothesis that Lys-233 stabilizes the transition state for tyrosine activation primarily through interactions with the pyrophosphate moiety of ATP (13). Replacement of the equivalent residue in human tyrosyl-tRNA synthetase, Ser-225, with alanine has little effect on the catalytic activity.

In contrast to serines 224 and 225 in human tyrosyl-tRNA synthetase, whose roles differ from the corresponding residues in B. stearothermophilus tyrosyl-tRNA synthetase, the role of Ser-226 in the human enzyme is similar to its counterpart (Thr-234) in B. stearothermophilus tyrosyl-tRNA synthetase. Specifically, replacement of Ser-226 with alanine in the human enzyme results in a 60-fold decrease in the forward rate constant for tyrosine activation, whereas replacement of Thr-234 by alanine in B. stearothermophilus tyrosyl-tRNA synthetase decreases the forward rate constant for tyrosine activation by 540-fold (13). In addition, replacement of Thr-234 by alanine in B. stearothermophilus tyrosyl-tRNA synthetase increases the affinity of the enzyme for ATP 3-fold, whereas replacement of Ser-226 with alanine has little effect on the initial binding of ATP to the human tyrosyl-tRNA synthetase. Thus, although their roles are similar, Thr-234 is significantly more important in the catalytic mechanism of B. stearothermophilus tyrosyl-tRNA synthetase than its counterpart, Ser-226, in the catalytic mechanism of the human enzyme. It is apparent from the kinetic analyses presented in this paper that, although the

# For the B. stearothermophilus tyrosyl-tRNA synthetase, Fersht et al. (26) have correlated the results of stopped-flow fluorescence experiments with data obtained from quenched-flow and pyrophosphate exchange assays to demonstrate that the change in the intrinsic fluorescence of the enzyme corresponds to the chemicalstep of the reaction. Whereas it is possible that the intrinsic fluorescence changes we observe for the human tyrosyl-tRNA synthetase correspond to precatalytic conformational changes, the similarity between the rate and dissociation constants for the B. stearothermophilus and human tyrosyl-tRNA synthetases suggests that this is not the case (18). The observation that the S224A and S226A variants of human tyrosyl-tRNA synthetase affect the observed rate constants for both the forward and reverse reactions supports this assumption.

# Dissociation and rate constants for each of the tyrosyl-tRNA synthetase variants
KMSKS motifs in the human and *B. stearothermophilus* tyrosyl-tRNA synthetases play similar roles, the extent to which KMSSS catalyzes the formation of tyrosyl-adenylate in the human enzyme is significantly less than that of its counterpart in *B. stearothermophilus* tyrosyl-tRNA synthetase.

**Potassium Does Not Interact with the KMSSS Sequence in the Human Tyrosyl-tRNA Synthetase**—If the KMSSS sequence in human tyrosyl-tRNA synthetase is less important in catalysis than its counterpart in the *B. stearothermophilus* enzyme, why do the two enzymes display similar kinetic properties? Previous investigations indicate that the loss of catalytic function by the KMSSS sequence is compensated for by the involvement of potassium in the catalytic mechanism of human tyrosyl-tRNA synthetase (19). Specifically, potassium has been shown to functionally compensate for the absence of the second lysine in the KMSSS sequence (19). Like Ser-224 and Ser-226, potassium stabilizes the transition state for tyrosine activation by interacting with the pyrophosphate moiety of ATP. These observations raise the question of whether potassium interacts with the serine residues in the KMSSS signature sequence in the human tyrosyl-tRNA synthetase. The replacement of either of these serine residues with alanine did not decrease the binding affinity of the human tyrosyl-tRNA synthetase for potassium, indicating that the KMSSS sequence does not form part of the potassium-binding site.

**Why Is the Second Lysine Absent from the KMSSSS Sequence?**—In eukaryotic tyrosyl- and tryptophanyl-tRNA synthetases, the second lysine in the KMSKS motif is replaced by either a serine or an alanine residue (28, 29). Given the role that Lys-233 plays in the catalysis of tyrosine activation in the *B. stearothermophilus* enzyme it is interesting that it is not conserved in the human tyrosyl-tRNA synthetase. In addition, Lys-233, which corresponds to the second lysine of the KMSKS signature sequence, is the most highly conserved residue among all Class I aminoacyl-tRNA synthetases (17). The observation that its replacement in the human enzyme, Ser-225, does not play a significant role in the catalysis of tyrosine activation raises the question of why this variation occurs in nature. In particular, given the ability of this lysine to stabilize the transition state for tyrosine activation, it is curious that, though the KMSKS motifs play similar roles in the two enzymes, the extent to which the KMSKS motif catalyzes the formation of tyrosyl-adenylate is significantly larger in the *B. stearothermophilus* synthetase. This hypothesis is currently being investigated.

