J. Biochem. 121, 244-250 (1997)

Lysyl-tRNA Synthetase from *Bacillus stearothermophilus*. Formation and Isolation of an Enzyme·Lysyladenylate Complex and Its Analogue[1]

Teisuke Takita, Satoshi Hashimoto, Yuji Ohkubo, Takanori Muto, Naofumi Shimizu, Tokuo Sukata, Kuniyo Inouye, Keitaro Hiromi, and Ben'ichiro Tonomura[2,3]

Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Kitashirakawa, Kyoto, Kyoto 606-01

Received for publication, September 17, 1996

The formation of an enzyme·lysyladenylate complex was studied with a highly purified lysyl-tRNA synthetase [L-lysine:tRNA^{Lys} ligase (AMP-forming); EC 6.1.1.6] from *Bacillus stearothermophilus*. The apparent dissociation equilibrium constants of the enzyme with L-lysine and ATP in the process of the complex formation were estimated to be 50.9 and 15.5 μM, respectively, at pH 8.0, 30°C, by fluorometric measurement. The isolated enzyme·lysyladenylate complex was relatively stable with a rate constant of decomposition of 1.7×10^{-5} s^{-1} at pH 8.5 and 0°C. The rate constant of transfer of L-lysine from the complex to *Escherichia coli* tRNA was 1.2×10^{-2} s^{-1} at pH 8.5 and 0°C. The effects of replacing L-lysine by several analogues on the complex formation were examined. L-Lysine hydroxamate, a strong inhibitor of the L-lysine dependent ATP-PP$_i$ exchange reaction, produced a stable complex with the enzyme and ATP, enzyme·lysinehydroxamate-AMP probably being formed. The binding stoichiometry of the assumed L-lysinehydroxamate-AMP per mol of the dimer enzyme was 1:1.

Key words: aminoacyl-tRNA synthetase, fluorescence titration, lysine hydroxamate, lysyladenylate, lysyl-tRNA synthetase.

Aminoacyl-tRNA synthetase (ARS) is a key figure ensuring the accuracy of translation of a genetic message into a protein structure (*1*). A high degree of substrate specificity is required for the reaction of ARS. Elucidation of the molecular mechanism by which this high degree of substrate specificity is exerted should be important for understanding the process of life.

The aminoacylation of tRNA usually involves two main steps (*1*): the activation of amino acids with ATP yielding an enzyme-aminoacyl adenylate complex (Eq. 1), and the transfer of the amino acid to tRNA from the enzyme-aminoacyl adenylate complex (Eq. 2).

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

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

where AA denotes an amino acid; E, the enzyme; and PP$_i$, inorganic pyrophosphate. An individual ARS specific for each of the 20 amino acids constituting proteins has to strictly discriminate its cognate amino acid and tRNA among like compounds *in vivo*.

ARSs are classified into two groups (Classes I and II) based on characteristic sequences in the primary structure. The catalytic domain of Class I enzymes is different from that of Class II enzymes (*2-4*). The three-dimensional structures of eleven of twenty ARSs have been or are being resolved by X-ray crystallographic analysis (*5*).

We have been interested in elucidating the molecular mechanism of substrate recognition by ARS, and have chosen as a target lysyl-tRNA synthetase [L-lysine: tRNA^{Lys} ligase (AMP-forming)], [EC 6.1.1.6], of *B. stearothermophilus* (*B.s.* LysRS), a Class II enzyme. A comprehensive review article on LysRS recently appeared (*6*). In the previous paper (*7*), we revealed for the first time, based on the results of fluorometric analysis, equilibrium dialysis, and kinetic analysis of the L-lysine dependent ATP-PP$_i$ exchange reaction, that the binding of the substrates, L-lysine and ATP, proceeded through a sequential ordered mechanism in which L-lysine was bound first to the enzyme. Accordingly, we have assumed that Eq. 1 should be expanded at least to Eqs. 3, 4, and 5 in the case of *B.s.* LysRS:

$$E+L\text{-}Lysine \rightleftharpoons E\cdot L\text{-}Lysine \quad (3)$$

$$E\cdot L\text{-}Lysine+ATP \rightleftharpoons E\cdot L\text{-}Lysine\cdot ATP \quad (4)$$

[1] This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, and from the Japan Foundation of Applied Enzymology.

[2] Present address: Research Institute for Biology-Oriented Science and Technology, Kinki University, 930 Nishimitani, Uchita-cho, Naga-gun, Wakayama 649-64.

[3] To whom correspondence should be addressed.

Abbreviations: ARS, aminoacyl-tRNA synthetase; *B.s.* ARS, ARS from *Bacillus stearothermophilus*; LysRS, lysyl-tRNA synthetase; SAEC, *S*-(2-aminoethyl)-L-cysteine; L-Lyshxt, L-lysine hydroxamate; $K_{d,app}$, apparent K_d; ^{3}H-L-lysine, [4,5-^{3}H]L-lysine; ^{3}H-ATP, [2,8-^{3}H]adenosine 5′-triphosphate; ^{32}P-ATP, adenosine 5′-[γ-^{32}P]-triphosphate. The other aminoacyl-tRNA synthetases are also abbreviated as the three-letter symbol for their specific amino acid followed by RS.

$$E \cdot L\text{-Lysine} \cdot ATP \rightleftharpoons E \cdot L\text{-Lys} \sim AMP + PP_i \quad (5)$$

In the present study, in order to verify this assumption, we have examined the formation of the enzyme·lysyladenylate complex by fluorometric measurement of the binding of ATP and L-lysine or its analogues when they exist together. We have also tried to isolate the enzyme·lysyladenylate complex and an enzyme·lysinehydroxamate-AMP complex to determine the binding stoichiometry.

