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# Lysyl-tRNA Synthetase from *Bacillus stearothermophilus*. Purification, and Fluorometric and Kinetic Analysis of the Binding of Substrates, L-Lysine and ATP[1]

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**Lysyl-tRNA synthetase [L-lysine:tRNA$^{Lys}$ ligase (AMP forming); EC 6.1.1.6] was purified from *Bacillus stearothermophilus* NCA1503 approximately 1,100-fold to homogeneity in PAGE. The enzyme is a homodimer of $M_r$ 57,700×2. The molar absorption coefficient, $\varepsilon$, at 280 nm is 71,600 $M^{-1} \cdot cm^{-1}$ at pH 8.0. Enzyme activity in the tRNA aminoacylation reaction and the ATP-$PP_i$ exchange reaction increases up to 50°C at pH 8.0, but is lost completely at 70°C. The pH-optima of the two reactions are 8.3 at 37°C. In the tRNA aminoacylation reaction, the $K_m$ values for L-lysine and ATP are 16.4 and 23.2 $\mu$M, respectively, and in the ATP-$PP_i$ exchange reaction, the $K_m$ values for L-lysine and ATP are 23.6 and 65.1 $\mu$M, respectively at 37°C, pH 8.0. Interaction of either L-lysine or ATP with the enzyme has been investigated by using as a probe the ligand-induced quenching of protein fluorescence and by equilibrium dialysis. These static analyses, as well as the kinetic analysis of the L-lysine dependent ATP-$PP_i$ exchange reaction indicate that the binding mode of L-lysine and ATP to the enzyme is sequential ordered (L-lysine first). The interaction of lysine analogues with the enzyme has also been investigated.**

**Key words: aminoacyl-tRNA synthetase, fluorescence titration, lysyl-tRNA synthetase, protein fluorescence, substrate binding order.**

An aminoacyl-tRNA synthetase (abbreviated as ARS) catalyzes the binding of an amino acid to the cognate tRNA, generally according to the following reaction scheme (*1*).

$$E + AA + ATP \rightleftharpoons E \cdot AA \sim AMP + PP_i \quad (1)$$

$$E \cdot AA \sim AMP + tRNA \rightleftharpoons E + AMP + AA\text{-}tRNA \quad (2)$$

where AA denotes the amino acid; E, ARS; $PP_i$, inorganic pyrophosphate; and E·AA∼AMP, an aminoacyl-adenylate-ARS complex. This tRNA aminoacylation reaction is critical for the fidelity of translation of the genetic information into protein structure. A high degree of substrate specificity for each heterogeneous substrate (amino acids, nucleotides, and tRNAs) is required. Elucidation of the mechanism of this strict discrimination of substrate structure is of vital importance. ARSs for the 20 kinds of amino acid that constitute proteins are not necessarily the same in their quaternary structures (monomers, dimers, and tetramers) (*2*), and they show considerable variations in primary structure, although they catalyze the same type of chemical reactions. Detailed studies on the structure-function relationship are necessary for each ARS specific to each of the 20 amino acids.

In recent years, determination of the primary structures of ARSs has rapidly progressed. The 20 kinds of ARSs can be divided into two classes according to their primary structure (*2-4*). Class I enzymes include those specific for cysteine, methionine, valine, leucine, isoleucine, arginine, glutamic acid, glutamine, tyrosine, and tryptophan, whereas Class II enzymes include those for glycine, alanine, serine, threonine, proline, histidine, lysine, aspartic acid, asparagine, and phenylalanine. Class I enzymes are characterized by the consensus signature sequences, HIGH and KMSKS, and Class II enzymes by three unique sequences, Motif 1, Motif 2, and Motif 3 (*2, 3*). Determination of the three-dimensional structure of ARS by X-ray crystallography has been successful with TyrRS of *Bacillus stearothermophilus* (*4, 5*); MetRS (*6*), SerRS (*7*), GlnRS (*8*), and LysRS (*lys U*-encoded) (*9*) of *Escherichia coli*; SerRS (*10*) and GluRS (*11*) of *Thermus thermophilus*; TrpRS (*12*), PheRS (*13*), and AspRS of *Saccharomyces cerevisiae* (*14*). Analysis of structure-function relationship has progressed rapidly. However, as more structural information becomes available, more precise kinetic information obtained with the purified enzymes is needed to analyze the molecular mechanism of the reaction. There is an apparent contradic-

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Abbreviations: Ant-ATP, anthraniloyl-ATP; ARS, aminoacyl-tRNA synthetase; *B.s.* ARS, ARS from *Bacillus stearothermophilus*; PMSF, phenylmethanesulfonyl fluoride; LysRS, lysyl-tRNA synthetase. The other aminoacyl-tRNA synthetases are also abbreviated as the three-letter code of their specific amino acid followed by RS.

tion, for example, between the results of structural analysis and those of kinetic analysis (*2, 3, 5*). Much effort is still required in enzyme chemistry.

We are interested in the molecular mechanism of the strict substrate specificity of ARSs and have chosen as the target LysRS of *B. stearothermophilus*. Several analogues of lysine are available, and our preliminary test revealed that LysRS activity in the crude extracts of *B. stearothermophilus* was much more stable than that of *E. coli*. These features should be advantageous for studies of the mechanism of substrate specificity.

There has been only one report on LysRS of *B. stearothermophilus* (abbreviated as *B.s.* LysRS) by Samuelsson and Lundvik (*15*), in which they described the purification procedures, amino acid composition, crystallization, and the effect of temperature on the reaction rate and $K_m$ values. Most of the studies on LysRS have been done with the *E. coli* (*16-20*) and yeast (*21, 22*) enzymes, but the reaction mechanism is not yet understood in detail for LysRS of any origin (*23*). As we wished to study *B.s.* LysRS more comprehensively, we first needed to establish a better purification procedure and to elucidate the basic properties of the enzyme.

In the present study, we have obtained a highly purified stable preparation of *B.s.* LysRS by means of a novel procedure giving a good yield, and we have characterized some basic properties of the enzyme. We have also examined the features of the binding of substrates, L-lysine and ATP, and of their analogues by means of ligand-induced quenching of protein fluorescence as a probe, as well as by equilibrium dialysis. In addition, the order of binding of these substrates to *B.s.* LysRS has been elucidated by steady-state kinetic analysis.

## MATERIALS AND METHODS

*Amino Acids and Lysine Analogues*—All L-amino acids were purchased from either Wako Pure Chemical Industries, Nakarai Tesque, or Takara Kohsan. Cadaverine, 6-amino-*n*-hexanoic acid, L-norleucine, L-ornithine, and D-lysine were purchased from Nakarai Tesque. L-Lysine hydroxamate, L-lysine amide, *Nα*-acetyl-L-lysine, *Nε*-methyl-L-lysine, *Nε*-acetyl-L-lysine, and 5-hydroxylysine (mixed DL and DL-allo) were obtained from Sigma Chemical. *S*-(2-Aminoethyl)-L-cysteine was the product of Wako Pure Chemical. *threo*-4-Hydroxy-L-lysine was purchased from Fluka Fine Chemicals.