**Concluding Remarks**—In this paper we provide evidence that the KMSSS sequence in human tyrosyl-tRNA synthetase is less important in catalysis than its counterpart in the *B. stearothermophilus* enzyme. The second lysine in the KMSSS motif does not play a significant role in the catalytic mechanism of the human tyrosyl-tRNA synthetase. This study suggests that inhibitors that interact with this motif may be effective in blocking the catalytic activity of the human enzyme, thereby potentially providing a therapeutic approach for the treatment of tyrosyl-tRNA synthetase-related disorders.
tif will have higher affinities for bacterial tyrosyl-tRNA synthetases than for their eukaryotic homologs.

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REFERENCES
1. Zelwer, C., Risler, J. L., and Brunie, S. (1982) J. Mol. Biol. 155, 63–81
2. Webster, T., Tsai, H., Kula, M., Mackie, G. A., and Schimmel, P. (1984) Science 226, 1315–1317
3. Hountondji, C., Dessen, P., and Blanquet, S. (1986) Biochimie (Paris) 68, 1071–1078
4. Brick, P., and Blow, D. M. (1987) J. Mol. Biol. 194, 287–297
5. Brick, P., Bhut, T. N., and Blow, D. M. (1989) J. Mol. Biol. 208, 83–98
6. Brunie, S., Zelwer, C., and Risler, J. L. (1990) J. Mol. Biol. 216, 411–424
7. Eriani, G., Delarue, M., Poch, O., Gangloff, J., and Moras, D. (1990) Nature 347, 203–206
8. Moras, D. (1992) Trends Biochem. Sci. 17, 159–164
9. Rould, M. A., Perona, J. J., Soll, D., and Steitz, T. A. (1989) Science 246, 1135–1142
10. Winter, G., Koch, G. L., Hartley, B. S., and Barker, D. G. (1983) Eur. J. Biochem. 132, 383–387
11. Fersht, A. R., Knill-Jones, J. W., Bedouelle, H., and Winter, G. (1988) Biochemistry 27, 1581–1587
12. First, E. A., and Fersht, A. R. (1993) Biochemistry 32, 13658–13663
13. First, E. A., and Fersht, A. R. (1993) Biochemistry 32, 13651–13657
14. First, E. A., and Fersht, A. R. (1993) Biochemistry 32, 13644–13650
15. First, E. A., and Fersht, A. R. (1995) Biochemistry 34, 5030–5043
16. Kleeman, T. A., Wei, D., Simpson, K. L., and First, E. A. (1997) J. Biol. Chem. 272, 14420–14425
17. Landes, C., Perona, J. J., Brunie, S., Rould, M. A., Zelwer, C., Steitz, T. A., and Risler, J. L. (1995) Biochimie (Paris) 77, 194–203
18. Austin, J., and First, E. A. (1992) J. Biol. Chem. 277, 14812–14820
19. Austin, J., and First, E. A. (1992) J. Biol. Chem. 277, 29243–29248
20. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Gene 77, 51–59
21. Laemmli, U. K. (1970) Nature 227, 680–685
22. Fersht, A. R. (1975) Biochemistry 14, 5–12
23. Appel, R. D., Bairoch, A., and Hochstrasser, D. F. (1994) Trends Biochem. Sci. 19, 254–260
24. Engel, P. C. (1996) Enzymology Labfax, 1st Ed., pp. 200–210, Academic Press, Inc., San Diego, CA and BIOS Scientific Publishers Ltd., Oxford, U. K.
25. Scatchard, G. (1949) Ann. N. Y. Acad. Sci. 51, 660–672
26. Fersht, A. R., Mulvey, R. S., and Koch, G. L. (1975) Biochemistry 14, 13–18
27. Wells, T. N., and Fersht, A. R. (1986) Biochemistry 25, 1881–1886
28. Ribas de Pouplana, L., Frugier, M., Quinn, C. L., and Schimmel, P. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 166–170
29. Brown, J. R., Robb, F. T., Weiss, R., and Doolittle, W. F. (1997) J. Mol. Evol. 45, 9–16