MATERIALS AND METHODS

Enzymes—*B.s.* LysRS was purified from *B. stearothermophilus* according to the method described previously (*7*). The enzyme is a homodimer, of which the molecular weight of the subunit is 57,700. The enzyme concentration was determined spectrophotometrically with a molar absorption coefficient, ε, at 280 nm of 71,600 $M^{-1} \cdot cm^{-1}$ at pH 8.0.

L-Lysine and Lysine Analogues—L-Lysine and *S*-(2-aminoethyl)-L-cysteine (SAEC) were products of Wako Pure Chemical Industries. Cadaverine, 6-amino-*n*-hexanoic acid, L-norleucine, L-ornithine, and D-lysine were purchased from Nacalai Tesque. L-Lysine hydroxamate (L-Lyshxt), L-lysine amide, and 5-hydroxylysine (mixed DL and DL-allo) were obtained from Sigma Chemical. *Threo*-4-hydroxy-L-lysine was purchased from Fluka Fine Chemicals.

Others—Anthraniloyl-ATP was synthesized according to the method of Hiratsuka (*8*). Adenosine 5′-[γ-^{32}P]triphosphate was obtained from Amersham International; [2,8-^{3}H]adenosine 5′-triphosphate (tetraammonium salt) from Moravek Biochemicals; [4,5-^{3}H]L-lysine from NEN Research Products; ATP (disodium salt) and α,β-methylene-ATP from Sigma Chemical; and glass microfiber filters (GF/C) from Whatman. Sephacryl S-200 and NAP-10 columns (columns prepacked with Sephadex G-25) were purchased from Pharmacia Fine Chemicals. Unfractionated tRNA from *Escherichia coli* MRE 600 was purchased from Boehringer Mannheim. All other chemicals were of reagent grade.

Fluorescence Titration—The purified LysRS was dialyzed, before the fluorescence measurement, against 100 mM Tris-HCl buffer (pH 8.0) containing 10 mM $MgCl_2$. Fluorometric titration was conducted at 30°C in the same buffer with a Hitachi Fluorescence Spectrophotometer 850. The excitation and emission wavelengths were 295 and 340 nm, respectively. When the fluorometric titration of a ligand was performed in the absence of the other ligand, K_d and ΔF_{max} were determined by the methods previously reported (*7*). When the fluorometric titration of a ligand, L_1, was carried out in the presence of a saturating amount of the other ligand, L_2, the apparent K_d ($K_{d,app}$) and ΔF_{max1} were determined according to Eqs. 6-8 using the nonlinear least-squares method (*9*).

$$E \cdot L_2 + L_1 \rightleftharpoons E \cdot L_2 \cdot L_1 \quad (6)$$

$$K_{d,app} = [E \cdot L_2][L_1]/[E \cdot L_2 \cdot L_1] \quad (7)$$

$$\Delta F(\%) = \Delta F_{max1}[L_1]_0/(K_{d,app} + [L_1]_0) \quad (8)$$

where $\Delta F(\%) = 100(F_{E \cdot L_2 \cdot L_1} - F_{E \cdot L_2})/F_E$; and $F_{E \cdot L_2 \cdot L_1}$, $F_{E \cdot L_2}$, and F_E are the fluorescence intensities at 340 nm of the enzyme-ligand$_2$-ligand$_1$ complex, the enzyme-ligand$_2$ complex, and the free enzyme, respectively.

Enzyme Assay—The activity of *B.s.* LysRS was measured by means of either the ATP-PP_i exchange reaction with [^{32}P]PP_i or the aminoacylation of tRNA with [4,5-^{3}H]L-lysine as described previously (*7*).

Filter Assay—After preincubation of the reaction mixture without the enzyme (20 μl) at 37°C for 2 min, the enzyme solution (20 μl) was added to it. The final reaction mixture comprised, in 40 μl: 100 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 4.65 μM LysRS, and either 200 μM [4,5-^{3}H]L-lysine (^{3}H-L-lysine) (0.5 Ci/mmol) plus 1 mM ATP or 1 mM L-lysine plus 200 μM [2,8-^{3}H]ATP (^{3}H-ATP) (0.5 Ci/mmol). After incubation at 37°C for several different times, the reaction mixtures were placed in an ice bath for 5 min to stop the reaction. An aliquot (up to 40 μl) of each reaction mixture was passed through a Whatman GF/C glass fiber filter that had been immersed in 100 mM Tris-HCl buffer (pH 8.0) containing 10 mM $MgCl_2$ overnight. The filter was washed six times each with 3 ml of ice-cold 100 mM Tris-HCl buffer (pH 8.0) containing 10 mM $MgCl_2$. After the filter had been dried under an IR lamp, the radioactivity remaining on the filter was measured with a Packard Liquid Scintillation Spectrometer Tri Carb 3255 similarly to in the case of tRNA aminoacylation. When adenosine 5′-[γ-^{32}P]triphosphate (^{32}P-ATP) was to be used, ^{3}H-ATP was substituted by ^{32}P-ATP.

Isolation of the Enzyme·Lysyladenylate Complex by Gel-Filtration—The reaction mixture comprised, in 0.4 ml: 100 mM Tris-HCl buffer (pH 8.0), 10 mM $MgCl_2$, 25 μM [^{3}H]-L-lysine (1.0 Ci/mmol), 1 mM ATP, and 2.2 μM *B.s.* LysRS. After incubation at 37°C for 4 min, the reaction mixture was applied to a Sephacryl S-200 column (ϕ 2×50 cm) equilibrated with 100 mM Tris-HCl buffer (pH 8.0) containing 10 mM $MgCl_2$, and eluted with the same buffer at 4°C. Each fraction of the eluate was subjected to the filter assay (see the previous section), measurement of the absorbance at 260 nm, and total radioactivity measurement. The total radioactivity was measured as follows: the sample solution was spotted on a glass fiber filter, which was dried under an IR lamp without washing, and then the radioactivity on the filter was measured as in the case of the filter assay.

