Transition State Stabilization by the N-terminal Anticodon-binding Domain of Lysyl-tRNA Synthetase*

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Lysyl-tRNA synthetase from *Bacillus stearothermophilus* (B. LysRS) (EC 6.1.1.6) catalyzes amination of tRNA\(^{\text{Lys}}\) with L-lysine, in which L-lysine was first activated with ATP to yield an enzyme (lysyladenylate complex), and then the lysine molecule was transferred from the complex to tRNA\(^{\text{Lys}}\). B. LysRS is a homodimer enzyme with a subunit that consists of two domains, an N-terminal tRNA anticodon-binding domain (TAB-ND: Ser\(^1\)-Pro\(^{144}\)) and a C-terminal Class II-specific catalytic domain (CAT-CD: Lys\(^{151}\)-Lys\(^{483}\)). CAT-CD alone retained catalytic activity, although at a low level; TAB-ND alone showed no activity. Size exclusion chromatography revealed that CAT-CD exists as a dimer, whereas TAB-ND was a monomer. The formation of a complex consisting of these domains was detected with the guidance of surface plasmon resonance. In accordance with this, the addition of TAB-ND to CAT-CD significantly enhanced both the L-lysine activation and the tRNA amination reactions. Kinetic analysis showed that deletion of TAB-ND resulted in a significant destabilization of the transition state of CAT-CD in the L-lysine activation reaction but had little effect on the ground state of substrate binding. A significant role of a cross-subunit interaction in the enzyme between TAB-ND and CAT-CD was proposed for the stabilization of the transition state in the L-lysine activation reaction.

AaRS\(^1\) catalyzes the ligation of an amino acid to the cognate tRNA, generally according to the following (Reaction Scheme 1),

\[
E + AA + ATP\rightarrow E\cdot AA + PP_i,
\]

\[
E\cdot AA + AMP + tRNA\rightarrow E + AMP + AA\cdot tRNA
\]

**Reaction Scheme 1**

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The abbreviations used are: aaRS, aminocyl-tRNA synthetase; B.s. LysRS, lysyl-tRNA synthetase from *Bacillus stearothermophilus*; CAT-CD, C-terminal catalytic domain of B.s. LysRS; E\(_{\text{app}}\), molar absorption coefficient at 280 nm; G\(^2\), binding standard free energy; \(\Delta G\), activation free energy; \(k_a\), association rate constant; \(k_d\), dissociation rate constant; \(K_{m}\), Michaelis constant for X; TAB-ND, N-terminal tRNA anticodon-binding domain of B.s. LysRS; EU, resonance unit; \(\lambda_{ex}\), the excitation wavelength; SPR, surface plasmon resonance; other aminocyl-tRNA synthetases were also abbreviated with the three-letter abbreviation of their specific amino acid followed by RS.

where AA denotes the amino acid; E, aaRS; PP\(_i\), inorganic pyrophosphate; and E\(-AA\)\(-AMP\), an aaRS:aminoacyladenylate complex. Because the tRNA amination reaction is critical for the fidelity of translation of genetic information into the structure of a protein, aaRSs must have gained a high degree of substrate specificity for each heterogeneous substrate during evolution. Recent progress in x-ray crystallographic analysis has revealed that aaRSs can be classified into two groups according to their active site topology. Class I aaRSs possess a catalytic core composed of a Rossmann fold, whereas Class II aaRSs possess a catalytic core consisting of antiparallel \(\beta\)-sheets (2). AaRSs are considered to have emerged at an early stage in the development of the contemporary system of protein synthesis (3). In addition, it was reported that several isolated catalytic domains of aaRS retained full or part of the amino acid activation activity (4–8). Together, these results have led to the proposal that the modern aaRS evolved from a primordial ancestor that had either of the two types of the class-defining domains, by getting the idiosyncratic region of each aaRS (3, 9, 10). In fact, the typical insertion in Class I aaRSs of the variable connective polypeptides (11) is responsible for the stabilizing transition state of methionine activation as well as methionine transfer to the 3′ end of tRNA in *Escherichia coli* MetRS (12), and also for the hydrolysis of misacylated Val-tRNA\(^{\text{Ile}}\) in *Thermus thermophilus* IleRS (13). On the other hand, the crystallographic structures of the aaRS\(_{\text{Glu}}\)-tRNA complex (8, 9, 14, 15) have revealed that an aaRS prepares a non-catalytic domain for the interaction with the anticodon stem/loop of the cognate tRNA and that each anticodon-binding domain shows considerable variation in its structure. Thus far, SerRS has been an exceptional case in which the anticodon stem/loop of the tRNA was not recognized by aaRS. In this system, the N-terminal non-catalytic domain interacts with an abnormally long variable arm of tRNA\(^{\text{Ser}}\) (16).