*Others*—ATP (disodium salt), *α,β*-methyleneATP, and $tRNA^{Lys}$ from *E. coli* were the products of Sigma Chemical; dialysis membrane Spectra/POR 7 (molecular weight cut off, 10,000) was from Spectrum Medical; charcoal (washed with hydrochloride) from Nakarai Tesque; adenosine from Oriental Yeast; isatoic anhydride from Tokyo Kasei; ADP, AMP, GTP, CTP, and UTP from P-L Biochemicals. Silica gel plates DC-Alufolien Kieselgel 60 F254 were purchased from E. Merck. [4,5-$^3$H]L-Lysine was the product of NEN Research Products; $^{32}$P-pyrophosphate was from Amersham International; [2,8-$^3$H]adenosine 5′-triphosphate tetraammonium salt from Moravek Biochemicals; streptomycin sulfate from Meiji Seika Kaisha. DEAE-Toyopearl 650M and AF Red Toyopearl 650 ML were the products of Tosoh; Dyematrex Red A and Dyematrex Blue A were from Amicon; lysine-Sepharose 4B, Sephacryl S-200 Superfine, and a Mono Q HR5/5 prepacked column from Pharmacia Fine Chemicals; glass microfiber filter (GF/C) from Whatman. Unfractionated tRNA from *E. coli* MRE 600 was purchased from Boehringer Mannheim. All other chemicals were of reagent grade.

*Synthesis of Anthraniloyl-ATP*—Ant-ATP was synthesized according to the method of Hiratsuka (*24*), lyophilized, and stored at −20°C until use.

*Preparation of tRNA Mixture*—Unfractionated tRNA mixture was prepared from *E. coli* K12 cells by the method of Zubay (*25*) with some modifications (*26*). The tRNA mixture obtained was dissolved in sterile water and stored at −20°C until use.

*Protein Concentration*—Protein concentration was measured either by the method of Lowry *et al.* (*27*) with crystalline bovine serum albumin as the standard, or spectrophotometrically with the molar absorption coefficient, $\varepsilon$, at 280 nm of 71,600 $M^{-1} \cdot cm^{-1}$ at pH 8.0 (see "RESULTS").

*Aminoacylation Reaction Assay*—The aminoacylation of tRNA with radioactive L-lysine was used to measure the activity of LysRS. The standard reaction mixture contained, in 0.5 ml: 100 mM Tris-HCl buffer (pH 8.0), 10 mM $MgCl_2$, 1 mM ATP, 100 $\mu$M $^3$H-L-lysine (40 mCi/mmol), and 20 $A_{260}$ units of tRNAmix obtained from *E. coli* K12. After preincubation at 37°C for 3 min, the reaction was started by the addition of 50 $\mu$l of the enzyme solution. The reaction was stopped, after incubation at 37°C for suitable time intervals, by adding 3 ml of cold 5% trichloroacetic acid. The mixture was kept at 0°C for 30 min and filtered through a Whatman GF/C glass fiber filter. The radioactivity remaining on the filter was measured in a Packard liquid scintillation spectrometer Tri Carb 3255 as described previously (*28*). One unit of enzyme is defined as the amount that incorporates 1 $\mu$mol of L-lysine into tRNA in 1 min under these conditions. The $K_m$ and $V_{max}$ were calculated by the nonlinear least-squares method (*29*).

*ATP-$PP_i$ Exchange Reaction Assay*—LysRS activity was also measured by use of the ATP-$PP_i$ exchange reaction according to the method of Seno *et al.* (*30*) with some modifications. The standard reaction mixture contained, in 0.5 ml: 100 mM Tris-HCl buffer (pH 8.0), 10 mM $MgCl_2$, 1 mM $^{32}$P-pyrophosphate (6.4 mCi/mmol), 1 mM L-lysine, and 1 mM ATP. After preincubation at 37°C for 3 min, the reaction was started by the addition of 50 $\mu$l of the enzyme solution. The reaction was stopped, after incubation at 37°C for suitable time intervals, by adding 7.5% perchloric acid containing 100 mM sodium pyrophosphate. ATP was adsorbed on charcoal by adding 0.1 ml of charcoal suspension (10% w/v). After 10 min at 0°C the charcoal was collected on a Whatman GF/C filter, and the radioactivity trapped on the filter was counted in the Packard liquid scintillation spectrometer Tri Carb 3255 as described previously (*28*). The $K_m$ and $V_{max}$ were calculated as in the case of the aminoacylation reaction.

*Purification of LysRS*—*B. stearothermophilus* NCA1503 cells were supplied by Unitika, Ltd., and stored at −20°C until use. All operations were done at 4°C unless otherwise mentioned. The standard buffer was 20 mM Tris-HCl buffer (pH 8.0) containing 2 mM EDTA, 10 mM 2-mercaptoethanol, 0.1 mM phenylmethanesulfonyl fluoride (abbreviated as PMSF), and 0.2% (v/v) isopropyl alcohol.

*Step 1. Cell extraction: B. stearothermophilus* NCA1503

cells (300 g) kept frozen at −20°C were suspended in 1 liter of the standard buffer. The suspension was sonicated for 30 min in an ice bath. The sonicated suspension was centrifuged at 12,000×*g* for 60 min at 4°C and the supernatant fraction was termed the crude extract (750 ml).

*Step 2. Streptomycin precipitation:* Streptomycin sulfate was added to the crude extract to the final concentration of 2.5% with stirring in an ice bath. After 30 min of stirring, this solution was centrifuged at 8,000×*g* for 30 min, and the supernatant fraction was collected (880 ml).

*Step 3. DEAE-Toyopearl 650 M chromatography:* The supernatant solution obtained in Step 2 was loaded onto a DEAE-Toyopearl 650 M column (6.5×21 cm) equilibrated with the standard buffer. The active fractions were obtained by stepwise elution with 100 mM Tris-HCl buffer (pH 7.0) containing 0.17 M NaCl, 2 mM EDTA, 10 mM 2-mercaptoethanol, 0.1 mM PMSF, and 0.2% (v/v) isopropyl alcohol.

*Step 4. Dyematrex Blue A column chromatography:* The active fraction obtained in Step 3 was loaded onto a Dyematrex Blue A column (3.2×23 cm) equilibrated with the standard buffer. The active fractions were obtained by stepwise elution at 0.35 M NaCl.

*Step 5. Dyematrex Red A column chromatography:* The active fraction obtained in Step 4 was loaded onto a Dyematrex Red A column (2.8×20 cm) equilibrated with the standard buffer. The active fractions were eluted stepwise at 0.45 M NaCl, concentrated by ultrafiltration with a Diaflo membrane YM10 and then dialyzed against the standard buffer at 4°C.

*Step 6. Lysine-Sepharose 4B chromatography:* The dialyzed solution obtained in Step 5 was loaded onto a lysine-Sepharose 4B column (2.4×13 cm) equilibrated with the standard buffer. The enzyme was eluted with a linear concentration gradient of NaCl in the standard buffer. The fractions with enzyme activity that appeared at NaCl concentrations from 40 to 60 mM were pooled.

*Step 7. AF-Red Toyopearl 650 ML chromatography:* The active fraction obtained in Step 6 was diluted twice with the standard buffer and loaded onto an AF-Red Toyopearl 650 ML column (2.8×17 cm) equilibrated with the standard buffer. The enzyme was eluted with a linear concentration gradient of NaCl from 0.35 to 0.7 M. The pooled fraction with enzyme activity was concentrated by ultrafiltration with a Diaflo membrane YM10 and then with a Centriprep-10 concentration tube.

*Step 8. Column chromatography on Mono Q HR5/5:* In some batches of purification, the final LysRS preparation at Step 7 revealed slight contamination on PAGE, and Step 8 was applied in such cases.