Stability of the LysRS·Lysyladenylate Complex and Transfer of L-Lysine from the Complex to tRNA—The LysRS·lysyladenylate complex labeled with [^{3}H]lysine was obtained by chromatographic separation at pH 8.5 on a Sephacryl S-200 column and held in an ice bath until use. For investigation of the stability of the LysRS·lysyladenylate complex, aliquots of the complex solution were subjected to the filter assay after different time intervals. The transfer of L-lysine to tRNA was started by adding unfractionated *E. coli* tRNA to the above mentioned LysRS·lysyladenylate complex at 0°C, pH 8.5, and stopped by adding 6 volumes of 5% trichloroacetic acid after different time intervals.

RESULTS

Fluorescence Change of B.s. LysRS on the Addition of ATP in the Presence of L-Lysine or Lysine Analogues—The addition of ATP to *B.s.* LysRS that had been saturated with L-lysine caused an apparent increase in the protein fluorescence; this may be expressed alternatively as that the fluorescence quenching of LysRS caused by the binding

of L-lysine (7) was restored on the addition of ATP (Fig. 1). This is different from the observation for *B.s.* ValRS (a monomer, Class I enzyme) reported previously, in which the addition of either L-valine or ATP lead to the quenching of protein fluorescence and the effects were additive (*10, 11*). This restoration may be regarded as a reflection of either ATP binding to LysRS (Eq. 4) or the formation of lysyladenylate on the enzyme (Eq. 5), or both, because the conditions employed were the same as those for the L-lysine dependent ATP-PP_i exchange reaction except for the absence of added PP_i. The apparent dissociation constant of ATP and the LysRS·L-lysine complex, $K_{d,app,A}$, was obtained by fitting to Eq. 8 to be 15.5 μM, while K_m for ATP in the ATP-PP_i exchange reaction was 65.1 μM (7).

Such fluorescence restoration by ATP was also observed when the enzyme was saturated with SAEC, a substrate of *B.s.* LysRS in the ATP-PP_i exchange reaction, and with L-Lyshxt and L-lysine amide, strong inhibitors of *B.s.* LysRS in the L-lysine dependent ATP-PP_i exchange reaction (data not shown), although the degree of restoration was different in each case. The $K_{d,app,A}$ values are 47.3 μM with SAEC, 3.0 μM with L-Lyshxt, and 4.0 μM with L-lysine amide (Table I).

The presence of EDTA inhibited the fluorescence restoration by ATP of the L-lysine-saturated enzyme (Fig. 2). α,β-Methylene ATP and anthraniloyl-ATP, neither of which is a substrate for the L-lysine-dependent ATP-PP_i exchange reaction, did not cause fluorescence restoration of *B.s.* LysRS saturated with L-lysine (data not shown).

Fluorometric Titration of LysRS with L-Lysine and Lysine Analogues in the Presence of Excess ATP—On the other hand, the addition of L-lysine to *B.s.* LysRS in the

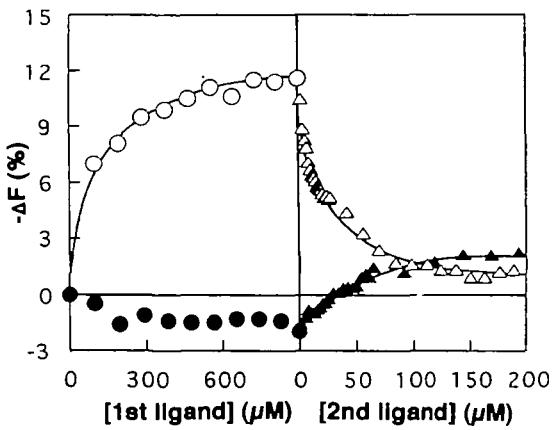

Fig. 1. **Fluorescence titration of LysRS with L-lysine and ATP.** ○, L-lysine; △, ATP in the presence of 1 mM L-lysine; ●, ATP; ▲, L-lysine in the presence of 1 mM ATP. The reaction mixture comprised 100 mM Tris-HCl buffer, pH 8.0, 10 mM $MgCl_2$, and 4.2 μM LysRS. λ_{ex}=295 nm, λ_{em}=340 nm, at 30°C. $K_{d,app,A}$=15.5±0.9 μM (△), $K_{d,app,L}$=50.6±3.1 μM (▲). The solid curves are the theoretical ones obtained according to Eq. 8 with the respective dissociation constants.

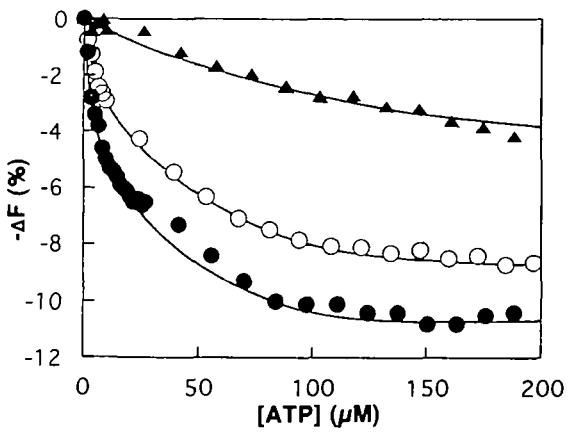

Fig. 2. **Effect of EDTA on the fluorescence titration with ATP of LysRS saturated with L-lysine.** Titration of LysRS with ATP in the presence of L-lysine and EDTA (●, 0 M; ○, 500 μM; ▲, 5 mM). The estimated $K_{d,app,A}$ are as follows: ●, 15.5±0.9 μM; ○, 25.7±1.6 μM; ▲, 443.3±22.7 μM. The reaction mixture comprised 100 mM Tris-HCl buffer, pH 8.0, 10 mM $MgCl_2$, 4.0 μM LysRS, 1 mM L-lysine, and various concentrations of EDTA. λ_{ex}=295 nm, λ_{em}=340 nm, at 30°C. The solid curves are the theoretical ones obtained according to Eq. 8 with the respective dissociation constants.

