Activation of D-Tyrosine by Bacillus stearothermophilus Tyrosyl-tRNA Synthetase

1. COOPERATIVE BINDING OF ATP IS LIMITED TO THE INITIAL TURNOVER OF THE ENZYME*

Received for publication, February 29, 2008. Published, JBC Papers in Press, March 4, 2008, DOI 10.1074/jbc.M801650200

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The activation of D-tyrosine by tyrosyl-tRNA synthetase has been investigated using single and multiple turnover kinetic methods. In the presence of saturating concentrations of D-tyrosine, the activation reaction displays sigmoidal kinetics with respect to ATP concentration under single turnover conditions. In contrast, when the kinetics for the activation reaction are monitored using a steady-state (multiple turnover) pyrophosphate exchange assay, Michaelis-Menten kinetics are observed. Previous investigations indicated that activation of L-tyrosine by the K233A variant of Bacillus stearothermophilus tyrosyl-tRNA synthetase displays sigmoidal kinetics similar to those observed for activation of D-tyrosine by the wild-type enzyme. Kinetic analyses indicate that the sigmoidal behavior of the D-tyrosine activation reaction is not enhanced when Lys-233 is replaced by alanine. This supports the hypothesis that the mechanistic basis for the sigmoidal behavior is the same for both D-tyrosine activation by wild-type tyrosyl-tRNA synthetase and activation of L-tyrosine by the K233A variant. The observed sigmoidal behavior presents a paradox, as tyrosyl-tRNA synthetase displays an extreme form of negative cooperativity, known as “half-of-the-sites reactivity,” with respect to tyrosine binding and tyrosyl-adenylate formation. We propose that the binding of D-tyrosine weakens the affinity with which ATP binds to the functional subunit in tyrosyl-tRNA synthetase. This allows ATP to bind initially to the nonfunctional subunit, inducing a conformational change in the enzyme that enhances the affinity of the functional subunit for ATP. The observation that sigmoidal kinetics are observed only under single turnover conditions suggests that this conformational change is stable over multiple rounds of catalysis.

Ribosomal protein synthesis is stereospecific, with only the L-stereoisomer being incorporated into the growing polypeptide chain. It was therefore surprising when Calendar and Berg (1) demonstrated that tyrosyl-tRNA synthetases (TyrRSs)2 from Escherichia coli and Bacillus subtilis catalyze the activation and attachment of D-tyrosine to tRNA\textsuperscript{\text{Tyr}} only 1–2 orders of magnitude less efficiently than for L-tyrosine. To further characterize the activation of D-tyrosine by tyrosyl-tRNA synthetase, we have used stopped-flow fluorescence spectroscopy to analyze the single turnover kinetics of this reaction (see accompanying paper (Ref. 32)). During the course of these investigations, we observed sigmoidal kinetics for the binding of ATP at saturating concentrations of D-tyrosine. This observation is reminiscent of a previous observation in which the K233A variant of Bacillus stearothermophilus tyrosyl-tRNA synthetase displays sigmoidal kinetics with respect to the binding of ATP (2).

The observation that tyrosyl-tRNA synthetase exhibits sigmoidal binding with respect to ATP in the D-tyrosine activation reaction presents a paradox. Analysis of tyrosine binding and tyrosyl-adenylate formation by equilibrium dialysis and active site titration clearly indicates that, as is the case for the activation of L-tyrosine, tyrosyl-tRNA synthetase displays half-of-the-sites reactivity with respect to both the binding of D-tyrosine and formation of the enzyme-bound D-Tyr-AMP intermediate. The observation that tyrosyl-tRNA synthetase exhibits sigmoidal binding with respect to ATP, however, suggests that the binding of ATP displays positive cooperativity. This raises the question “how can an enzyme with only one functional active site (because of half-of-the-sites reactivity) display positive cooperativity with respect to the binding of one of its substrates?” In the case of the K233A variant of B. stearothermophilus tyrosyl-tRNA synthetase, it was postulated that there is an intrinsic cooperativity between the two subunits of the tyrosyl-tRNA synthetase dimer with respect to the binding of ATP (2). In the wild-type enzyme, this intrinsic cooperativity is not observed because ATP binds initially to the functional subunit. Because the tyrosine activation assay monitors formation of the TyrRS-Tyr-AMP intermediate, any subsequent binding of ATP to the nonfunctional subunit will not be observed. In the tyrosyl-tRNA synthetase K233A variant, however, the affinity of the functional subunit for ATP is decreased, allowing it to bind initially to the inactive subunit. Because ATP binds initially to the inactive subunit, it is possible to observe the effect that binding of ATP to the inactive subunit has on the activation of tyrosine at the functional subunit. In other words, in the K233A tyrosyl-tRNA synthetase variant, the initial binding of ATP to the inactive subunit permits the binding of ATP to both subunits to be observed by monitoring formation of the tyrosyl-adenylate intermediate.

* This work was supported, in whole or in part, by National Institutes of Health Grant GM68070 from NIGMS. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: TyrRS, tyrosyl-tRNA synthetase; PP\textsubscript{i}, pyrophosphate; MESG, 2-amino-6-mercapto-7-methylpurine riboside.
D-Tyrosine Induces Cooperative ATP Binding in TyrRS

In this study, we test the hypothesis that the sigmoidal binding of ATP during D-tyrosine activation has the same molecular basis as the sigmoidal binding of ATP that is observed during the activation of L-tyrosine by the K23A variant of tyrosyl-tRNA synthetase. In addition, we demonstrate that this sigmoidal activity is observed when D-tyrosine activation is monitored using single turnover (i.e., pre-steady-state) kinetics but not when D-tyrosine activation is monitored using multiple turnover (i.e., steady-state) kinetics. Finally, we demonstrate that the ATP bound to the nonfunctional subunit can be exchanged with ATP in solution. Taken together, these observations suggest that the binding of ATP during the first catalytic cycle induces a conformational change in tyrosyl-tRNA synthetase that is stable over multiple rounds of catalysis and does not require that ATP remains bound to the nonfunctional subunit.