Recently, it has been suggested that the interface between the anticodon-binding domain and the catalytic domain plays an important role in the tRNA-dependent conformational change in the active site, not only in GlnRS, which requires the cognate tRNA for the amino acid activation reaction (17), but also in MetRS, which does not require it (3, 18). Furthermore, it has been reported that an accurate anticodon-aaRS interaction was essential for enhancement of the catalytic constant of amination (10, 19–21). These results suggest that in some aaRSs, the signal generated by the appropriate interaction between the anticodon of tRNA and the anticodon-binding domain was transmitted to the active site through either the aaRS or the tRNA molecule (10, 17, 18). In contrast to the tRNA-dependent domain-domain interaction, the role of the interaction between the anticodon-binding domain and the catalytic domain has never been reported in the absence of tRNA. Thus far, only for dimeric HisRS (Class IIa), both the catalytic domain and the non-catalytic domain (this domain has not yet been proven to be...
the anticodon-binding domain) have been purified separately (7). In this system, it was shown that in both the tRNA-independent amino acid activation and the aminoacylation reactions, the C-terminal non-catalytic domain contributed significantly to the stabilization of the transition state, but not to the ground state of substrate binding. In this case, however, because the catalytic domain of HisRS was purified as monomer, it was not clear whether the observed destabilization of the transition state upon deletion of the non-catalytic domain was because of the dissociation of the catalytic domain into a monomer or because of the loss of the interaction between the catalytic domain and the non-catalytic domain.

In the course of the studies on *B. stearothermophilus* Lysyl-tRNA synthetase (AMP forming); EC 6.1.1.6, a Class II enzyme, from *Bacillus stearothermophilus* (22–25), we have dissected the enzyme into N-terminal and C-terminal domains. By analogy with the crystallographic structure of *Escherichia coli* LysRS(U) (26), homodimeric *B. stearothermophilus* LysRS (molecular mass of the subunit is 57,273 Da (25)) was considered to have a simple modular organization in which the C-terminal catalytic domain (CAT-CD) was joined through a short flexible linker to the N-terminal anticodon-binding domain (TAB-ND) (Fig. 1). In this study, we have succeeded in purifying CAT-CD as a dimer. The aim of this study was to elucidate the role of the interaction between the catalytic domain and the anticodon-binding domain in the tRNA-independent l-lysine activation reaction, and to elucidate the mechanism of the transition state stabilization in the enzymatic reaction.

**EXPERIMENTAL PROCEDURES**

### Proteins—

The recombinant form of wild-type *B. stearothermophilus* LysRS was purified from *E. coli* BL21(DE3) cells by the method reported previously (25). The enzyme concentration was determined spectrophotometrically with an extinction coefficient at 280 nm of 70,600 M⁻¹ cm⁻¹ at pH 8.0, 25 °C (25). Streptomycin sulfate inhibitor was a gift from Dr. B. Tonomura. Superoxide dismutase from bovine erythrocytes was the product of Wako Pure Chemical. Gel filtration molecular weight markers were purchased from Sigma.