TABLE I. **Fluorometric parameters of the binding of ATP, L-lysine, and its analogues with *B.s.* LysRS.**

	In the absence of ATP[a]		In the presence of ATP[b]		ATP binding[c]		$K_{d,app,L}/K_d$
	K_d (μM)	$-\Delta F_{max}$ (%)	$K_{d,app,L}$ (μM)	$-\Delta F_{max}$ (%)	$K_{d,app,A}$ (μM)	ΔF_{max} (%)	(%)
L-Lysine	20.4± 1.8[d]	16.1	50.9± 3.2	5.0	15.5 ±0.9	12.8	250
α-Carboxyl group modified							
Cadaverine	19,900 ± 900	15.6	120 ± 18	4.1	/[e]	/	0.60
L-Lysine hydroxamate	704 ± 79	11.0	4.0± 0.6	4.8	2.98±0.54	9.31	0.57
L-Lysine amide	4,260 ± 210	16.6	48.9± 3.7	10.2	3.98±0.55	5.51	1.2
α-Amino group modified							
6-Amino-*n*-hexanoic acid	21,000 ±2,160	8.2	–[f]	0	–	0	–
ε-Amino group modified							
L-Norleucine	–	0	–	0	–	0	–
Others							
S-(2-Aminoethyl)-L-cysteine	197 ± 24	14.1	421 ± 35	5.6	47.3 ±2.6	9.13	213
L-Ornithine	14,700 ± 900	8.2	–	0	/	/	–
D-Lysine	3,180 ± 180	10.2	–	0	–	0	–
5-Hydroxylysine (mixed DL and DL-allo)	18,600 ± 600	19.0	–	0	–	0	–
Threo-4-hydroxy-L-lysine	3,230 ± 170	8.9	11,200 ±1,300	6.1	/	/	347

$[E]_0$=4.2 μM, λ_{ex}=295 nm, λ_{em}=340 nm, pH 8.0, 30°C. [a]Cited from the previous report (7). *B.s.* LysRS was titrated with L-lysine and its analogues. [b]*B.s.* LysRS was titrated with L-lysine and its analogues in the presence of 1 mM ATP. [c]*B.s.* LysRS was titrated with ATP in the presence of excess L-lysine and its analogues. [d]$[E]_0$=0.88 μM. [e]Not measured. [f]– indicates that a fluorescence change could not be detected.

presence of 1 mM ATP caused quenching of the protein fluorescence to a lesser extent than in the case of the absence of ATP (Fig. 1). The final level of the relative fluorescence intensity coincided more or less with the level after the fluorescence restoration by ATP of the L-lysine-saturated LysRS. The apparent dissociation constant of L-lysine in the presence of an excess amount of ATP calculated with Eq. 8, $K_{d,app,L}$, is 50.9 μM, while K_m for L-lysine was 23.6 μM in the ATP-PP_i exchange reaction (7) (Table I).

The fluorometric titration of LysRS was also carried out with lysine analogues in the presence of 1 mM ATP. The estimated $K_{d,app,L}$ and ΔF_{max} are listed in Table I together with K_d obtained in the absence of ATP as reported previously (7). SAEC and *threo*-4-hydroxy L-lysine, which were substrates for the ATP-PP_i exchange reaction, caused quenching of the fluorescence of LysRS in the presence of ATP, though to a lesser extent than in the absence of ATP. Each $K_{d,app,L}$, *i.e.* 421 μM for SAEC and 11.2 mM for *threo*-4-hydroxy L-lysine, is slightly larger than the respective K_d obtained in the absence of ATP. 5-Hydroxy-L-lysine and L-ornithine did not cause a fluorescence change in the presence of ATP, although they were substrates for the ATP-PP_i exchange reaction. A fluorescence change was not detected, in the presence of ATP, with either D-lysine, 6-amino-*n*-hexanoic acid, or norleucine, which were inhibitors of the ATP-PP_i exchange reaction. All the α-carboxyl group-modified analogues of L-lysine tested: cadaverine, L-Lyshxt and L-lysine amide, which were inhibitors of the ATP-PP_i exchange reaction, caused fluorescence quenching of LysRS in the presence of ATP, and each estimated $K_{d,app,L}$ is considerably smaller than the respective K_d obtained in the absence of ATP (Table I).

Filter Assay—The formation of lysyladenylate on *B.s.* LysRS was examined with [^{3}H]L-lysine or [^{3}H]ATP by trapping the complex on a glass fiber filter. Appreciable radioactivity was detected on the filter only when LysRS, L-lysine, and ATP were present together, and the radioactivity trapped on the glass fiber filter was proportional to the enzyme concentration used.

When the enzyme was added to a reaction mixture containing [^{3}H]ATP and either L-lysine or L-Lyshxt, appreciable radioactivity was detected on the filter in both cases (Fig. 3). However, when [^{3}H]ATP was replaced by [γ-^{32}P]ATP (^{32}P-ATP), no net radioactivity was detected in either case (Fig. 3). This implies that the enzyme•lysyladenylate complex (LysRS•L-Lys~AMP) or an enzyme•lysinehydroxamate-AMP (or -ADP) complex had been formed and trapped on the filter.