EXPERIMENTAL PROCEDURES

Materials

Reagents were purchased from the following sources: D-[14C]tyrosine (American Radiolabeled Chemicals Inc.); L-[14C]tyrosine (Moravek Biochemicals); tetrasodium [32P]pyrophosphate (PerkinElmer Life Sciences); β-mercaptoethanol and inorganic pyrophosphatase (Sigma); nitrocellulose filters (Schleicher & Schuell); Source 15Q resin and NAP-25 columns (GE Healthcare); DispoEquilibrium biodialyzer (The Nest Group, Inc.); RNA/DNA midi kit (Qiagen); and EnzChek pyrophosphatase assay kit (Invitrogen). All other reagents were purchased from Fisher. Grafit version 5.0.6 (Erithacus Software Ltd.) and Kaleidograph version 3.6 (Synergy Software) were used to fit the kinetic data.

Purification of Recombinant Tyrosyl-tRNA Synthetase and tRNA\textsuperscript{32}Tyr

Purification of the wild-type and variant B. stearothermophilus tyrosyl-tRNA synthetases was performed as described in the accompanying paper (Ref. 32). Purification of the recombinant human tyrosyl-tRNA synthetase was carried out as described in Kleeman et al. (3). Briefly, tyrosyl-tRNA synthetase was expressed with an amino-terminal His\textsubscript{6} tag/S tag in E. coli BL21DE3 pLysS cells harboring the pHYSTS3-WT plasmid (3). The E. coli cells were lysed by sonication in Buffer A (50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole, and 1 mM phenylmethylsulfonyl fluoride, pH 8), 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. Human tyrosyl-tRNA synthetase was eluted with Buffer A containing 250 mM imidazole, and the peak fractions were dialyzed overnight against Buffer B (20 mM Tris, pH 7.5, 0.1 mM EDTA, and 10 mM β-mercaptoethanol) plus 0.1 mM tetrasodium pyrophosphate to remove any bound tyrosyl-adenylate, followed by dialysis against Buffer B. The protein solution was then loaded onto a Source 15Q-Sepharose anion exchange high performance liquid chromatography column and eluted with a gradient of 0–1 M NaCl in Buffer B. The peak eluting at 395 mM NaCl was collected and dialyzed against two changes of Buffer C (50 mM Tris, pH 7.5, 20 mM β-mercaptoethanol, and 10 mM MgCl\textsubscript{2}) followed by dialysis against Buffer C plus 10% glycerol (v/v). SDS-PAGE analysis showed a single band that migrates with a molecular mass of 66,000 Da. The concentration of the purified tyrosyl-tRNA synthetase was determined using a filter-based active-site titration assay, in which the incorporation of [14C]tyrosine into the enzyme-bound tyrosyl-adenylate intermediate is monitored (4). Comparison of the tyrosyl-tRNA synthetase concentration determined by active site titration with that determined by A\textsubscript{280} (5) indicates that >95% of the purified protein was active tyrosyl-tRNA synthetase.

The tRNA\textsuperscript{Tyr} substrate was obtained by in vitro transcription from a FokI-linearized pGFX-WT plasmid as described previously (6, 7). A typical 5-ml reaction contained 200 mM HEPES, pH 7.5, 20 mM MgCl\textsubscript{2}, 40 mM dithiothreitol, 4 mM spermidine, 100 μg/ml bovine serum albumin, 6.25 mM of each NTP, 0.06 mg/ml FokI-linearized pGFX-WT plasmid, 200 units/ml RNasin, 0.4 unit/ml inorganic pyrophosphatase, and 0.3 mg/ml T7 RNA polymerase. In vitro transcribed tRNA\textsuperscript{Tyr} was purified using a RNA/DNA midi kit (Qiagen) and annealed by incubation at 80 °C, followed by slow cooling until the sample reached room temperature. MgCl\textsubscript{2} was added to a final concentration of 10 mM. A nitrocellulose filter assay, in which the incorporation of [14C]tyrosine into the Tyr-tRNATyr\textsuperscript{Tyr} product is monitored, was used to determine the concentration of tRNA\textsuperscript{Tyr}. In this assay, tyrosyl-tRNA synthetase is preincubated with 10 mM ATP and 50 μM [14C]tyrosine at 25 °C for 5 min. The substrate tRNA\textsuperscript{Tyr} is then added; 10-μl aliquots are removed from the reaction at various time points, quenched by the addition of 5% trichloroacetic acid, and then passed through nitrocellulose filters, which retain the [14C]tyrosine bound in the Tyr-tRNATyr\textsuperscript{Tyr} complex but not free [14C]tyrosine. Greater than 95% of the tRNA\textsuperscript{Tyr} sample was aminocylated as judged by comparison of the tRNA\textsuperscript{Tyr} concentration calculated based on its absorbance at 260 nm (ε\textsubscript{260} = 906,100 M\textsuperscript{−1} cm\textsuperscript{−1}) with that calculated based on the incorporation of [14C]tyrosine into the Tyr-tRNATyr\textsuperscript{Tyr} product.