### Chemicals—

L-lysine and [3H]pyrophosphate were the products of Amersham Biosciences; ATP (disodium salt) was from Sigma; glass filter (GF/C) was from Whatman Ltd.; DEAE-Toyopearl 650M from Tosoh. BIAcore chemical acti-

### Construction of Plasmids—

**B. stearothermophilus** LysRS (25), the anticodon-

### Protein Expression and Purification—

**B. stearothermophilus** LysRS was designed to be expressed in the *E. coli* with an N-terminal T7 Tag sequence as TAB-ND and CAT-CD, respectively (Table 2). Two sense N-terminal primers with BamHI restriction sites, 5′-TA-

### N-terminal Amino Acid Sequence Analysis—

The amino acid se-

### SPR Analysis—

Real-time interaction between TAB-ND and CAT-CD was detected with the guidance of surface plasmon resonance (SPR) at 25 °C using a BIAcore 2000. Activation of the carboxylmethylated dextran-

### UV Absorption, Fluorescence, and CD Spectra—

All measurements were done in 100 mM Tris-HCl buffer (pH 8.0) containing 10 mM MgCl₂, UV absorption spectra and far-UV CD spectra were measured at 25 °C with a Shimadzu spectrophotometer UV-2200 and a Shimadzu-Avicir circular dichroism spectrophotometer model 202, respectively. Fluorescence spectra were measured at 30 °C with a Hitachi fluorescence spectrophotometer 850.
Enzyme concentrations were 0.5 nM from 5 to 100 nM TRP-ND plus 10 nM CAT-CD. The radioactivity in both assays measured by using the L-lysine-dependent ATP-PPi exchange reaction of E. coli LysRS(Δ) in a subunit are represented in magenta and cyan, respectively, whereas the corresponding domains in the other subunit are in orange and yellow, respectively. B, view from the opposite side from A.

\[
\frac{R_0}{R} = k_0(t_c - t_0)
\]  

(Eq. 2)

where \(R_0\) was \(R\) at time \(t_c\), and \(R_s\) is \(R\) at an arbitrary starting time, \(t_0\). The \(k_0\) value was obtained as the linear slope of the \(\ln(R/R_0)\) versus \((t_c-t_0)\) plot.

**ATP-PPi Exchange Reaction**—The l-lysine activation reaction was measured by using the l-lysine-dependent ATP-PPi exchange reaction at pH 8.0, 37 °C, as reported previously (22). The standard reaction mixture contained in 0.5 ml: 100 mM Tris-HCl buffer (pH 8.0), 10 mM MgCl₂, ATP, l-lysine, and PPI (9.1 mM/cmol). Kinetic parameters were calculated by the nonlinear least-squares method with KaleidaGraph (Synergy software). \(K_{m,ATP}\) was estimated over a range of ATP concentrations from 10 to 200 μM, whereas \(K_{m,PPi}\) was estimated over a range of ATP concentrations from 20 to 500 μM. \(K_{m,PPi}\) was estimated over a range of PPI concentrations from 30 to 500 μM. In all cases, the initial concentration of the other two substrates was 1 mM. 1 mM might not be the saturating concentration for ATP (see Table II), but this concentration was chosen after considering the ratio of ATP to Mg²⁺.

**Aminoacylation Reaction**—The tRNA aminoacylation reaction was measured at pH 8.0, 37 °C, as reported previously (22). The standard reaction mixture contained in 0.5 ml: 100 mM Tris-HCl buffer (pH 8.0), 10 mM MgCl₂, ATP, l-(H)lysine (40 μM/mmol), and 20 A₂₆₀ units of E. coli tRNA. \(K_{m,tRNA}\) was estimated over a range of l-lysine concentrations from 5 to 100 μM in the presence of 1 mM ATP and 20 A₂₆₀ units of tRNA, whereas \(K_{m,ATP}\) was estimated over a range of ATP concentrations from 5 to 300 μM in the presence of 1 mM l-lysine and 20 A₂₆₀ units of tRNA. Enzyme concentrations were 0.5 nM B.s. LysRS, 300 nM CAT-CD, and 150 nM TAB-ND plus 10 nM CAT-CD. The radioactivity in both assays was measured in an Aloka Liquid Scintillation Counter LSC-5100.