The concentrated sample in Step 7 was applied to a Mono Q HR5/5 column equilibrated with 100 mM Tris-HCl buffer (pH 8.0) in a Pharmacia FPLC system at room temperature. The enzyme was eluted with a linear concentration gradient of NaCl from 0 to 1.0 M. The active fractions were pooled and dialyzed against the standard buffer, and this was stored at 4°C as the final preparation.

*Molecular Weight Determination*—The molecular weight of LysRS was measured both by gel filtration with a Sephacryl S-200 column (2.5×79 cm) and by SDS-PAGE with a Pharmacia Phast System with a PhastGel Gradient 8-25.

*Amino Acid Composition Analysis*—The purified LysRS was hydrolyzed *in vacuo* at 110°C either in 6 N HCl for 24, 48, and 72 h or in 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole for 24 h. Amino acid analysis was conducted using the ninhydrin reaction on a Hitachi 835 amino acid analyzer.

*Effect of pH*—In the aminoacylation reaction and the ATP-$PP_i$ exchange reaction, the enzyme activity was measured at 37°C in the pH range of 5-10 as described above, except that 10 mM piperazine-glycylglycine buffer or 100 mM glycine-NaOH buffer was used. All buffers contained 0.1 M NaCl and 10 mM $MgCl_2$. The enzyme concentrations were 0.8 and 4.0 nM in the aminoacylation and the ATP-$PP_i$ exchange reaction, respectively. To examine the pH stability, the enzyme solutions (11 nM) at different pH values were incubated at 37°C for 1-6 h. In the pH range 4.3-9.3, 10 mM piperazine-glycylglycine buffer was used. At pH 3.3 and 10.1, 100 mM glycine-HCl buffer and glycine-NaOH buffer were used, respectively. All buffers contained 0.1 M NaCl and 10 mM $MgCl_2$. The enzyme activity was measured in the aminoacylation reaction under the conditions described above at pH 8.0, 37°C, $[E]_0 = 1.0$ nM.

*Effect of Temperature*—The effect of temperature on the enzyme activity was investigated in the range of 0 to 70°C in the aminoacylation reaction and the ATP-$PP_i$ exchange reaction, both at pH 8.0. The enzyme concentration used was 1.7 nM in the aminoacylation reaction and 4.0 nM in the ATP-$PP_i$ exchange reaction. To examine heat stability, the enzyme solution [11 nM in 10 mM piperazine-glycylglycine buffer (pH 8.0) containing 0.1 M NaCl and 10 mM $MgCl_2$] was incubated in the range of 20 to 60°C for 1-6 h. The remaining activity was measured in the aminoacylation reaction under the conditions described above at pH 8.0, 37°C, $[E]_0 = 1.0$ nM.

*Fluorescence Spectroscopy*—Fluorescence spectra were measured at 30°C with a Hitachi 850 fluorescence spectrophotometer. The excitation spectra were obtained with the fixed emission wavelength of 340 nm, and the emission spectra were recorded with excitation at 280 and 295 nm. The purified LysRS was dialyzed, before the fluorescence measurement, against 100 mM Tris-HCl buffer (pH 8.0) containing 10 mM $MgCl_2$.

Fluorescence titration of *B.s.* LysRS with ligands was conducted at 30°C in the same buffer. The excitation wavelength and emission wavelength were 295 and 340 nm, respectively, when the quenching of LysRS fluorescence by the addition of L-lysine or ATP was measured. The excitation wavelength and emission wavelength were 375 and 428 nm, respectively, when quenching of Ant-ATP fluorescence was measured. The absorbance of the enzyme solution was less than 0.1 $cm^{-1}$ at the excitation wavelength, 295 nm. The fluorescence intensity was corrected for dilution of LysRS due to addition of the ligand solutions when the quenching of LysRS was measured. The enzyme solution was placed in a quartz cell thermostated at 30°C and then the ligand solutions were added to the enzyme solution with a micro syringe and the mixture was stirred. After 2 min, the fluorescence intensity was measured.

The values of the dissociation constant ($K_d$) for the enzyme-ligand complex (E·L) and of the fluorescence intensity decrease ($\Delta F_{max}$) at 340 nm (as a percentage) that would be observed when the enzyme was saturated with the ligand were determined by assuming a simple bimolecular

binding equilibrium between the enzyme, E, and ligand, L, (Eqs. 3, 4, and 5) using the nonlinear least-squares method (*29*).

$$E + L \rightleftharpoons E \cdot L \quad (3)$$

$$K_d = [E][L]/[E \cdot L] \quad (4)$$

$$\Delta F(\%) = \Delta F_{max}[L]_0/(K_d + [L]_0) \quad (5)$$

where $[L]_0$ is the total concentration of the ligand, $[L]_0 = [L] + [E \cdot L]$, and $\Delta F$ is the fluorescence intensity change observed at 340 nm when a certain amount of the ligand is added. This is expressed as a percentage of the fluorescence intensity of the enzyme; namely $\Delta F(\%) = 100 \times (F_{E \cdot L} - F_E)/F_E$, where $F_{E \cdot L}$ and $F_E$ are the fluorescence intensities at 340 nm of the enzyme-ligand complex and the enzyme, respectively.

*Equilibrium Dialysis*—All equilibrium dialysis experiments were done in a 4°C compartment. The buffer used for the experiments was 100 mM Tris-HCl buffer (pH 8.0) containing 10 mM $MgCl_2$. Each dialysis chamber was separated by membrane, Spectra/POR 7. In the examination of the binding between LysRS and L-lysine, one chamber (100 $\mu$l) contained 13.9 $\mu$M LysRS and the other (100 $\mu$l) contained $^3$H-L-lysine in the concentration range of 1 $\mu$M to 1 mM without ATP. For binding between LysRS and ATP, one chamber contained the same concentration of LysRS and the other contained $^3$H-ATP in the same concentration range without L-lysine. The apparatus was slowly rotated for 30 h at 4°C, then samples were withdrawn from each chamber and the radioactivity of each sample was measured in a Packard liquid scintillation spectrometer Tri Carb 3255. In the preliminary tests we found that 30 h was long enough for equilibrium to be reached under the conditions employed.

*Kinetic Determination of the Order of Substrate Binding*—Cole and Schimmel (*31*) and Santi *et al.* (*32*) have established procedures for the kinetic analysis of the ATP-$PP_i$ exchange reaction to determine the order of the binding of substrates among three possibilities: (a) AA binds first, (b) ATP binds first, and (c) AA and ATP bind in a random sequence. The effects of the substrate concentration on the initial velocity ($v$) of the ATP-$PP_i$ exchange reaction were examined at pH 8.0 and 37°C according to their procedures. The concentration of one substrate (called the "variable substrate" for convenience) was varied at each of several fixed concentrations of the other substrate (called the "fixed substrate" for convenience). The effects of dead-end inhibitors (substrate analogues), cadaverine and adenosine, on the reaction rate were also examined. Graphic patterns of the double-reciprocal plots, $1/v$ *versus* 1/[variable substrate], at several concentrations of the fixed substrate or the dead-end inhibitors were examined. In each case, replots of the slopes and the vertical intercepts in the double-reciprocal plots were made.