Kinetic Procedures

Pre-steady-state Kinetic Analysis—Pre-steady-state kinetic analyses were performed at 25 °C in Tris buffer containing 144 mM Tris, pH 7.78, and 10 mM MgCl\textsubscript{2} (standard Tris buffer). Stopped-flow fluorescence was used to monitor single turnover kinetics for the ATP dependence of the D-tyrosine activation reaction (8). In these studies an SX 18.MV stopped-flow spectrophotometer (Applied Photophysics) was used to monitor the decrease in fluorescence of the B. stearothermophilus and human tyrosyl-tRNA synthetases that is associated with formation of the TyrRS-Tyr-AMP complex (λ\textsubscript{ex} = 295 nm, λ\textsubscript{em} > 320 nm) (8, 9). In the ATP dependence experiments, one syringe contained the enzyme (0.3–0.5 μM), inorganic pyrophosphatase (1 unit/ml), and saturating concentrations of D-tyrosine (1.5 mM) in standard Tris buffer. The other syringe contained inorganic pyrophosphatase (1 unit/ml), 1.5 mM D-tyrosine, and 0.1–80 mM MgATP in standard Tris buffer. Equal volumes from each syringe were mixed, and the decrease in the intrinsic fluorescence of the protein was monitored. The addition of inorganic pyrophosphatase prevents the reverse reaction from occurring once the TyrRS-Tyr-AMP complex has formed. In assays where NaCl is present, the assay solution was...
made up such that the final solution contained 0.5 mM NaCl. The equilibrium constant for the dissociation of ATP from the TyrRS-d-Tyr-AMP complex (Kd,ATP) and the forward rate constant for the activation of d-tyrosine (k₆) were calculated from a plot of kobs versus ATP concentration for the d-tyrosine activation reaction. All assays were replicated 3–5 times using tyrosyl-tRNA synthetase from at least three separate enzyme purifications.

Pre-steady-state kinetic data were fit to a single exponential floating end point equation using the Applied Photophysics stopped-flow software package to determine the observed rate constants (kobs) as shown in Equation 1,

\[ F = (F_0 - F_t)e^{-k_{obs} \times t} + F_t \]  
(Eq. 1)

where F is the measured fluorescence at time t, kobs is the apparent first order rate constant for the approach of F to its final value F₀, and F₀ corresponds to the fluorescence at time t = 0. Rate and dissociation constants were determined by plotting kobs versus substrate concentration. Data that displayed a hyperbolic dependence on substrate concentration were fit to Equation 2 (10),

\[ k_{obs} = \frac{k_3[S]_T}{(K_a + [S]_T)} \]  
(Eq. 2)

where kobs is the observed rate constant; k₃ is the forward rate constant for the formation of tyrosyl-adenylate; [S]₉ is the total substrate concentration, and Kₐ is the dissociation constant for the substrate of interest. An Eadie-Hofstee transformation was used to determine how well the data fit Equation 2 (11, 12) to create Equation 3,

\[ k_{obs} = k_3 - \frac{k_9k_{obs}}{[S]_T} \]  
(Eq. 3)

where kobs, k₃, k₉, and [S]₉ are defined as above. Data that displayed a sigmoidal dependence with respect to the substrate concentration were fit to the Adair equation, Equation 4 (13),

\[ k_{obs} = \frac{k_3(\alpha[ATP] + \beta[ATP]^2)}{1 + (\alpha + \gamma)[ATP] + \beta[ATP]^2} \]  
(Eq. 4)

where kobs is the observed rate constant for the formation of tyrosyl-adenylate; k₃ is the forward rate constant for tyrosyl-adenylate formation, and α, β, and γ are fitting constants. If the k₃ values for the monoligated and biligated species are assumed to be identical, then α corresponds to K₁, β corresponds to K₂, and γ corresponds to K₃. To determine the extent of cooperativity, the data were fit to the Hill equation, Equation 5 (14),

\[ \log\left(\frac{k_{obs}}{k_3 - k_{obs}}\right) = n_h\log[ATP] - \log K_{d,ATP} \]  
(Eq. 5)

where kobs/(k₃ - kobs) is the fraction of ATP-binding sites that are occupied; Kd,ATP is the dissociation constant for ATP, and nH is the Hill coefficient.

**Steady-state Pyrophosphate Exchange Assay**—The Kₘ values for ATP and pyrophosphate were determined using a pyrophosphate exchange assay (8). In this assay, [32P]pyrophosphate is incorporated into ATP through Reaction 1,

\[ \text{TyrRS} \cdot \text{Tyr-AMP} + \text{PP} \rightarrow \text{TyrRS} + \text{Tyr} + \text{ATP} \]  
(REACTION 1)

where TyrRS represents tyrosyl-tRNA synthetase and “·” and “-” represent noncovalent and covalent interactions, respectively. In this assay, *B. stearothermophilus* tyrosyl-tRNA synthetase (0.25 μM) is incubated with d-tyrosine (1.5 mM), [32P]pyrophosphate (0.2 Ci/mol), and ATP in standard Tris buffer. For determination of the Kₘ for ATP, the concentration of pyrophosphate is 2 mM, and the concentration of ATP varies from 0.2 to 10 mM. For determination of the Kₘ for pyrophosphate, the concentration of MgATP is 10 mM, and the concentration of [32P]pyrophosphate varies from 4.0 to 400 μM. Aliquots (10 μl) are periodically taken and quenched with 3.5% perchloric acid containing 1% charcoal, filtered through GF/C filters, and washed four times with pyrophosphate wash buffer (10 mM pyrophosphate, pH 2.0) and one time with 100% ethanol. The incorporation of [32P] into ATP is monitored by measuring the amount of [32P]ATP adsorbed on charcoal by scintillation counting. The Michaelis-Menten equation (15, 16) was used to determine Kₘ and kcat values (Equation 6) and the Eadie-Hofstee transformation (11, 12) was used to determine how well the data fit the Michaelis-Menten model (Equation 7),