**Area of Domain-Domain Interactions in E. coli LysRS(U)**—B.s. LysRS consists of 493 amino acid residues and the amino acid sequence has 52% homology to E. coli LysRS(U) (504 residues) (25). Therefore, to obtain information on the domain-domain interactions of B.s. LysRS, each contact area between the domains of E. coli LysRS(U) was estimated based on the 2.5-Å resolution structure of the complex with the substrate l-lysine (26) (Fig. 1). A dimer of E. coli LysRS(U) was chosen, and hydrogens were added to the groups ionized at pH 8. After removing the l-lysine substrate and water molecules, the accessible surface area of the free dimer enzyme was estimated with Insight II/Homology (Biosym Technologies, San Diego, CA) by rolling a sphere of 1-4 Å radius corresponding to a water molecule around the enzyme (27). In the same way, the accessible surface areas of \(N_A\), \(N_B\), \(C_A\), and \(C_B\), the standard markers used were: phosphorylase b, 97,400; bovine serum albumin, 66,267; aldolase, 42,400; carbonic anhydrase, 30,000; trypsin inhibitor, 20,100; lysozyme, 14,400.

**RESULTS**

**Purification and Structures of TAB-ND and CAT-CD**—Both TAB-ND and CAT-CD were more highly expressed in E. coli B121(DE3) cells compared with the intact enzyme, and were purified to homogeneity as judged by SDS-PAGE (Fig. 2). Their migrations on SDS-PAGE were consistent with the molecular mass of TAB-ND, 17,965 Da, and that of CAT-CD, 41,286 Da (Table I). N-terminal amino acid sequences of TAB-ND and CAT-CD were determined as ASMTGQGQQMGRGSGMSHEEL and ASMTGGQGQMGGRSGKDEQRY, respectively, in complete accordance with those expected from their DNA sequences (Table I). The underlined regions show the sequence from Ser¹ to Leu⁹ of TAB-ND and from Lys³¹ to Tyr¹⁵⁷ of CAT-CD. Size exclusion chromatography (Supplemental Materials) indicated that TAB-ND existed as a monomer but CAT-CD was a dimer under the conditions used, because the molecular masses of TAB-ND and CAT-CD were estimated to be 13,500 and 88,300 Da, respectively (Supplemental Materials).

**UV Absorption Spectra and Fluorescence Emission Spectra**—UV absorption spectra of TAB-ND, CAT-CD, and the intact B.s. LysRS are shown in the Supplemental Materials. The spectra of TAB-ND have a shoulder at 280 nm in analogy to tyrosine spectra. Estimated ε₂₈₀ values at pH 7.8, 25 °C, 5,200 M⁻¹ cm⁻¹ for the TAB-ND monomer and 45,000 M⁻¹ cm⁻¹ for the CAT-CD dimer were closely consistent with the expected values of 4,783 and 5,988 M⁻¹ cm⁻¹, respectively, which were calculated by the numbers and ε₂₈₀ values of the Phe, Tyr, and Trp residues. Fluorescence emission spectra of TAB-ND, CAT-CD, and the intact B.s. LysRS are shown in the Supplemental Materials. The emission spectra of TAB-ND at an excitation wavelength of 280 nm has a λ_max value of 304 nm, which was calculated by the numbers and ε₂₈₀ values of the Phe, Tyr, and Trp residues. Fluorescence spectra of TAB-ND, CAT-CD, and the intact B.s. LysRS are shown in the Supplemental Materials. The emission spectra of TAB-ND at an excitation wavelength of 280 nm has no λ_max value of 304 nm, which was calculated by the numbers and ε₂₈₀ values of the Phe, Tyr, and Trp residues. Fluorescence emission spectra of TAB-ND, CAT-CD, and the intact B.s. LysRS are shown in the Supplemental Materials. The emission spectra of TAB-ND at an excitation wavelength of 280 nm has a λ_max value of 304 nm, which was calculated by the numbers and ε₂₈₀ values of the Phe, Tyr, and Trp residues. Fluorescence emission spectra of TAB-ND, CAT-CD, and the intact B.s. LysRS are shown in the Supplemental Materials.
TAB-ND and CAT-CD (515 residues) was larger than that of B.s. LysRS (493 residues) because of the inclusion of the N-terminal T7 tag sequence (Table I). It may be reasonable that the ellipticity of the synthesized spectra of the complex of TAB-ND and CAT-CD was somewhat negatively larger than those of B.s. LysRS. On the other hand, despite having the same number of amino acid residues, the ellipticity of the observed spectra of TAB-ND plus CAT-CD was negatively larger than the synthesized one, suggesting that the interaction between TAB-ND and CAT-CD affects the secondary structures of either or both domains.