## RESULTS

*Purification, Molecular Weight, and Amino Acid Composition*—Three mg of highly purified LysRS was obtained from 300 g of frozen cells of *B. stearothermophilus*. The yield of activity in this novel purification procedure was about 30% with an overall purification of 1,100-fold (Table I). A single band was obtained both with PAGE and with SDS-PAGE. The molecular weight of LysRS was estimated to be 120,000 by gel filtration on a Sephacryl-S200 column and 60,000 by SDS-PAGE. The amino acid composition determined here is essentially consistent with the one reported by Samuelsson and Lundvik (*15*), except for some difference in the content of L-glutamic acid plus L-glutamine and that of L-alanine, and the minimum molecular weight calculated on this composition is about 57,700. Only one N-terminal amino acid sequence (Ser-His-Glu) has been obtained (Takita *et al.*, to be published). From those results we conclude that *B.s.* LysRS is a homodimer.

*UV Absorption Spectra and Molar Absorption Coefficient*—The UV absorption spectrum of LysRS was recorded at 25°C, pH 8.0 ($\lambda_{max} = 277$ nm, $\lambda_{min} = 250$ nm, $A_{min}/A_{max} = 0.44$). The observed molar absorption coefficient, $\varepsilon$,

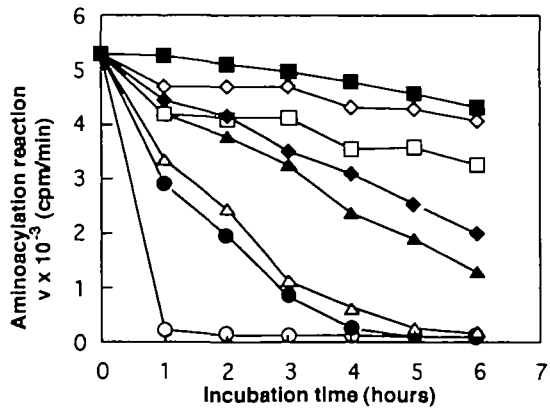


Fig. 1. **pH-stability of LysRS from *B. stearothermophilus*.** The remaining enzyme activity was measured in the aminoacylation reaction at pH 8.0 and 37°C, $[E]_0 = 1.0$ nM: (○), pH 3.3; (●), pH 4.4; (△), pH 5.4; (▲), pH 6.6; (□), pH 7.3; (■), pH 8.3; (◇), pH 9.3; (◆), pH 10.1. See the text for the detailed conditions.

TABLE I. **Purification of LysRS from *B. stearothermophilus*.**

| Purification step | Total protein (mg) | Total activity (units) | Specific activity[a] (units/g) | Yield (%) | Purification fold |
|---|---|---|---|---|---|
| Crude extract | 11,900 | 19.43 | 1.63 | 100 | 1 |
| Streptomycin sulfate precipitation | 5,350 | 11.85 | 2.22 | 61 | 1.4 |
| DEAE-Toyopearl 650 M | 2,000 | 10.88 | 5.44 | 56 | 3.3 |
| Dyematrex Blue A | 67.8 | 8.74 | 129 | 45 | 79 |
| Dyematrex Red A | 9.95 | 7.76 | 780 | 40 | 478 |
| Lysine-Sepharose 4B | 4.56 | 6.98 | 1,530 | 36 | 938 |
| AF-Red Toyopearl 650 ML | 3.15 | 5.64 | 1,790 | 29 | 1,098 |

[a]One unit is defined as the amount of the enzyme that forms 1 $\mu$mol of [$^3$H]Lys-tRNA in 1 min at 37°C.

at 280 nm is 71,600 $M^{-1}\cdot cm^{-1}$ ($A_{280}^{1\%}=6.18$) based on the molecular weight of the enzyme as a dimer, 115,400. The value is consistent with that of 71,700 $M^{-1}\cdot cm^{-1}$ ($A_{280}^{1\%}=6.19$) at pH 7.1, estimated from the content of the aromatic amino acids (3 Trp, 16 Tyr, and 24 Phe/57,700 protein).

*pH-Activity Profile and pH-Stability*—The pH-activity profile of our highly purified *B.s.* LysRS (data not shown) in the ATP-$PP_i$ exchange reaction is broader in the alkaline pH region than that of the aminoacylation reaction, though the optimum pH is 8.3 in both reactions. Examination of pH stability (Fig. 1) indicates that under our assay conditions for evaluating the pH-activity relationship (pH 6-10, 37°C, 5 min), denaturation of *B.s.* LysRS is negligible.

*Optimum Temperature and Thermostability*—The optimal temperature of *B.s.* LysRS is 50°C in both the aminoacylation reaction and the ATP-$PP_i$ exchange reaction at pH 8, and both enzyme activities were completely lost at 70°C (data not shown). Since both activities similarly decreased at above 50°C, this heat inactivation is not due to the denaturation of tRNA. *B.s.* LysRS is stable below 50°C at pH 8.0 for 5 h. At 60°C, however, the aminoacylation activity is lost completely after 4 h. We compared the thermostability of the aminoacylation reaction in the crude extracts of *E. coli* K-12 and *B. stearothermophilus* NCA-1503 at 50°C at pH 8.0. *E. coli* LysRS activity was completely lost within 5 min, while *B.s.* LysRS activity was unaffected after 60 min.

*Kinetic Parameters*—The $K_m$ values for L-lysine in the aminoacylation reaction and the ATP-$PP_i$ exchange reaction at pH 8.0 and 37°C were 16.4±3.9 and 23.6±2.1 $\mu$M, respectively, over the range of L-lysine concentration of 5-200 $\mu$M. The $K_m$ values for ATP at pH 8.0 and 37°C were 23.2±4.0 $\mu$M in the aminoacylation reaction over the range of ATP concentration of 25-400 $\mu$M, and 65.1±5.3 $\mu$M in the ATP-$PP_i$ exchange reaction over the ATP concentration range of 10-1,000 $\mu$M. Substrate inhibition by ATP was observed at concentrations of more than 400 $\mu$M in the aminoacylation reaction, but no such inhibition was detected in the ATP-$PP_i$ exchange reaction. The apparent $k_{cat}$ values were estimated as 3.2 $s^{-1}$ in the aminoacylation reaction and 40.9 $s^{-1}$ in the ATP-$PP_i$ exchange reaction.

*Fluorescence Spectrometry*—Figure 2 shows the fluorescence excitation and emission spectra of *B.s.* LysRS at pH 8.0 and 37°C. They are typical for a protein containing both tyrosine and tryptophan residues: $\lambda_{max}$ of excitation is 282 nm for the emission at 340 nm, and $\lambda_{max}$ of emission is 328 nm with excitation at 280 and 335 nm with excitation at 295 nm. A small shoulder was detected around 320 nm in the emission spectrum on excitation at 295 nm. At the excitation wavelength of 295 nm and emission wavelength of 340 nm, the relative fluorescence quantum yield of the enzyme was 0.27 when the relative quantum yield of *N*-acetyl-L-tryptophan ethyl ester was assumed to be 0.2 (*33*).