\[ \frac{v_0}{v} = \frac{k_{cat}[E][S]_T}{(K_a + [S]_T)} \]  
(Eq. 6)

\[ \frac{v_0}{v} = k_{cat}[E][S]_T \]  
(Eq. 7)

where v₀ is the initial rate; [E] is the total concentration of tyrosyl-tRNA synthetase in the assay, and [S]₉ is the total concentration of the substrate of interest (either pyrophosphate or ATP).

**Filter Binding Assay**—Formation of the enzyme-bound tyrosyl-adenylate complex from [α-32P]ATP and d- or L-tyrosine was monitored using a nitrocellulose filter assay. Assay solutions were made up such that, after addition of wild-type *B. stearothermophilus* tyrosyl-tRNA synthetase, the assay solution contained 144 mM Tris, pH 7.78, 10 mM MgCl₂, 1 unit/ml inorganic pyrophosphatase, 10 mM β-mercaptoethanol, 10 mM [α-32P]ATP (12 Ci/mmol), and either 1.0 mM d-tyrosine or 50 μM L-tyrosine. Assays were incubated at 25 °C, and prewarmed enzyme was added to initiate the reaction. Aliquots were withdrawn periodically over a period of 10 min, filtered through nitrocellulose filters, washed three times with 3 ml of ice-cold buffer composed of 144 mM Tris, pH 7.78, 10 mM MgCl₂, and 10 mM β-mercaptoethanol. TyrRS-Tyr-[α-32P]AMP bound to the nitrocellulose filter was assayed by scintillation counting.

**Equilibrium Binding Studies**—Equilibrium dialysis was performed using a modification of the method previously described by Fersht (17). Briefly, one chamber of each dialysis cell contained 60 μM tyrosyl-tRNA synthetase, d-tyrosine (1.5 mM), or l-tyrosine (200 μM) and 1 unit/ml inorganic pyrophosphatase in the standard Tris buffer (chamber A). The other chamber (chamber B) of each dialysis cell contained d-tyrosine.
**RESULTS**

**ATP Displays Cooperative Binding during the Activation of D-Tyrosine**—The dependence of the initial rate for formation of TyrRS-D-Tyr-AMP with respect to the concentration of ATP was measured under single turnover conditions. As the value of $K_d^{D-Tyr}$ for *B. stearothermophilus* tyrosyl-tRNA synthetase is 102 μM and the concentration of D-tyrosine in the assay is 1.5 mM, ~95% of the enzyme in the assay contains bound D-tyrosine prior to the binding of ATP. In other words, under the assay conditions used (i.e. 1.5 mM D-tyrosine), dissociation of ATP from the TyrRS-Tyr-AMP complex is being monitored. Under single turnover conditions, the activation of D-tyrosine by *B. stearothermophilus* tyrosyl-tRNA synthetase exhibits a sigmoidal dependence with respect to ATP concentration (Fig. 1, panel A). Rate and dissociation constants obtained by fitting the data to the Adair equation (Equation 4) are shown in Table

\[
\alpha = \frac{n_b [ATP]_{\text{free}}}{K_d^{ATP} + [ATP]_{\text{free}}} 
\]

where $\alpha = [ATP]_{\text{bound}}/[E]_i; K_d^{ATP}$ is the dissociation constant for ATP; $n_b$ is the number of binding sites, and $[E]_i$ is the total tyrosyl-tRNA synthetase concentration (18, 19). Because tyrosyl-tRNA synthetase is present in the equilibrium dialysis chambers, when $[\alpha-^{32}P]ATP$ is present, $[ATP]_{\text{bound}}$ can include both Tyr-[\alpha-^{32}P]AMP bound to the functional subunit and $[\alpha-^{32}P]ATP$ bound to the nonfunctional subunit (assuming the ATP bound to the nonfunctional subunit has a sufficiently high affinity). In the case of $[\gamma-^{32}P]ATP$, only the binding of ATP will be monitored because the label is lost on formation of the TyrRS-Tyr-AMP intermediate. To ensure that the results using $[^{32}P]ATP$ are consistent with previous results obtained using $[^{14}C]tyrosine$, the experiment was repeated using assay mix with $L-[^{14}C]tyrosine$ and 13 μM tyrosyl-tRNA synthetase, and the concentration of tyrosine in each chamber was determined.