Isolation of the LysRS•Lysyladenylate Complex by Gel Filtration, and Transfer of Lysine from the Complex to tRNA—The enzyme•lysyladenylate complex labeled with [^{3}H]lysine was isolated by gel filtration on a Sephacryl S-200 column. The eluate fractions that were active in the filter assay described above could be completely separated from those of free L-lysine and free ATP at pH 8.0 and 4°C. The isolated LysRS•lysyladenylate complex was kept at 0°C at pH 8.5 for 12 h, during which time the filter-assay activity was intermittently examined. The activity decreased gradually (Fig. 4), the half-life time of the complex decomposition being about 700 min ($k = 1.7 \times 10^{-5}$ s^{-1}) under these conditions. The transfer of [^{3}H]lysine from the complex to tRNA was followed, as shown in Fig. 4 (inset). k_{app} of the transfer reaction was 1.2×10^{-2} s^{-1} at 0°C and pH 8.5.

Chasing the Radioactivity out of the LysRS•Lysyladenylate Complex—The radioactivity of the LysRS•lysyladenylate complex labeled with [^{3}H]ATP was chased out with cold ATP added to the elution buffer during gel filtration, but not with the added cold L-lysine (Fig. 5A). When the complex was labeled with [^{3}H]lysine, the radioactivity was chased out with the added cold L-lysine but not with the added cold ATP (data not shown). However, for the *B.s.* LysRS•lysinehydroxamate-AMP complex labeled with [^{3}H]ATP, the radioactivity was not chased out with either cold Lyshxt or with cold ATP added to the buffer (Fig. 5B).

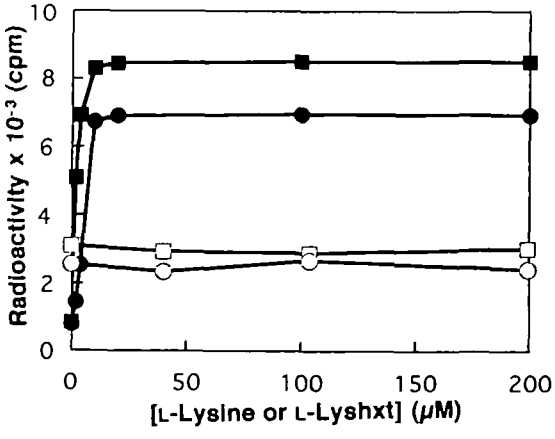

Fig. 3. **Formation of a LysRS-Lyshxt-AMP complex, as judged with the filter assay.** The reaction mixture comprised 100 mM Tris-HCl buffer, pH 8.0, 10 mM $MgCl_2$, 4.2 μM LysRS, either L-lysine or L-Lyshxt, and 200 μM ATP labeled with either [2,8-^{3}H]-ATP or [γ-^{32}P]ATP. (●) and (○) represent the trapped radioactivity when L-lysine was used with [^{3}H]ATP and [^{32}P]ATP, respectively. (■) and (□) represent the trapped radioactivity when L-Lyshxt was used with [^{3}H]ATP and [^{32}P]ATP, respectively. See the text ("MATERIALS AND METHODS") for details of the filter assay.

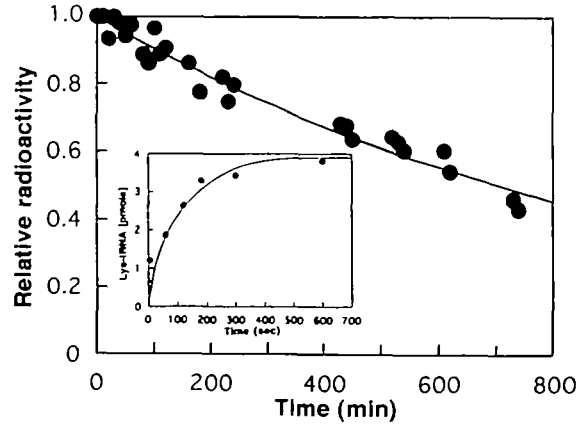

Fig. 4. **Stability of the LysRS•lysyladenylate complex, and the transfer of lysine to tRNA.** The solid curve is the theoretical first-order reaction curve with $k_{app} = 1.7 \times 10^{-6}$ s^{-1} ($t_{1/2} = 700$ min). The inset figure shows the transfer of [^{3}H]lysine from the LysRS•lysyladenylate complex to tRNA. [LysRS•lysyladenylate]$_0$ = 18.5 nM, tRNA concentration, $A_{260} = 30$. The solid line is the theoretical first-order reaction curve with $k_{app} = 1.2 \times 10^{-2}$ s^{-1}. See the text ("MATERIALS AND METHODS") for the detailed procedures.