**SPR Measurement**—Fig. 3A shows the progress curves of the interaction between free TAB-ND and immobilized CAT-CD on a sensor chip. In the association phase (0–180 s), the intensity of SPR was increased, indicating the formation of the complex, and in the dissociation phase (180–485 s), the intensity of SPR was decreased, indicating that TAB-ND dissociates from the immobilized CAT-CD. By non-linear fitting of the association phase to Equation 1, the values of \( k_a [\text{TAB-ND}]_0 + k_d \) and \( k_d [\text{TAB-ND}]_0 \) were estimated, and the linear plot of \( k_d [\text{TAB-ND}]_0 + k_d \) versus [TAB-ND] \(_0\) is shown in Fig. 3B. Estimated \( k_a \) and \( k_d \) were 48,800 ± 2,300 M\(^{-1}\) s\(^{-1}\) and 0.0008 ± 0.0009 s\(^{-1}\). When \( k_d \) was estimated by linear fitting in the plot of \( \ln(R_j/R_0) \) versus \( t_j - t_0 \) according to Equation 2, curved lines which indicate a slowdown of the dissociation rate were observed (Supplemental Materials). Therefore, \( k_d \) values were estimated from the slope of the tangent to the initial straight parts (±20 s). The averaged \( k_d \) value was 0.0012 ± 0.0009 s\(^{-1}\), which agrees with the value estimated above in the association phase. With \( k_a = 48,800 \text{ M}^{-1}\text{s}^{-1} \) and \( k_d = 0.0008 \text{ s}^{-1} \), the \( k_d \) (\( = k_d/k_a \)) value was calculated to be 16.4 ± 1.2 ns (Table II). When the interaction between free CAT-CD and immobilized TAB-ND was also investigated in a similar way, binding of CAT-CD to immobilized TAB-ND was observed, but CAT-CD did not dissociate from the immobilized TAB-ND (data not shown). This may be because of the bivalent property of dimeric CAT-CD (see Supplemental Materials) in binding to TAB-ND.

**Enzymatic Activities of TAB-ND and CAT-CD**—Neither the L-lysine-dependent ATP-PPi exchange activity nor the tRNA aminocacylation activity could be measured with TAB-ND, whereas CAT-CD retained both activities at a very low level. The kinetic constants of CAT-CD in the ATP-PPi exchange reaction were estimated with 500 nM CAT-CD (Table II). The \( k_{cat} \) value of CAT-CD was 470-fold smaller than that of the intact B.s. LysRS, whereas \( K_m \) values of CAT-CD (\( K_m \text{Lys}^\text{r}, \ K_m \text{ATP}, \text{and} \ K_m \text{PPi} \)) were comparable with those of the intact enzyme. In the presence of 20 \( \mu \text{M} \) units of \( E. \text{coli} \) tRNA, the kinetic constants of CAT-CD in the aminocacylation reaction were estimated with 300 nM CAT-CD (Table II). The \( k_{cat} \) value of CAT-CD was 29,000-fold smaller than that of the intact enzyme, whereas \( K_m \) values of CAT-CD (\( K_m \text{Lys}^\text{r} \) and \( K_m \text{ATP} \)) were comparable with those of the intact enzyme. Because it is known that \( T. \text{thermophilus} \) LysRS interacts with the anticodon region of the tRNA transcripts through the N-terminal anticodon-binding domain (15), we consider that the affinity of CAT-CD for tRNA\(^{1\text{rRNA}}\) must be weakened significantly. In the case of AspRS, which belongs to the same subclass as LysRS (Class IIb), the deletion of the anticodon-binding domain resulted in a 100-fold increase in the \( K_m \) for tRNA\(^{Asp} \) but \( k_{cat} \) remained unchanged (29). Although the estimated \( k_{cat} \) and \( K_m \) of CAT-CD in the aminocacylation reaction were apparent values because of the possible nonsaturating conditions of tRNA, it should be noted that CAT-CD alone can bind to \( E. \text{coli} \) tRNA and catalyze aminocacylation without the N-terminal anticodon-binding domain.