**Steady-state Pyrophosphate Release Assay**—Pyrophosphate release was assayed at 25°C using the EnzChek™ pyrophosphate assay kit (Invitrogen). A 100-μl reaction mix contained 144 mM Tris, pH 7.8, 10 mM MgCl₂, 200 μM 2-amino-6-mercapto-7-methylpurine riboside (MESG), 0.1 unit of purine nucleoside phosphorylase, 0.003 unit of inorganic pyrophosphatase, 1.0 mM D-tyrosine, 0.25 μM *B. stearothermophilus* tyrosyl-tRNA synthetase, 30 μM in vitro transcribed *B. stearothermophilus* tRNA$_{D}$, and MgATP (0.5–10 mM). The reaction was initiated by the addition of enzyme, and the increase in absorbance at 360 nm was monitored spectrophotometrically for 60 min (20). This increase in absorbance is proportional to the consumption of phosphate ($P_i$) by the MESG/purine nucleoside phosphorylase reaction. Spontaneous hydrolysis of ATP was negligible under the assay conditions used. The Michaelis-Menten equation (Equation 6) was used to determine the $K_m$ and $k_{cat}$ values.
TABLE 1
Kinetic constants for the activation of D-tyrosine

Equilibrium constants for the dissociation of ATP from the TyrRS-D-Tyr-ATP complex were determined by fitting the data to the Adair equation (13) for all variants except the wild-type and K233A variants of B. stearothermophilus (B. st.) tyrosyl-tRNA synthetase in the presence of 0.5 M NaCl, which were fit to a hyperbolic equation (10).

| Variant   | Source | [NaCl] | $n_u$ | $K_{d,ATP}^{ATP}$ | $K_{d,ATP}^{k_1}$ | $k_3$ |
|-----------|--------|--------|-------|-------------------|-------------------|-------|
| Wild type | B. st. | 0.0    | 2.4 (±0.4) | 29 (±3) | 0.5 (±0.1) | 13 (±1) |
| Wild type | B. st. | 0.5    | 6.9 (±0.4) | NA       | 1.3 (±0.1) |       |
| Wild type | Human  | 0.0    | 2.5 (±0.1) | 24 (±5) | 0.6 (±0.1) | 3.7 (±0.3) |
| Wild type | Human  | 0.5    | 21 (±0.3) | 29 (±9) | 4.9 (±0.4) | 1.5 (±0.1) |
| K233A     | B. st. | 0.0    | 2.4 (±0.4) | 33 (±10) | 2.6 (±0.8) | 0.7 (±0.1) |
| K233A     | B. st. | 0.5    | NA      | 14 (±1) | NA       | 1.6 (±0.1) |

* Values are taken from 2.

1. Fitting the data to the Hill equation (Equation 5) results in a linear plot with a Hill coefficient of 2.4 (±0.4) (Fig. 1, panel A, inset). The value for the forward rate constant ($k_3$) obtained from the Hill plot is 13 (±1) s⁻¹, whereas the average value for the equilibrium constant for the dissociation of ATP from the TyrRS-D-Tyr-ATP complex ($K_{d,ATP}^{ATP}$) is 3.6 (±0.5) mM.

To determine whether the sigmoidal behavior is also observed in eukaryotic tyrosyl-tRNA synthetases, the ATP dependence of D-tyrosine activation by human tyrosyl-tRNA synthetase was investigated. Human tyrosyl-tRNA synthetase was found to display sigmoidal kinetics with respect to ATP concentration under conditions that are identical to those used for the B. stearothermophilus enzyme (Fig. 1, panel B). Rate and dissociation constants obtained by fitting the data to the Adair equation are shown in Table 1. Fitting the data for human tyrosyl-tRNA synthetase to the Hill equation (Equation 5) resulted in a Hill coefficient of 2.5 (±0.1), and $k_3$ and average $K_{d,ATP}^{ATP}$ values of 3.7 (±0.3) s⁻¹ and 3.2 (±0.2) mM, respectively.

The Cooperative Binding of ATP Is Dependent on Ionic Strength—It has previously been observed that the addition of 0.5 M NaCl abolishes the sigmoidal binding behavior of ATP during the activation of L-tyrosine by the tyrosyl-tRNA synthetase K233A variant (1). To determine whether ionic strength has a similar effect on the sigmoidal binding of ATP observed for the activation of D-tyrosine by the K233A variant of B. stearothermophilus tyrosyl-tRNA synthetase, the human tyrosyl-tRNA synthetase displays hyperbolic kinetics (Fig. 2, panel A). Fitting the data to a hyperbolic rate equation (Equation 2) gives a forward rate constant ($k_3$) of 1.3 (±0.1) s⁻¹ and a dissociation constant for ATP ($K_{d,ATP}^{ATP}$) of 6.9 (±0.4) mM.

In contrast to B. stearothermophilus tyrosyl-tRNA synthetase, the human tyrosyl-tRNA synthetase displays sigmoidal kinetics with respect to the concentration of ATP even in the presence of 0.5 M NaCl (Fig. 2, panel B). Rate and dissociation constants obtained by fitting the data to the Adair equation (Equation 4) are shown in Table 1. Fitting these data to the Hill equation (Equation 5) results in a Hill coefficient of 2.1 (±0.3) and $k_3$ and average $K_{d,ATP}^{ATP}$ values of 1.5 (±0.1) s⁻¹ and 11.4 (±0.8) mM, respectively (Fig. 2, panel B, inset).

The Sigmoidal Behavior with Respect to ATP Binding Is Not Enhanced in the Tyrosyl-tRNA Synthetase K233A Variant—To test the hypothesis that the source of the cooperative binding of ATP in the D-tyrosine activation reaction is the same as that observed for the activation of L-tyrosine by the K233A variant of B. stearothermophilus tyrosyl-tRNA synthetase, the activation of D-tyrosine by the K233A variant of B. stearothermophilus tyrosyl-tRNA synthetase was investigated. In the presence of a saturating (1.5 mM) concentration of D-tyrosine, the K233A variant shows sigmoidal kinetics with respect to the binding of ATP for the activation of D-tyrosine with a Hill coefficient of 2.4 (±0.4) mM (Fig. 3, panel A). The $k_3$ and average $K_{d,ATP}^{ATP}$ values obtained from the Hill plot are 0.7 (±0.1) s⁻¹ and 7.4 (±0.4) mM, respectively. Rate and dissociation constants obtained by fitting the data to the Adair equation (Equation 4) are shown in Table 1.