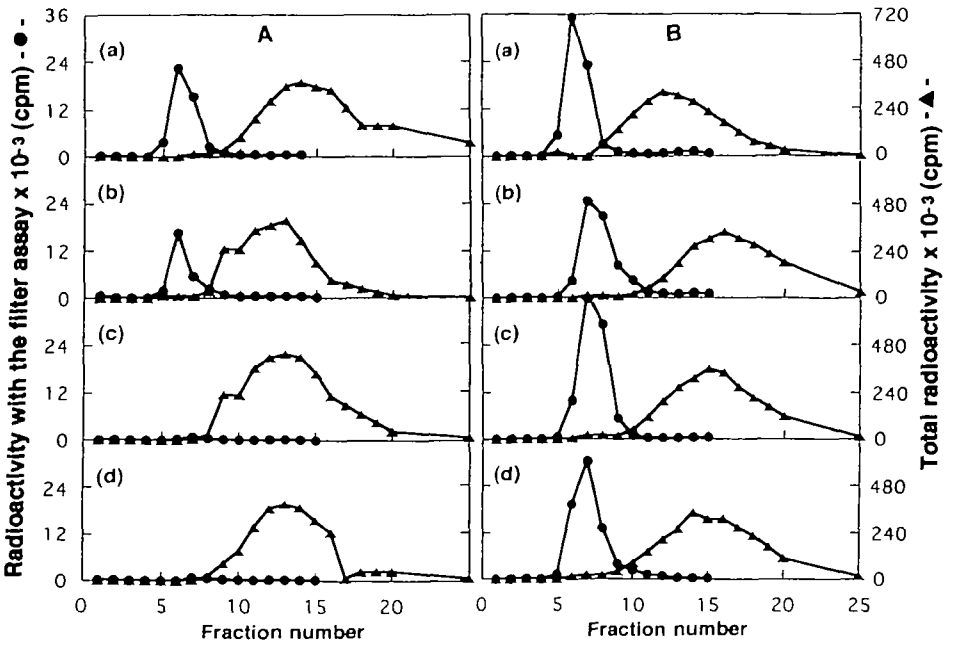

Fig. 5. **Chasing out of the radioactivity of the [^{3}H]AMP labeled LysRS·amino acid·AMP complex.** (A) LysRS·lysyladenylate complex. The reaction mixture comprised 100 mM Tris-HCl buffer (pH 8.0), 10 mM $MgCl_2$, 1 mM L-lysine, 200 μM [^{3}H]ATP (1.2 Ci/mmol), and 1.85 μM LysRS. The reaction mixture was incubated at 37°C for 3 min and held in an ice bath until use, and then it was applied to a NAP-10 column equilibrated with one of the following buffers: (a) 100 mM Tris-HCl buffer (pH 8.0) containing 10 mM $MgCl_2$; (b) (a)+1 mM L-lysine; (c) (a)+1 mM ATP; (d) (a)+1 mM L-lysine+200 μM ATP. The elution buffer was the same as the equilibrating buffer in each case. (B) LysRS·L-Lyshxt-AMP complex. The reaction mixture and buffers were the same as in (A) except for the replacement of 1 mM L-lysine by 1 mM L-Lyshxt. The reaction mixture was treated similarly to as in (A).

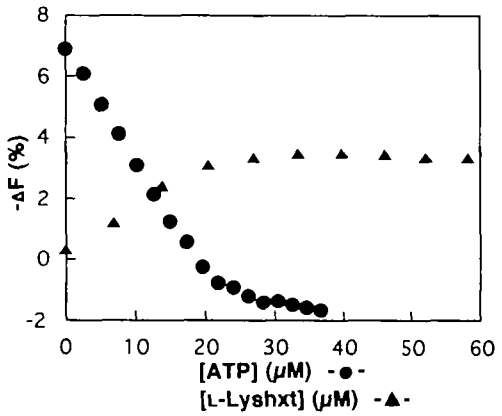

Fig. 6. **Binding stoichiometry of L-Lyshxt-AMP and *B.s.* LysRS on fluorescence titration.** Titration of LysRS with ATP in the presence of 2.4 mM L-Lyshxt (●). Titration of LysRS with L-Lyshxt in the presence of 1 mM ATP (▲). The reaction mixture comprised 100 mM Tris-HCl buffer, pH 8.0, and 10 mM $MgCl_2$. λ_{ex} = 295 nm, λ_{em} = 340 nm, at 30°C. $[E]_0$ = 22.1 μM as a dimer.

These results show that the formation of the LysRS·lysyladenylate complex is reversible, whereas the formation of the LysRS·lysinehydroxamate-AMP complex is practically irreversible. The latter coincides with that we could not detect an L-Lyshxt dependent ATP-PP_i exchange reaction (*7*).

Binding Stoichiometry of L-Lyshxt-AMP and B.s. LysRS—B.s. LysRS was titrated with ATP using the fluorescence change as a probe in the presence of excess L-Lyshxt, and also titrated with L-Lyshxt in the presence of excess ATP. In both cases the fluorescence difference changed linearly and reached a plateau (Fig. 6), and from the inflection point of each curve, the stoichiometry of binding was estimated to be 1 mol each of L-Lyshxt and ATP, and consequently Lyshxt-AMP, per mol of dimer LysRS.

DISCUSSION

Substrate-Induced Fluorescence Change of B.s. LysRS in the Presence of Both L-Lysine and ATP—The addition of ATP to *B.s.* LysRS saturated with either L-lysine (Fig. 1), SAEC or threo-4-hydroxy-L-lysine (data not shown), substrates for the ATP-PP_i exchange reaction, resulted in an apparent increase in protein fluorescence or restoration of the protein fluorescence from the quenched state caused by the binding of the amino acid substrates. The fluorescence restoration observed reflects the binding of ATP to LysRS complexed with an amino acid substrate, and is consistent with the previous conclusion regarding the substrate binding expressed as Eqs. 3 and 4.

Similar fluorescence restoration by ATP was observed when it was added to the enzyme complexed with lysine analogues of which the α-carboxyl group was modified. The analogues were cadaverine, L-Lyshxt, and L-lysine amide (data not shown); all were inhibitors of the L-lysine dependent ATP-PP_i exchange reaction by *B.s.* LysRS (*7*). The addition of EDTA to remove Mg^{2+} ions decreased the binding strength of ATP (Fig. 3), and the addition, in place of ATP, of α,β-methylene ATP, an inhibitor, in which the triphosphate moiety was supposed to take on a stretched conformation by analogy with β,γ-methylene ATP (*12, 13*), did not cause a fluorescence change. These facts may suggest that the triphosphate moiety of ATP must be in the right conformation to be properly bound to the enzyme to cause the fluorescence restoration. The observed $K_{d,app,A}$ values (Table I) are smaller for the α-carboxyl modified analogues than for the substrates of the ATP-PP_i exchange reaction. This is probably due to the lack of repulsion between the negative electrostatic charges of carboxylate and phosphate in the former cases.