**Enhancement of the Enzymatic Activity of CAT-CD Induced by TAB-ND**—The addition of TAB-ND to CAT-CD enhanced both the L-lysine-dependent ATP-PPi exchange activity and the tRNA aminocacylation activity as compared with CAT-CD alone (Table II, Fig. 4). The kinetic parameters of the TAB-ND/CAT-CD complex in the ATP-PPi exchange reaction were estimated with 75 nM TAB-ND plus 5 nM CAT-CD (Table II). Under these conditions, most of the CAT-CD exists in a complex with TAB-ND (Fig. 3). The estimated \( k_{cat} \) value of the complex was 170-fold larger than that of CAT-CD alone, whereas the \( K_m \) values of the complex were comparable with those of CAT-CD but even closer to those of the intact B.s. LysRS. Similarly, the kinetic parameters of the complex in the aminocacylation reaction were estimated with 150 nM TAB-ND plus 10 nM CAT-CD. The estimated \( k_{cat} \) of the complex was 390-fold larger than that of CAT-CD, whereas the \( K_m \) values of
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TABLE II

Kinetic and static parameters of B.s. LysRS, CAT-CD, and the TAB-ND-CAT-CD complex

|                  | ATP-PPi exchange reaction | Aminoacylation reaction |
|------------------|---------------------------|-------------------------|
|                  | $k_{cat}$ ($s^{-1}$) | $K_{m,Lys}$ ($\mu$M) | $K_{m,ATP}$ ($\mu$M) | $K_{m,PPi}$ ($\mu$M) |
| B.s. LysRS       | 46.4 ± 1.0a             | 14.1 ± 1.8             | 227 ± 10              | 50.2 ± 5.3             |
| TAB-ND           | ND                       | 20.9 ± 4.5             | 60.8 ± 4.8            |
| CAT-CD           | 0.098 ± 0.016c          | 57.2 ± 19.3            | 18.3 ± 3.0            |
| TAB-ND + CAT-CD  | 17.1 ± 0.6e             | 13.2 ± 2.5             | 35.7 ± 5.3            |
|                  | 14.7 ± 0.7f             |                        |                       |

|                  | $k_{cat}$ ($s^{-1}$) | $K_{m,Lys}$ ($\mu$M) | $K_{m,ATP}$ ($\mu$M) |
| B.s. LysRS       | 3.48 ± 0.26a         | 20.9 ± 4.5            | 60.8 ± 4.8            |
| TAB-ND           | ND                     |                        |                       |
| CAT-CD           | 0.00012 ± 0.00002d    | 60.5 ± 21.3            | 90.2 ± 24.5           |
| TAB-ND + CAT-CD  | 0.047 ± 0.002e        | 12.7 ± 4.1             | 52.0 ± 8.4            |
|                  | 0.041 ± 0.005f        |                        |                       |

$K_d$ of TAB-ND and CAT-CD

|                  | ATP-PPi exchange reaction | Aminoacylation reaction |
|------------------|---------------------------|-------------------------|
| SPR              | 16.4 ± 1.2a               | 30.7 ± 4.0'              |
|                  |                           | 41.8 ± 15.2'             |

$^a$ $k_{cat}$ values were determined in the presence of 1 mM l-lysine plus 1 mM PPI.

$^b$ $[E]_0$ = 5 nM.

$^c$ ND, not determined.

$^d$ 75 nM TAB-ND + 5 nM CAT-CD.

$^e$ Estimated in Fig. 4.

$^f$ $k_{cat}$ values were determined in the presence of 1 mM l-lysine plus 20 $A_{260}$ units of tRNA.

$^g$ $[E]_0$ = 0.5 nM.