Addition of L-lysine to *B.s.* LysRS resulted in a decrease of the fluorescence intensity (Fig. 2), and the enzyme could be titrated with L-lysine, using the fluorescence quenching as a probe (Fig. 3). This titration curve was fitted to Eq. 5, and $K_d$ and $\Delta F_{max}$ were calculated to be 20.4±1.8 $\mu$M and −16.2±0.3%, respectively (Table II). On the other hand, the addition of ATP, other nucleotides, or Ant-ATP caused no appreciable change in the fluorescence intensity (Table II). These results are consistent with the results of

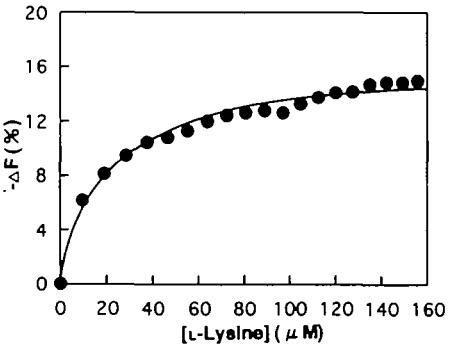


Fig. 3. **Fluorescence titration of LysRS with L-lysine.** $[E]_0=0.88$ $\mu$M, 10 mM $MgCl_2$, $\lambda_{ex}=295$ nm, $\lambda_{em}=340$ nm, pH 8.0, 30°C. The solid line is the theoretical curve drawn according to Eq. 5 with $K_d=20.1$ $\mu$M and $\Delta F_{max}=-16.2\%$.

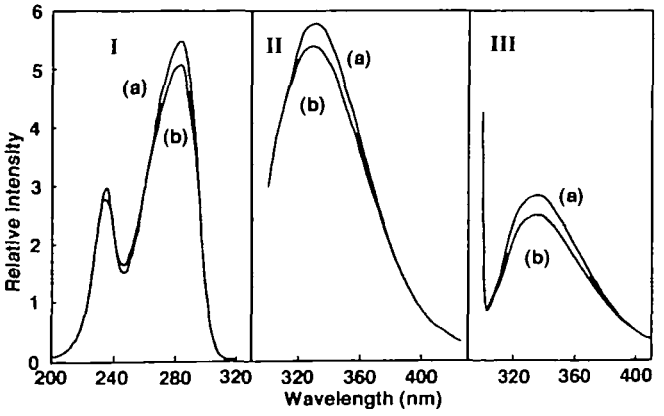


Fig. 2. **Fluorescence spectra of LysRS from *B. stearothermophilus* and effect of adding L-lysine.** I, excitation spectra for the emission at 340 nm; II, emission spectra with excitation at 280 nm; III, emission spectra with excitation at 295 nm. $[E]_0=4.9$ $\mu$M; pH 8.0, 30°C; (a), [L-lysine] = 0 $\mu$M; (b), [L-lysine] = 227 $\mu$M.

TABLE II. **Fluorescence titration of LysRS with L-lysine, ATP, and some other ligands.**

| Ligands | $K_d$ ($\mu$M) | $-\Delta F_{max}$ (%) |
|---|---|---|
| L-Lysine | 20.4± 1.8 | 16.2 |
| L-Glycine | 21,000 ±5,100 | 8.7 |
| L-Arginine | —[a] | 0 |
| L-Glutamine | 30,000 ±16,000 | 23.4 |
| L-Methionine | 24,400 ±11,000 | 14.1 |
| L-Histidine | — | 0 |
| L-Proline | — | 0 |
| L-Serine | — | 0 |
| ATP | — | 0 |
| ADP | — | 0 |
| AMP | — | 0 |
| GTP | — | 0 |
| CTP | — | 0 |
| UTP | — | 0 |
| $\alpha,\beta$-MethyleneATP | — | 0 |
| Ant-ATP[b] | — | 0 |

$[E]_0=0.88$ $\mu$M, $\lambda_{ex}=295$ nm, $\lambda_{em}=340$ nm, pH 8.0, 30°C. [a]—indicates that no fluorescence change could be detected. [b] $[E]_0=4.2$ $\mu$M, $\lambda_{ex}=375$ nm, $\lambda_{em}=428$ nm, pH 8.0, 30°C.

TABLE III. **Fluorescence titration and ATP-$PP_i$ exchange reaction of LysRS with lysine analogues.**

| | Fluorescence titration | | ATP-$PP_i$ exchange reaction | | | | $K_i/K_d$ (%) | $K_m/K_d$ (%) |
|---|---|---|---|---|---|---|---|---|
| | | | Kinetic parameters[a] | | Relative inhibition (%)[c] | Inhibitor constant $K_i$ ($\mu$M) | | |
| | $K_d$ ($\mu$M) | $-\Delta F_{max}$ (%) | $K_m$ ($\mu$M) | $k_{cat}$ ($s^{-1}$) | | | | |
| L-Lysine | 20.4± 1.8* | 16.2* | 23.6 ± 2.1 | 40.9[e]±0.6 | / | / | / | 115 |
| Other 19 amino acids | (see Table III) | | —[b] | — | N.D.[d] | N.D. | | / |
| $\alpha$-Carboxyl group modified | | | | | | | | |
| Cadaverine | 19,900±900 | 15.6 | — | — | 85 | 77 ± 8 | 0.4 | / |
| L-Lysine hydroxamate | 704± 79 | 11.0 | — | — | 100 | 0.6[e]± 0.2 | 0.08 | / |
| L-Lysine amide | 4,260±210 | 16.6 | — | — | 95 | 30[e]± 4 | 0.7 | / |
| $\alpha$-Amino group modified | | | | | | | | |
| 6-Amino-*n*-hexanoic acid | —** | 0 | — | — | 44 | 480 ±40 | | |
| $N\alpha$-Acetyl-L-lysine | — | 0 | — | — | 0 | / | / | / |
| $\varepsilon$-Amino group modified | | | | | | | | |
| L-Norleucine | — | 0 | — | — | 0 | / | / | / |
| $N\varepsilon$-Methyl-L-lysine | — | 0 | — | — | 0 | / | / | / |
| $N\varepsilon$-Acetyl-L-lysine | — | 0 | — | — | 13 | N.D. | / | |
| Others | | | | | | | | |
| D-Lysine | 3,180±180 | 10.2 | — | — | 68 | 120[e]±20 | 3.7 | / |
| *S*-(2-Aminoethyl)-L-cysteine | 197± 24 | 14.1 | 69.3[e]± 8.7 | 10.2[e]±0.5 | N.D. | N.D. | | 35.2 |
| *threo*-4-Hydroxy-L-lysine | 3,230±170 | 8.9 | 898 ± 68 | 36.6 ±1.1 | N.D. | N.D. | | 27.8 |
| 5-Hydroxylysine (mixed DL and DL-allo) | 18,600±600 | 19.0 | 5,450 ± 260 | 57.1 ±0.9 | N.D. | N.D. | | 29.3 |
| L-Ornithine | 14,700±900 | 8.2 | 11,900[e]±2,040 | 29.8[e]±2.1 | N.D. | N.D. | | 80.9 |

Fluorescence titration. $[E]_0=4.2\ \mu M$, $\lambda_{ex}=295$ nm, $\lambda_{em}=340$ nm, pH 8.0, 30°C. *$[E]_0=0.88\ \mu M$. **—indicates that no fluorescence change could be detected. ATP-$PP_i$ exchange reaction. [a]$[E]_0=15.9$ nM, 1 mM ATP, 1 mM $PP_i$, pH 8.0, 37°C. [b]—indicates that no ATP-$PP_i$ exchange activity could be detected. [c]Individual inhibitions were represented as follows: $(V-V_i)\times 100/V$, where $V$ is the initial velocity in the absence of lysine analogues and $V_i$ is that in the presence of 4 mM analogues. [d]N.D. indicates that this assay was not conducted. [e]These values appeared in a conference proceedings (*34*), and are cited here by permission of the New York Academy of Sciences.