The addition of L-lysine to *B.s.* LysRS in the presence of ATP resulted in a slight decrease in the protein fluorescence (Fig. 1). There must have been no enzyme-ATP complex to

start with in this case according to the order of binding of the two substrates, as proved previously, and as soon as the enzyme•L-lysine complex was formed on the addition of L-lysine, ATP must have been bound to this complex (Eqs. 3 and 4). The smaller degree of fluorescence quenching, thus, must be the outcome of the binding of both substrates. This interpretation is in accord with the kinetic observations with the stopped-flow method (Takita, T. *et al.*, to be published).

The differences in the magnitude of $K_{d,app,L}$ obtained in the presence of ATP, and of K_d obtained in the absence of ATP (Table I) should be a measure of the effect of ATP on the binding of L-lysine and its analogues to the enzyme. The ratios of the two dissociation constants ($K_{d,app,L}/K_d$) are compared in Table I. For L-lysine, SAEC, and *threo*-4-hydroxy-L-lysine, $K_{d,app,L}$ is 2- to 3-fold larger than the respective K_d; thus, the effect of ATP co-existence slightly reduces the binding strength of amino acid substrates, which may be favorable for the following reactions. On the other hand, for lysine analogues in which the α-carboxyl group is modified, $K_{d,app,L}$ is much smaller (0.5-1%) than the respective K_d (Table I). Thus, the presence of ATP greatly enhances the apparent binding of these inhibitors.

The LysRS•Lysyladenylate Complex—We found that filtration with glass fiber filters could be used for the detection and quantitative analysis of the LysRS• lysyladenylate complex at pH 8.0. The radioactivity was trapped only when LysRS, L-lysine, and ATP were all present together in the reaction mixture. Nitrocellulose and DEAE-cellulose filters were used to trap other enzyme•aminoacyladenylate complexes (*14, 15*); however, under our conditions, a glass fiber filter exhibits more sensitive detection of the LysRS•lysyladenylate complex than either a nitrocellulose or DEAE-cellulose filter. The LysRS•lysyladenylate complex can be isolated by gel filtration by applying the filter assay for detection. The isolated complex is relatively stable, the half-life time of the decomposition being about 11 h at 0°C and pH 8.5 (Fig. 4), which is shorter than the value reported for the *E. coli* LysRS•lysyladenylate complex (30 h at 4°C and pH 8.0) (*16*). The estimated k_{app} of the transfer reaction (Fig. 4, inset), 0.012 s^{-1}, is similar to the value for *E. coli* IleRS under comparable conditions (*17*).

A Complex Formed from LysRS, ATP, and L-Lyshxt—The above results with the filter assay (Fig. 3) indicate that the filter-trapped complex with L-Lyshxt was LysRS•L-Lyshxt-AMP or LysRS•L-Lyshxt-ADP. By analogy to the LysRS•lysyladenylate complex, we may assume that the complex is LysRS•L-Lyshxt-AMP. Thus, this looks contradictory, at least superficially, with the fact that we could not detect an L-Lyshxt dependent ATP-PP_i exchange reaction (*7*). However, this apparent inconsistency can be explained by assuming that the formation of the LysRS•Lyshxt-AMP complex is practically irreversible, as suggested by the results of radioactivity chasing-out experiments (Fig. 5, A and B).

A recent study on the structure of *T. thermophilus* SerRS complexed with seryladenylate analogues (*13*) showed that a serinehydroxamate-AMP complex was formed enzymatically from ATP and serine hydroxamate in the enzyme crystal, that the linkage between serine hydroxamate and AMP was -P-O-N(H)- rather than -P-N(OH)-, as deduced from the results of ^{31}P-NMR studies, and that the enzyme serinehydroxamate-AMP complex was more stable as to hydrolysis than the enzyme seryladenylate complex. These results support the interpretation of our observation on the reaction products of *B.s.* LysRS, ATP, and L-Lyshxt.

If one takes it into consideration that a stable covalent bond may have been formed between L-Lyshxt and AMP, the linear relationship between ΔF and the ligand concentration under the conditions in Fig. 6 is rational. It has been shown that 1 mol of L-Lyshxt-AMP is formed per mol of dimer LysRS. In the previous report we showed that there were two equivalent binding sites for L-lysine per dimer enzyme (*7*). Accordingly, "half of the sites" of the enzyme reactivity seems to have started at this step.

Structural Considerations—*B.s.* LysRS and *E. coli* LysRS (U), which is coded by the gene, *lysU*, exhibit more than 50% homology in primary structure (Takita, T. *et al.*, to be published), and the three-dimensional structure of the *B.s.* enzyme is expected to be very similar to that of the *E. coli* enzyme. The structure of *E. coli* LysRS (U) complexed with L-lysine has been resolved at 2.8 Å resolution (*18*). Onesti *et al.* (*18*) have described that a model of ATP binding to *E. coli* LysRS (U) can be built easily, although no experimental X-ray crystallographic data are available. The structure of ATP found in the yeast AspRS•$tRNA^{Asp}$•ATP complex (*19*) can be simply pasted into the structure of the *E. coli* LysRS (U) complexed with L-lysine without any manual adjustment. This is likely to mean that the ATP binding site is in an open state in LysRS complexed with L-lysine. This agrees with our conclusion that the binding of L-lysine to *B.s.* LysRS enables ATP to enter the binding site of the enzyme (Eqs. 3 and 4). The triphosphate backbone of this ATP molecule is said to be in a bent conformation (*19*), which is stabilized by charged residues of the enzyme and Mg^{2+} ions. Our results of fluorometric study in the presence of EDTA (Fig. 3) and with α,β-methylene ATP in place of ATP seem to be in accordance with this feature of the ATP conformation.