In the presence of 0.5 M NaCl, the tyrosyl-tRNA synthetase K233A variant displays hyperbolic kinetics with respect to the...
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ATP concentration for the activation of d-tyrosine (Fig. 3, panel B). Under these conditions, the values for the forward rate constant, $k_{f}$, and dissociation constant for ATP, $K_d^{\text{ATP}}$, are 1.6 ($\pm 0.1$) s$^{-1}$ and 14 ($\pm 1$) mM, respectively.

**Cooperative Binding of ATP Is Not Observed in Steady-state Kinetic Assays**—Previous investigations using steady-state kinetic methods indicate that the E. coli and B. subtilis tyrosyl-tRNA synthetases display classical Michaelis-Menten kinetics with respect to the binding of ATP for the aminoacylation of tRNA$^\text{Tyr}$ by d-tyrosine (1, 21). This suggests that the cooperative binding of ATP is only observed during the initial turnover of the enzyme. To test this hypothesis, the activation of d-tyrosine by B. stearothermophilus tyrosyl-tRNA synthetase was monitored using a steady-state pyrophosphate exchange assay. Using this assay, classical Michaelis-Menten kinetics were observed for the activation of d-tyrosine. Fitting the data to the Michaelis-Menten equation (Equation 6) gives a $K_{\text{cat}}$ value of 3.06 ($\pm 0.05$) s$^{-1}$ and a $K_m^{\text{ATP}}$ value of 1.0 ($\pm 0.2$) mM at a saturating d-tyrosine concentration (Fig. 4).

The observation that sigmoidal kinetics are only observed under single turnover conditions raises the possibility that when ATP binds to the nonfunctional subunit of tyrosyl-tRNA synthetase, it remains bound through subsequent rounds of catalysis (i.e. it is a nonexchangeable ATP). To test this hypothesis, the binding of ATP to B. stearothermophilus tyrosyl-tRNA synthetase was monitored using a filter binding assay. In this assay, the incorporation of $[\alpha-\text{P}]$ATP into the enzyme-bound tyrosyl-adenylate intermediate is monitored. If ATP is not released after binding to the nonfunctional subunit, then the ratio of $\text{P}$ to tyrosyl-tRNA synthetase in this assay will be 2:1, because one molecule of $\text{P}$ will be incorporated as Tyr-$\text{P}$AMP (at the functional subunit), and one molecule will be incorporated as $[\alpha-\text{P}]$ATP (at the nonfunctional subunit). In contrast, if ATP is released from the nonfunctional subunit after one catalytic cycle, then the ratio of $\text{P}$ to tyrosyl-tRNA synthetase will be 1:1. Analysis of this filter binding assay indicates that the ratio of $\text{P}$ to tyrosyl-tRNA synthetase is 1:1, indicating that the ATP bound to the nonfunctional subunit is released after d-tyrosine has been activated (Table 2).

To confirm that ATP does not remain bound to the nonfunctional subunit after formation of the TyrRS-Tyr-AMP

**FIGURE 3. Activation of d-tyrosine by the K233A variant of B. stearothermophilus tyrosyl-tRNA synthetase.** Typical plots for the activation of d-tyrosine in the absence (panel A) and presence (panel B) of 0.5 M NaCl are shown for the K233A variant of B. stearothermophilus tyrosyl-tRNA synthetase. The concentration of d-tyrosine in these assays was 1.5 mM. Initial rates were determined under single turnover conditions as described in Fig. 1. Data for the activation of d-tyrosine in the absence of 0.5 M NaCl are fit to the Adair equation (Equation 4), with the Hill plot (Equation 5) shown as an inset. Data for the activation of d-tyrosine in the presence of 0.5 M NaCl are fit to a hyperbolic equation (Equation 2), with the Eadie-Hofstee transformation (Equation 3) shown as an inset.

**FIGURE 4. Activation of d-tyrosine under steady-state conditions.** A typical plot for the dependence of the initial rate for the activation of d-tyrosine with respect to ATP concentration under steady-state conditions is shown for B. stearothermophilus tyrosyl-tRNA synthetase. The reaction kinetics were monitored using a pyrophosphate exchange assay in which the incorporation of $[^{32}\text{P}]$PP into ATP is monitored. The concentrations of tyrosyl-tRNA synthetase, d-tyrosine, and pyrophosphate in the assay are 0.25 mM, 1.5 mM, and 2.0 mM, respectively. The data are fit to the Michaelis-Menten equation (Equation 6) with the Eadie-Hofstee transformation (Equation 7) shown as an inset.

**TABLE 2**

| Experimental procedure          | $[^{32}\text{P}]/\text{TyrRS}$ |
|---------------------------------|--------------------------------|
| Filter binding assay            | 0.91 ($\pm 0.05$)              |
| Equilibrium dialysis            | 0.94 ($\pm 0.09$)              |

*a The concentration of tyrosyl-tRNA synthetase was determined by filter binding assay using $[^{14}\text{C}]$tyrosine as substrate.
intermediate, equilibrium dialysis was performed. In these experiments, one equilibrium dialysis chamber contained *B. stearothermophilus* tyrosyl-tRNA synthetase and either L- or D-tyrosine at saturating concentrations. The other chamber contained either L- or D-tyrosine and 10 mM [α-32P]ATP. Inorganic pyrophosphatase was present in both chambers to drive formation of the TyrRS-Tyr-AMP intermediate to completion. The results of these experiments confirm the incorporation of a single molecule of [α-32P]ATP into the TyrRS-Tyr-AMP intermediate (Table 2).