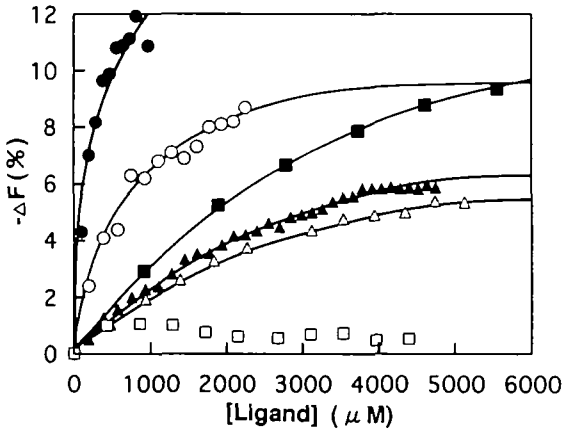


Fig. 4. **Fluorescence titration of LysRS with L-lysine analogues.** $[E]_0=4.2\ \mu M$, 10 mM $MgCl_2$, $\lambda_{ex}=295$ nm, $\lambda_{em}=340$ nm, pH 8.0, 30°C. *S*-(2-Aminoethyl)-L-cysteine (●), L-lysine hydroxamate (○), L-lysine amide (■), *threo*-4-hydroxy-L-lysine (△), D-lysine (▲), and 6-amino-*n*-hexanoic acid (□). The solid lines are the theoretical curves drawn according to Eq. 5 with a $K_d$ of 197 $\mu$M (●), 704 $\mu$M (○), 4,260 $\mu$M (■), 3,230 $\mu$M (△), and 3,180 $\mu$M (▲).

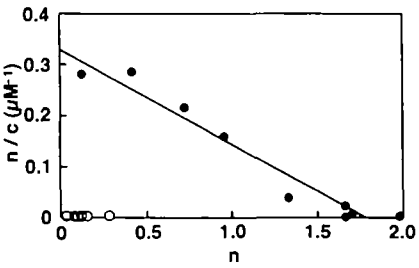


Fig. 5. **Scatchard plots for the binding of either L-lysine or ATP to LysRS as measured by equilibrium dialysis.** $[E]_0=13.9\ \mu M$, 10 mM $MgCl_2$, pH 8.0, 4°C, L-lysine binding (●), ATP binding (○). The solid line for L-lysine binding (●) was obtained by the least-squares method. $K_d=5.44\ \mu M$ and $n=1.79$.

the equilibrium dialysis study that will be presented below. Accordingly the lysine-induced quenching of *B.s.* LysRS fluorescence can be regarded as representing the ligand-enzyme binding.

*Fluorescence Titration of LysRS with Other Amino Acids*—The ligand induced quenching of LysRS fluorescence was measured with several amino acids. The fluorescence change was observed with L-glycine, L-glutamine, and L-methionine among the amino acids tested (Table II). The $K_d$s for these amino acids were larger than that of L-lysine by more than 1,000-fold, though $\Delta F_{max}$ was rather similar to that of L-lysine.

*Fluorescence Titration of LysRS with Lysine Analogues*—Lysine analogues whose addition to *B.s.* LysRS caused quenching of the enzyme fluorescence can be classified into 2 groups (Table III). The first group contains $\alpha$-carboxyl group-modified compounds: L-lysine hydroxamate, L-lysine amide, and cadaverine. The second group contains D-lysine and the side-chain-modified compounds: *S*-(2-aminoethyl)-L-cysteine, *threo*-4-hydroxy-L-lysine, 5-hydroxylysine, and L-ornithine. Figure 4 shows the titration curves with *S*-(2-aminoethyl)-L-cysteine, L-lysine-hydroxamate, and D-lysine, for which the $K_d$'s were calculated by means of Eq. 5 to be 197±24, 704±79, and 3,180±180 $\mu$M, respectively.

*Equilibrium Dialysis*—The binding of L-lysine and ATP to *B.s.* LysRS were examined separately by the equilibrium dialysis method at pH 8.0 and 5°C, with $^3$H-labeled

TABLE IV. **Effects of substrates and inhibitors on the ATP-$PP_i$ exchange reaction.**

| Fixed substrate or inhibitor | L-Lys | ATP | Cadaverine | | Adenosine | |
|---|---|---|---|---|---|---|
| Variable substrate | ATP | L-Lys | ATP | L-Lys | ATP | L-Lys |
| Obtained pattern | B | A | B | B | C | B |
| Ordered: AA first | B | A | B | B | C | B |
| Ordered: ATP first | A | A | A | C | B | A |
| Ordered: ATP first[a] | A | B | B | C | B | B |
| Random | A | A | B | A | B | A |

A: Intersection is to the left of the vertical axis. B: Intersection is on the vertical axis. C: Parallel. [a]Rapid equilibrium (*31, 32*).

substrates. Figure 5 indicates that $1.79 \pm 0.28$ mol of L-lysine are bound to a mole of dimer enzyme with an apparent $K_d$ of $5.44 \pm 0.41$ $\mu$M, and that ATP binding did not take place in the ATP concentration range of 1 $\mu$M–1 mM. These findings are consistent with the results of fluorometric studies shown above (Table II).

*ATP-$PP_i$ Exchange Reaction Activity*—All 20 constituent L-amino acids of proteins were tested for amino acid-dependent ATP-$PP_i$ exchange reaction catalyzed by *B.s.* LysRS. No activity was detected with 19 amino acids (*i.e.*, all except L-lysine) either at 1 or 10 mM (Table III). Lysine analogues were also examined in the same manner. The activity was detected with *S*-(2-aminoethyl)-L-cysteine, L-ornithine, 5-hydroxylysine, and *threo*-4-hydroxy-L-lysine. The estimated $K_m$ and $k_{cat}$ values are listed in Table III.

The enzymatic, L-lysine-dependent synthesis of 5′,5′-diadenosine tetraphosphate ($Ap_4A$) was observed by HPLC with a Novapack $C_{18}$ column (data not shown) when 100 nM *B.s.* LysRS was incubated with 2 mM L-lysine and 5 mM ATP in 20 mM Tris-HCl buffer (pH 7.6) containing 150 mM KCl and 130 $\mu$M $ZnCl_2$ at 37°C for 2–6 h.

*Inhibition of the ATP-$PP_i$ Exchange Reaction by Lysine-Analogues*—The inhibitory effects of lysine analogues on the L-lysine-dependent ATP-PPi exchange reaction were examined (Table III). Relative inhibition is represented as a percentage of the initial rate according to Eq. 6.

$$\text{Inhibition } (\%) = (v - v_i) \times 100/v \qquad (6)$$

where $v$ is the initial rate without inhibitor and $v_i$ is the initial rate in the presence of the inhibitor at 4 mM.

Those compounds in which the $\alpha$-carboxyl group was modified strongly inhibited the reaction. Among the compounds in which the $\varepsilon$- or $\alpha$-amino group was modified, only 6-amino-*n*-hexanoic acid caused appreciable inhibition. D-Lysine effectively inhibited the reaction. The estimated inhibitor constants ($K_i$s) are shown in Table III.