LysRS is classified into the same subclass as AspRS among the Class II enzymes (*3, 4, 20, 21*). The amino acid residues bound to ATP and the L-aspartic acid substrate in yeast AspRS are functionally well conserved in *E. coli* LysRS. It has been assumed from an X-ray study with yeast AspRS that the postulated binding site of L-aspartic acid should be blocked when ATP is in its binding site and that the substrate binding should be the sequential ordered mechanism in which L-aspartic acid is bound first (*19*). This agrees with our conclusion regarding the order of substrate binding in *B.s.* LysRS, reported in the previous paper (Eqs. 3 and 4), and with the observation on ATP binding presented in this paper, implying that the topology of the substrate binding site of *B.s.* LysRS may be similar to that of yeast AspRS.

We are grateful to Unitika, Ltd. for the kind supply of *B. stearothermophilus* NCA1503 cells. We also thank Professor K. Soda of Kyoto University for letting us use the jar fermenters for the culture of *E. coli*.

REFERENCES

1. Berg, P. (1961) Specificity in protein synthesis. *Annu. Rev. Biochem.* **30**, 293-324
2. Eriani, G., Delarue, M., Poch, O., Gangloff, J., and Moras, D.

(1990) Partition of tRNA synthetases into two classes based on mutually exclusive sets of sequence motifs. *Nature* **347**, 203-206
3. Delarue, M. and Moras, D. (1992) *Nucleic Acid and Molecular Biology* (Eckstein, F. and Lilley, D.M.J., eds.) Vol. 6, pp. 203-224, Springer-Verlag, Berlin, Heidelberg
4. Carter, C.W., Jr. (1993) Cognition, mechanism, and evolutionary relationships in aminoacyl-tRNA synthetases. *Annu. Rev. Biochem.* **62**, 715-748
5. Cusack, S. (1995) Eleven down and nine to go. *Nature Struct. Biol.* **2**, 824-831
6. Freist, W. and Gauss, D.H. (1995) Lysyl-tRNA synthetase. *Biol. Chem. Hoppe-Seyler* **376**, 451-472
7. Takita, T., Ohkubo, Y., Shima, H., Muto, T., Shimizu, N., Sukata, T., Ito, H., Saito, Y., Inouye, K., Hiromi, K., and Tonomura, B. (1996) Lysyl-tRNA synthetase from *Bacillus stearothermophilus*. Purification, and fluorometric and kinetic analysis of the binding of substrates, L-lysine and ATP. *J. Biochem.* **119**, 680-689
8. Hiratsuka, T. (1983) New ribose-modified fluorescent analogues of adenine and guanine nucleotides available as substrates for various enzymes. *Biochim. Biophys. Acta* **742**, 496-508
9. Sakoda, M. and Hiromi, K. (1976) Determination of the best-fit values of kinetic parameters of the Michaelis-Menten equation by the method of least squares with Taylor expansion. *J. Biochem.* **80**, 547-555
10. Kakitani, M., Tonomura, B., and Hiromi, K. (1986) Valyl-tRNA synthetase from *Bacillus stearothermophilus*. Purification and binding with the substrates, L-valine and ATP. *Agric. Biol. Chem.* **50**, 2437-2444
11. Kakitani, M., Tonomura, B., and Hiromi, K. (1987) Fluorometric study on the interaction of amino acids and ATP with valyl-tRNA synthetase from *Bacillus stearothermophilus*. *J. Biochem.* **101**, 477-484
12. Biou, V., Yaremchuk, A., Tukalo, M., and Cusack, S. (1994) The 2.9 Å crystal structure of *T. thermophilus* seryl-tRNA synthetase complexed with $tRNA^{Ser}$. *Science* **263**, 1404-1410
13. Belrhali, H., Yaremchuk, A., Tukalo, M., Larsen, K., Berthet-Colominas, C., Leberman, R., Beijer, B., Sproat, B., Als-Nielsen, J., Grübel, G., Legrand, J.-F., Lehmann, M., and Cusack, S. (1994) Crystal structure at 2.5 angstrom resolution of seryl-tRNA synthetase complexed with two analogues of seryl adenylate. *Science* **263**, 1432-1436
14. Yarus, M. and Berg, P. (1970) On the properties and utility of a membrane filter assay in the study of isoleucyl-tRNA synthetase. *Anal. Biochem.* **35**, 450-465
15. Bartmann, P., Hanke, T., and Holler, E. (1976) Rapid determination of an amino acid:tRNA ligase·aminoacyl adenylate complex on DEAE-cellulose filter disks. *Anal. Biochem.* **70**, 174-180
16. Waldenström, J. (1968) Some properties of lysyl ribonucleic acid synthetase from *Escherichia coli*. *Eur. J. Biochem.* **5**, 239-245
17. Eldred, E.W. and Schimmel, P.R. (1972) Investigation of the transfer of amino acid from a transfer ribonucleic acid synthetase-aminoacyl adenylate complex to transfer ribonucleic acid. *Biochemistry* **11**, 17-23
18. Onesti, S., Miller, A.D., and Brick, P. (1995) The crystal structure of the lysyl-tRNA synthetase (LysU) from *Escherichia coli*. *Structure* **3**, 163-176
19. Cavarelli, J., Eriani, G., Rees, B., Ruff, M., Boeglin, M., Mitschler, A., Martin, F., Gangloff, J., Thierry, J.C., and Moras, D. (1994) The active site of yeast aspartyl-tRNA synthetase: structural and functional aspects of the aminoacylation reaction. *EMBO J.* **13**, 327-337
20. Eriani, G., Dirheimer, G., and Gangloff, J. (1990) Aspartyl-tRNA synthetase from *Escherichia coli*: cloning and characterisation of the gene, homologies of its translated amino acid sequence with asparaginyl- and lysyl-tRNA synthetases. *Nucleic Acids Res.* **18**, 7109-7118
21. Cusack, S., Härtlein, M., and Leberman, R. (1991) Sequence, structural and evolutionary relationships between class 2 aminoacyl-tRNA synthetases. *Nucleic Acids Res.* **19**, 3489-3498