Although ATP does not remain bound to the nonfunctional subunit, it is conceivable that it phosphorylates the nonfunctional subunit prior to dissociating. To test this hypothesis, the above filter binding and equilibrium dialysis assays were repeated using [γ-32P]ATP in place of [α-32P]ATP. The results of these experiments indicate that ATP bound to the nonfunctional subunit does not phosphorylate the enzyme (data not shown).

**The Sigmoidal Behavior with Respect to ATP Binding Is Not Coupled to the High Affinity Binding of Pyrophosphate** — When D-tyrosyl-adenylate is bound to tyrosyl-tRNA synthetase, pyrophosphate binds with a 14-fold higher affinity than when L-tyrosyl-adenylate is bound (see accompanying paper (32)). If the sigmoidal binding of ATP is because of D-tyrosine decreasing the affinity of the functional subunit for ATP, the increased affinity for pyrophosphate may be a consequence of this altered conformation. If this is the case, one would predict that, in the presence of D-tyrosine, the binding of pyrophosphate would stabilize the low ATP affinity conformation of the functional subunit. In other words, the high affinity binding of the pyrophosphate moiety may be coupled to the sigmoidal binding of ATP observed during the activation of D-tyrosine. One prediction of this hypothesis is that reaction conditions that give rise to hyperbolic kinetics in the presence of D-tyrosine will display a decreased affinity for pyrophosphate. In particular, in the presence of 0.5 M NaCl, the $K_{d}^{PP}$ value (where $K_{d}^{PP}$ is the equilibrium constant for the dissociation of pyrophosphate from the TyrRS-Tyr-AMP-PP$_i$ complex) determined in the presence of D-tyrosine should be similar to that determined in the presence of L-tyrosine. To test this hypothesis, the effect that 0.5 M NaCl has on the pyrophosphorylation of the TyrRS$_{L}$-Tyr-AMP and TyrRS$_{D}$-Tyr-AMP intermediates was determined. The addition of 0.5 M NaCl was found to decrease the affinity of *B. stearothermophilus* tyrosyl-tRNA synthetase for pyrophosphate by 4- and 10-fold, respectively, when L- and D-tyrosine are present (Fig. 5 and Table 3). The observation that, in the presence of 0.5 M NaCl, tyrosyl-tRNA synthetase binds pyrophosphate ~6-fold more tightly when D-tyrosine is present suggests that the sigmoidal binding of ATP is not coupled to the increased affinity for pyrophosphate.

To further demonstrate that the high affinity binding of pyrophosphate is not coupled to the observed sigmoidal behavior, the $K_m$ value for the dissociation of pyrophosphate from the TyrRS$_{D}$-Tyr-AMP-PP$_i$ complex was determined under steady-state (multiple turnover) conditions. Because classical Michaelis-Menten kinetics are observed under steady-state conditions, one would predict that if the high affinity binding of pyrophosphate is coupled to the sigmoidal behavior, then the affinity of the enzyme for pyrophosphate will not be increased when D-tyrosine is present in the steady-state assay. The $K_m$ for the dissociation of pyrophosphate from the enzyme-bound D-Tyr-AMP-PP$_i$ complex is 0.032 (±0.004) mM (Fig. 6). This is similar

**FIGURE 5. NaCl decreases the affinity of the TyrRS-Tyr-AMP complex for pyrophosphate.** The binding of pyrophosphate to *B. stearothermophilus* tyrosyl-tRNA synthetase was determined by monitoring the conversion of TyrRS-Tyr-AMP + pyrophosphate to TyrRS + Tyr + ATP in the presence of 0.5 M NaCl using single turnover conditions. The reaction of pyrophosphate with the TyrRS$_{L}$-Tyr-AMP and TyrRS$_{D}$-Tyr-AMP intermediates are shown in panels A and B, respectively. The data are fit to a hyperbolic equation (2) with the Eadie-Hofstee transformation (3) shown as an inset.

**TABLE 3**

| Rate and dissociation constants for the pyrophosphorylation of TyrRS-Tyr-AMP |
|------------------|------------------|------------------|------------------|
| Enzyme complex   | [NaCl] (M)       | $k_{\text{s}}$  | $k_{\text{d}}^{\text{PPi}}$ |
| TyrRS$_{D}$-Tyr-AMP$^a$ | 0.0 (±0.6)      | 0.043 (±0.001)  | 110,000          |
| TyrRS$_{D}$-Tyr-AMP$^b$ | 0.5 (±1)        | 0.4 (±0.1)     | 14,550           |
| TyrRS$_{L}$-Tyr-AMP$^c$ | 0.0 (±0.6)      | 0.61 (±0.1)    | 27,200           |
| TyrRS$_{L}$-Tyr-AMP$^c$ | 0.5 (±1)        | 2.5 (±0.1)     | 5,900            |

$^a$ Values are taken from accompanying paper (32).