*Kinetic Analysis of the Order of Binding of L-Lysine and ATP to B.s. LysRS*—When L-lysine was the variable substrate with different but fixed concentrations of ATP in the ATP-$PP_i$ exchange reaction, the point of intersection in double-reciprocal plots of the reaction rate and the variable substrate concentration was to the left of the vertical axis, a pattern similar to that seen in non-competitive type inhibition. The replot of the slopes of the plots *versus* 1/[ATP] showed a straight line passing through the origin. On the other hand, when ATP was the variable substrate with different but fixed concentrations of L-lysine, the point of intersection of the double reciprocal plots was on the vertical axis, a pattern similar to that of competitive inhibition. The replot of the slopes *versus* 1/[L-Lysine] showed a straight line that does not pass through the origin. These patterns are consistent with those of the ordered bireactant mechanism, where an amino acid is first bound to the enzyme (*31, 32*) (Table IV).

Inhibition studies are useful to determine the order of substrate binding. Cadaverine and adenosine were used as dead-end inhibitors against L-lysine and ATP, respectively. Cadaverine acts as a competitive inhibitor, regardless of whether the variable substrate is L-lysine or ATP. Adenosine acts as an uncompetitive inhibitor of L-lysine and as a competitive inhibitor of ATP. These patterns also suggest that L-lysine is first bound to LysRS (*31, 32*) (Table IV). The inhibitor constants ($K_i$) of cadaverine and adenosine are $77.1 \pm 8.1$ and $552 \pm 43$ $\mu$M, respectively. We think this is the first report that has proved by kinetic analysis the occurrence of sequential ordered substrate binding (amino acid first) in the reaction of Eq. 1 (see "DISCUSSION").

## DISCUSSION

*Purification, Molecular Weight, Amino Acid Composition, and Basic Properties*—We have presented a novel purification procedure with a good yield of *B.s.* LysRS (Table I). There has been one report describing the purification of *B.s.* LysRS (*15*) and several reports on the purification of *E. coli* LysRS (*16–20*) and yeast LysRS (*21, 22*). The molecular weight and the amino acid composition of *B.s.* LysRS obtained here are essentially consistent with those previously reported (*15*). The values of $K_m$s in the ATP-$PP_i$ exchange reaction (23.6 $\mu$M for L-lysine and 65.1 $\mu$M for ATP, at pH 8.0, 37°C) are comparable to those previously reported (60 $\mu$M for L-lysine and 20 $\mu$M for ATP, at pH 7.0, 40°C) (*15*). Accordingly, we assume that we have isolated the same enzyme as that reported by Samuelsson and Lundvik (*15*), despite considerably different purification procedures. The molecular weight, quaternary structure (a homodimer), and pH profile of the enzyme activity of *B.s.* LysRS are essentially similar to those of *E. coli* LysRS (*16, 35*). However, *B.s.* LysRS is more thermostable than the *E. coli* enzyme.

*Binding of L-Lysine and ATP to B.s. LysRS*—The present results of fluorescence titration with the substrates of *B.s.* LysRS are different from those reported previously with *B.s.* ValRS (a monomer, Class I enzyme), in which the addition of either L-valine or ATP led to a quenching of protein fluorescence and the effects were additive (*28, 36*). We found with *B.s.* LysRS that the addition of L-lysine to the enzyme led to a decrease of the protein fluorescence (Figs. 2 and 3) but that the addition of ATP did not cause any appreciable fluorescence change. The fluorometrically estimated $K_d$ for L-lysine ($20.4 \pm 1.8$ $\mu$M) agrees with the $K_m$ for L-lysine in the aminoacylation reaction ($16.4 \pm 3.9$ $\mu$M) and in the ATP-$PP_i$ exchange reaction ($23.6 \pm 2.1$ $\mu$M), and roughly with $K_d$ obtained in the equilibrium dialysis ($5.44 \pm 0.41$ $\mu$M) (Fig. 5). This indicates that the observed fluorescence quenching is a genuine reflection of L-lysine binding to *B.s.* LysRS, and that a tryptophan residue may be located in the vicinity of the L-lysine binding site of *B.s.* LysRS. A small bump is apparent in the fluorescence titration curve (Fig. 3) at around 100 $\mu$M L-lysine. This was a reproducible observation, but its significance remains unclear.

The results of the equilibrium dialysis study (Fig. 5) suggest that 2 mol of L-lysine is bound to 1 mol of dimer *B.s.* LysRS in the range of 1 $\mu$M to 1 mM L-lysine with the same binding constants. The apparently identical two subunits of *B.s.* LysRS seem equivalent also in function at this stage of the reaction. This is similar to the observation with *E. coli* LysRS (*37*).

That the addition of ATP alone to the enzyme did not cause any fluorescence change may suggest either that ATP is not bound to LysRS in the absence of L-lysine or that ATP alone is bound to the enzyme but no tryptophan residues are located in the vicinity of the ATP-binding site. The former possibility is favored because Ant-ATP, a fluorescent derivative, did not show any change of its own fluorescence when added to *B.s.* LysRS. A considerable change of Ant-ATP fluorescence was observed when the ATP derivative was added to *B.s.* ValRS (*38*). The results of the equilibrium dialysis study (Fig. 5) support this conclusion. In the case of *E. coli* LysRS, however, 2 mol of ATP was reported to be bound without L-lysine (*37*). It is interesting that the primary structure of *B.s.* LysRS (Takita *et al.*, to be published) shows a fairly high homology (more than 50%) to *E. coli* LysRS, and yet the binding mechanism of ATP might be different in the two LysRSs. The recent report on the three-dimensional structure of *E. coli* LysRS (u) complexed with L-lysine (*9*) provided no experimental data for ATP binding.

*Discrimination of Amino Acids*—It was reported with *E. coli* LysRS (*17*) that supplementation of each of the 19 L-amino acids other than L-lysine that constitute proteins caused no inhibition of the tRNA aminoacylation reaction, and that no aminoacyl-tRNA could be detected upon substitution of $^{14}$C-L-lysine with other $^{14}$C-labeled amino acids. The question arises, which step of the reaction, Eq. 1 or 2, contributes more to this specificity of LysRS for the amino acid substrate?

In the present study, the results of fluorometric titration suggest that *B.s.* LysRS may discriminate L-lysine from the other 19 protein-constituting amino acids mainly at the binding step, since there was only with very weak interaction with a few other amino acids (Table II). The fluorometric titration experiments with amino acids were also performed in the presence of ATP, when aminoacyladenylate could be formed; however, no indication of binding could be obtained except for L-lysine (data not shown). It was reported that *B.s.* ValRS bound L-threonine, L-isoleucine, L-glutamic acid, L-leucine, and D-valine, and misactivated L-threonine (*36, 39*). However, *B.s.* LysRS recognized only L-lysine as a substrate in the ATP-$PP_i$ exchange reaction (Table III), completely discriminating L-lysine from the other 19 amino acids at the step of lysyladenylate formation (Eq. 1). Freist *et al.* investigated discrimination between L-lysine and the other 19 amino acids in the aminoacylation reaction with the native and the phosphorylated yeast LysRS and reported that L-cysteine was transferred to $tRNA^{Lys}$ effectively by both preparations of LysRS (*40*).

*Discrimination of Lysine Analogues*—The significance of three functional groups of the lysine molecule, the $\varepsilon$-amino group, $\alpha$-amino group, and $\alpha$-carboxyl group, in the substrate binding was examined by fluorometric titration. All $\alpha$-carboxyl group-modified analogues, L-lysine amide, L-lysine hydroxamate, and cadaverine, caused a decrease of the fluorescence of *B.s.* LysRS upon being added (Table III). The difference in $K_d$ between L-lysine and cadaverine shows that the $\alpha$-carboxyl group is important, but not indispensable for the binding to *B.s.* LysRS. The comparison of $K_d$ of L-lysine amide with that of L-lysine hydroxamate suggests the importance of the hydroxyl group introduced in L-lysine hydroxamate.