$^h$ 300 nM.

$^i$ 150 nM TAB-ND + 10 nM CAT-CD.

$^j$ Estimated in Fig. 3.

Fig. 4. Enhancement of the catalytic activity of CAT-CD by binding to TAB-ND. ○, the l-lysine-dependent ATP-PPi exchange reaction catalyzed by 5 nM CAT-CD in the presence of 1 mM l-lysine, 1 mM ATP, and 1 mM PPI at pH 8.0, 37 °C. ○, the tRNA aminoacylation reaction catalyzed by 10 nM CAT-CD in the presence of 1 mM l-lysine, 1 mM ATP, and 20 $A_{260}$ units of tRNA at pH 8.0, 37 °C. The solid lines show the theoretical curves obtained by regression analysis according to Equation 3. Estimated $K_d$ and $k_{cat}$ values were: 30.7 ± 4.0 nM and 14.7 ± 0.7 s$^{-1}$ (○); 41.8 ± 15.2 nM and 0.041 ± 0.005 s$^{-1}$ (○), respectively.

we can estimate the $K_d$ value for the complex of TAB-ND and CAT-CD assuming a simple bimolecular binding equilibrium in that two TAB-ND monomers bind to a CAT-CD dimer independently,

$$(\text{CAT-CD}_m)_2 + 2\text{TAB-ND} \rightleftharpoons (\text{TAB-ND-CAT-CD})_2$$

where TAB-ND, CAT-CDm, TAB-ND-CAT-CDm are the TAB-ND monomer, the CAT-CD monomer, and the complex of TAB-ND monomer and CAT-CD monomer, respectively. Assuming that the binding of TAB-ND to one CAT-CDm in a CAT-CD dimer does not affect the activity of the other CAT-CDm, the enzymatic reaction velocity observed was represented as follows under saturating substrate conditions (see Supplemental Materials).

$$v = \frac{k_{cat2}([\text{TAB-ND}]_0 + [\text{CAT-CD}_m]_0 + K_d)}{4}$$

$$k_{cat2} \sqrt{[\text{TAB-ND}]_0 + [\text{CAT-CD}_m]_0 + K_d} - 4[\text{CAT-CD}_m]_0[\text{TAB-ND}]_0$$

(Eq. 3)

By fitting the data in Fig. 4 to Equation 3, $K_d$ and $k_{cat2}$ were estimated as 30.7 ± 4.0 nM and 14.7 ± 0.7 s$^{-1}$ (36.8 pmol s$^{-1}$ per 2.5 pmol of CAT-CD), respectively, in the ATP-PPi exchange reaction. On the other hand, in the aminoacylation reaction, $K_d$ and $k_{cat2}$ were estimated as 41.8 ± 15.2 nM and 0.041 ± 0.005 s$^{-1}$ (0.20 pmol s$^{-1}$ per 5 pmol of CAT-CD), respectively (Table II).

DISCUSSION

The general structural organization of E. coli LysRS(U) (26) was illustrated in Fig. 1. The subunit consists of three...
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TABLE III

Activation energies and binding energies for B. s. LysRS, CAT-CD, and the TAB-ND-CAT-CD complex

The activation free energy and the binding free energies were calculated with the kinetic parameters of the l-lysine-dependent ATP-PPi exchange reaction in Table II.

|               | $\Delta G^{\text{act}}$ | $\Delta G^{\text{act}}_{\text{Lys}}$ | $\Delta G^{\text{act}}_{\text{ATP}}$ | $\Delta G^{\text{act}}_{\text{PPi}}$ |
|---------------|------------------------|--------------------------------------|-----------------------------------|-----------------------------------|
| B. s. LysRS   | 15.81                  | −6.88                                | −5.17                             | −6.10                             |
| CAT-CD       | 19.60                  | −6.02                                | −4.73                             | −6.72                             |
| TAB-ND-CAT-CD| 16.42                  | −6.92                                | −5.30                             | −6.31                             |

$\Delta G^{\text{act}}$, the activation free energy at the step of $k_{\text{cat}}$, $\Delta G^{\text{act}} = RT \ln(k_{\text{cat}}/h – \ln K_{\text{cat}})$, $k$ is the Boltzmann constant and $h$ is the Planck constant.