$^b$ Values are taken from Ref. 26.
to the dissociation constant for pyrophosphate determined under single turnover conditions (Table 3). These dissociation constants are 14- and 31-fold less than the corresponding values for the dissociation of pyrophosphate from the TyrRS–d-tyrosyl-tRNA synthetase in a high affinity conformation (with respect to the binding of ATP). To test this hypothesis, a steady-state pyrophosphate release assay was used to monitor the kinetics for the activation of d-tyrosine with respect to the concentration of ATP. In this assay, the pyrophosphate moiety in the TyrRS-d-tyrosyl-tRNA synthetase complex is transferred to tRNA, and pyrophosphate is released during each round of catalysis. The results of this assay indicate that the Michaelis-Menten kinetics observed under steady-state conditions are not dependent on the binding of pyrophosphate to the enzyme (Fig. 7). Taken together, these results support the hypothesis that the binding of ATP to the nonfunctional subunit induces a conformational change in the enzyme that remains stable over multiple turnovers of the enzyme and is independent of the presence of bound ATP or pyrophosphate.

**DISCUSSION**

The observation that tyrosyl-tRNA synthetase displays sigmoidal kinetics with respect to the concentration of ATP strongly suggests that ATP binds in a cooperative manner during the activation of d-tyrosine. This presents a paradox, however, as tyrosyl-tRNA synthetase displays half-of-the-sites reactivity with respect to the binding of ATP. Two lines of evidence support the hypothesis that the sigmoidal kinetics observed for the activation of d-tyrosine have
the same mechanistic basis as that observed for the activation of L-tyrosine by the K233A variant of tyrosyl-tRNA synthetase. First, the Hill coefficient for the activation of n-tyrosine is identical for both the wild-type and K223A variants of B. stearothermophilus tyrosyl-tRNA synthetase. This indicates that the sources of the sigmoidal activity in n-tyrosine activation and the K233A variant are not additive. Second, when 0.5 M NaCl is present in the assay mix, hyperbolic kinetics are observed for both the activation of L-tyrosine by the K233A variant of B. stearothermophilus tyrosyl-tRNA synthetase and the activation of n-tyrosine by both the wild-type enzyme and the K233A variant.

In the presence of 0.5 M NaCl, the activation of n-tyrosine displays hyperbolic kinetics with respect to the concentration of ATP. This can be explained by the observation that increasing the ionic strength of the assay mix increases the affinity with which ATP binds to the functional subunit. This increased affinity for ATP at higher ionic strengths may result from either weakening of ionic bonds that stabilize the low affinity conformation of the functional subunit or strengthening the hydrophobic interactions between ATP and the active site of the functional subunit. In either case, the increased affinity of the functional subunit for ATP in the presence of NaCl allows ATP to bind initially to the functional subunit. This prevents the effect that the binding of ATP to the nonfunctional subunit has on the reaction kinetics from being observed.

The observation that human tyrosyl-tRNA synthetase still displays sigmoidal activity with respect to the ATP concentration in the presence of 0.5 M NaCl suggests that although the molecular basis for sigmoidal activity is conserved, the effect that ionic strength has on the affinity of the functional subunit for ATP is not the catalytic mechanisms of the human and B. stearothermophilus tyrosyl-tRNA synthetases differ in that, unlike the bacterial enzyme, human tyrosyl-tRNA synthetase requires the binding of a potassium cation for optimal activity (Kd = 32 mM) (9). Furthermore, in the human enzyme, the second lysine in the KMSKS signature sequence is replaced by a serine residue (3). This lysine residue is one of the most highly conserved amino acids in the class I aminoacyl-tRNA synthetase family (24, 25). In B. stearothermophilus tyrosyl-tRNA synthetase, the second lysine in the KMSKS signature sequence, Lys-233, has been shown to interact with the pyrophosphate moiety of ATP on formation of the transition state for the tyrosine activation reaction (26). This is the same residue that, when replaced by alanine, results in a tyrosyl-tRNA synthetase variant that displays sigmoidal kinetics with respect to ATP concentration. In human tyrosyl-tRNA synthetase, the potassium cation plays the same functional role as Lys-233 in B. stearothermophilus tyrosyl-tRNA synthetase (27). It is possible that the different response to 0.5 M NaCl by the human and B. stearothermophilus tyrosyl-tRNA synthetases is related to either the absence of the second lysine in the KMSKS signature sequence in the human enzyme or its requirement for potassium cation.

It is intriguing that sigmoidal behavior is observed under single but not multiple turnover conditions. This implies that there is a conformational change that occurs during the first turnover of the enzyme that is essentially irreversible during the time course of the steady-state assays. One possibility is that the ATP bound to the inactive subunit is nonexchangeable. Nonexchangeable ATP molecules have previously been observed, notably in E. coli F1-ATPase (28). However, active site titration and equilibrium dialysis experiments failed to demonstrate the presence of a second, nonexchangeable, ATP bound to the enzyme in the presence of tyrosyl-adenylate. This suggests that the binding of ATP to the inactive subunit induces a conformational change in the enzyme that is kinetically irreversible over the time course of the steady-state assay. Bacteriorhodopsin offers a precedent for a ligand-induced conformational change. Correct folding of this integral membrane protein occurs only when retinal is bound (29). Using 13C NMR, Yamaguchi et al. (30) have shown that the removal of retinal by hydroxylamine bleaching does not cause reversion of bacteriorhodopsin to the partially folded form. The authors speculate that the retinal-induced irreversible folding step may lead to formation of the bacteriorhodopsin trimer. In this regard, it is interesting to note that dimerization is essential for activity in tyrosyl-tRNA synthetase (31). Whether the ATP bound to the nonfunctional subunit acts through changes in the dimer interface of tyrosyl-tRNA synthetase remains to be determined.

Acknowledgments—We thank Jason Manning, Chuka Ifeanyi, and Tara Andrews for technical assistance in protein purification. We thank Gyanesh Sharma for assistance with the pyrophosphorolysis experiments.

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