In the cases of the $\alpha$-amino group-modified compounds, the fluorescence of *B.s.* LysRS was not decreased by the addition of 6-amino-*n*-hexanoic acid or $N\alpha$-acetyl-L-lysine below 20 mM. These results suggest that the positive charge of the $\alpha$-amino group is important for the binding to the enzyme. The fluorescence intensity of *B.s.* LysRS did not change at all upon addition of any of the $\varepsilon$-amino group-modified analogues, suggesting that the $\varepsilon$-amino group is indispensable for the recognition of L-lysine at the binding step.

The recent X-ray crystallographic analysis of *E. coli* LysRS (u) complexed with L-lysine (*9*) shows that these 3 functional groups of L-lysine construct hydrogen-bond networks with the enzyme, indicating the importance of these groups for the substrate recognition. Some lysine analogues that have these three functional groups were also examined in the fluorometric titration (Table III). They were L-ornithine, in which the length of the side chain is shorter than in L-lysine; D-lysine; and compounds that have modification on the side-chains, *i.e.*, *S*-(2-aminoethyl)-L-cysteine, *threo*-4-hydroxy-L-lysine, and 5-hydroxylysine (mixed DL and DL-allo). Comparison of $K_d$ of L-ornithine with that of D-lysine indicates that the position of the amino group of the side-chain is more important than the enantiomeric configuration. The results with the analogues having side-chain modification show that the hydrophobicity of the methylene groups of the side-chain seems to be important in the binding process.

Among those compounds that were suggested by the fluorescence titration to be bound to *B.s.* LysRS, *S*-(2-aminoethyl)-L-cysteine, *threo*-4-hydroxy-L-lysine, 5-hydroxylysine, and L-ornithine were substrates of the ATP-$PP_i$ exchange reaction (Table III). The $K_m$ for *S*-(2-aminoethyl)-L-cysteine is much smaller than those for the other three analogues, and this agrees with the results of fluorometric titration experiments. We could not detect appreciable activity of the D-lysine-dependent ATP-$PP_i$ exchange reaction by *B.s.* LysRS. It was reported with *E. coli* LysRS (*16*) that *S*-(2-aminoethyl)-L-cysteine and 5-hydroxylysine could replace L-lysine in the L-lysine-dependent ATP-$PP_i$ exchange reaction with $K_m$ values of 1.4 and 300 $\mu$M, respectively, and that DL-ornithine was not a substrate, but an inhibitor. It was also reported with *E. coli* LysRS (*17*) that 4-oxalysine, 2,6-diamino-4-hexynoic acid, and *trans*-4-dehydrolysine were substrates in the ATP-$PP_i$ exchange reaction, and that *cis*-4-hydroxylysine was not a substrate, but a competitive inhibitor for the reaction.

Each of the $\alpha$-carboxyl group-modified analogues strongly inhibited the L-lysine-dependent ATP-$PP_i$ exchange reaction (Table III). It was reported with *E. coli* LysRS (*41*) that L-lysine amide, L-lysine hydrazide, L-lysine methyl ester, and L-lysine ethyl ester competitively inhibited the aminoacylation reaction and that the $K_i$ values for these derivatives were 7.7, 400, 100, and 66 $\mu$M, respectively, while $K_m$ for L-lysine was 2.5 $\mu$M. It was also reported with TyrRSs of *E. coli* and *Bacillus subtilis* (*42*) that L-tyrosine

analogues in which the carboxyl group was modified competitively inhibited the ATP-$PP_i$ exchange reaction and that the $K_i$ values were quite small (in some cases even smaller than $K_m$ for L-tyrosine). These results suggest that the $\alpha$-carboxyl group-modified analogues are good competitive inhibitors for all ARSs. For the $\varepsilon$-amino group- and $\alpha$-amino group-modified analogues, however, no appreciable inhibition was observed except for 6-amino-*n*-hexanoic acid (Table III). The comparison of either $K_m$ or $K_i$ estimated in the ATP-$PP_i$ exchange reaction with $K_d$ observed in the fluorescence titration (Table III) indicates that the binding of those analogues to the enzyme is enhanced by the presence of ATP.

*Order of Substrate Binding*—ARSs were divided into two groups based on the kinetically determined order of substrate binding in the ATP-$PP_i$ exchange reaction (*43*). One group included ARSs that followed the sequential ordered mechanism (ATP binds first): LeuRS and SerRS of *E. coli* B, TrpRS of bovine pancreas, and ValRS of yeast. The other group included ARSs that followed the random sequence mechanism: PheRS of *E. coli* B, *E. coli* K12, and yeast; TyrRS of *E. coli*, MetRS of *E. coli*, and ValRS of *B. stearothermophilus*. Freist *et al.* investigated the order of substrate addition to ARSs in the aminoacylation reaction and reported that the addition of inorganic pyrophosphatase changed the aminoacylation mechanisms of ARSs (*44, 45*). In either case, however, the addition of the amino acid and ATP followed either a random or an ordered (ATP first) mechanism.

As far as we know, no ARS that follows the sequential ordered binding mechanism (amino acid first) has been identified by kinetic analysis except for our present study on *B.s.* LysRS (Table IV). Our kinetic conclusion is entirely consistent with the results of fluorescence titration (Table II) and equilibrium dialysis (Fig. 5) and with the recent report on X-ray crystallographic analysis of *E. coli* LysRS (u) complexed with L-lysine (*9*).

From the conclusion thus obtained, the activation of L-lysine by *B.s.* LysRS (Eq. 1) should be expanded at least to the following three steps:

$$E + \text{L-lysine} \rightleftharpoons E \cdot \text{L-lysine} \quad (7)$$

$$E \cdot \text{L-lysine} + ATP \rightleftharpoons E \cdot \text{L-lysine} \cdot ATP \quad (8)$$

$$E \cdot \text{L-lysine} \cdot ATP \rightleftharpoons E \cdot \text{L-lysine-AMP} + PP_i \quad (9)$$

The structural features that make *B.s.* LysRS follow this unique sequential order are of interest.

The three-dimensional structure of TyrRS, a Class I enzyme, from *B. stearothermophilus* complexed with tyrosyladenylate suggests that L-tyrosine has to bind before ATP, otherwise its binding is blocked (*5*). The three-dimensional structure of yeast AspRS complexed with aspartyladenylate, which is a Class II enzyme and is considered to belong to the same subclass as LysRS, indicates that the access of L-aspartic acid to the enzyme is blocked when ATP is in place and that L-aspartic acid has to enter the active site first (*14*).

On the other hand, kinetic analyses of the *B.s.* TyrRS reaction implied a random order mechanism (*46, 47*), and this is in apparent contradiction to the structural information described above (*2, 5*). We can consider that the random order mechanism is the most general to represent the binding of two substrates, and that the sequential ordered mechanisms are the extreme cases in which the equilibrium of a step is shifted extremely in one direction. We have shown here that *B.s.* LysRS follows the sequential ordered mechanism (L-lysine first), but it is noteworthy that there are a few hints to suggest that *E. coli* LysRS may follow the random order mechanism. One of the two active fractions of *E. coli* LysRS bound L-lysine and ATP independently (*37*), and the affinity labeling of *E. coli* LysRS with adenosine di or triphosphopyridoxals proceeded in the absence of L-lysine (*48*).

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