$\Delta G^{\text{act}}_{\text{Lys}}$, difference in the activation free energy of $k_{\text{cat}}$, $\Delta G^{\text{act}} = \Delta G^0 (x) – \Delta G^0 (\text{B. s. LysRS})$.

$\Delta G^{\text{act}}_{\text{ATP}}$, difference in the binding free energy of $K_{\text{d}}$.

Fig. 5. Energy diagrams in the ATP-PPi exchange reaction.

Energy diagrams were drawn with the values of $\Delta G_0$ and $\Delta G^0$ in Table III assuming that the binding processes for l-lysine, ATP, and PPi were in rapid equilibrium and that the forward reaction toward the transition state, $E$-Lys-ATP $\rightarrow$ [E-Lys-ATP] $\rightarrow$ E, was the rate-determining step. Black (---), B. s. LysRS; red (----), CAT-CD; blue (-- -- --), TAB-ND-CAT-CD complex.

domains: (a) the tRNA anticodon-binding domain in the N terminus; (b) the catalytic site domain in the C terminus; and (c) a short region connecting them. The dimerization of E. coli LysRS(U) was sustained by three domain-domain interactions: (i) between the catalytic domains; (ii) between the anticodon-binding domain and the catalytic domain of the other subunit; (iii) between the anticodon-binding domain and the catalytic domain of the same subunit. These contact areas were calculated to be: 5,000 Å² (i), 2,100 Å² (ii), and 500 Å² (iii), respectively. Size exclusion chromatography revealed that CAT-CD exists as a dimer, whereas TAB-ND was a monomer (Supplemental Materials). These results agree with the x-ray crystallographic structure of E. coli LysRS(U) (26), in which there was no interaction between the two N-terminal domains in the dimer, whereas the two C-terminal domains have significant interactions with each other (contact area 5,000 Å²). Prokaryotic MetRS (Class I) (4), AlaRS (Class II) (5), HisRS (Class II) (7), and SerRS (Class II) (6), which are all dimeric enzymes, were converted into monomeric catalytic domains upon deletion of the non-catalytic domain, in contrast to the present case, in which CAT-CD retains a dimeric structure. This indicates that the interaction between the catalytic domains of B. s. LysRS was considerably stronger than that of the other aaRSs, which has enabled us to investigate the role of the interaction between the anticodon-binding domain and the catalytic domain in the catalytic reaction of B. s. LysRS.

The result of SPR measurements (Fig. 3) and the increase in the CAT-CD catalytic activity by the addition of TAB-ND (Fig. 4) indicates that the interaction between the anticodon-binding domain and the catalytic domain was achieved in the TAB-ND-CAT-CD complex as it was in the intact B. s. LysRS. The $K_d$ value of the complex, 30.7 ± 4.0 nm (37 °C), which was estimated by the increase in the ATP-PPi exchange reaction activity of CAT-CD in the complex (Fig. 4), was comparable with 16.4 ± 1.2 nm (25 °C), which was estimated from the SPR measurements (Fig. 3, Table II). The values of the free energy of binding between CAT-CD and TAB-ND were calculated with these $K_d$ values to be −10.7 kcal mol⁻¹ (37 °C) and −10.6 kcal mol⁻¹ (25 °C).

If we assume that the differences in the kinetic parameters between CAT-CD and the TAB-ND-CAT-CD complex reflect the interactions between the anticodon-binding domain and the catalytic domain, and that the differences between the TAB-ND-CAT-CD complex and the intact B. s. LysRS reflect the covalent link between the two domains in a subunit, then their energetic contributions to the $l$-lysine-dependent ATP-PPi exchange reaction can be estimated according to Scheme 2, where E denotes B. s. LysRS, CAT-CD, or the TAB-ND-CAT-CD complex, and $k_{\text{cat}}$ and $K_m$ are the kinetic parameters in the ATP-PPi exchange reaction.

E$\rightarrow$E$\cdot$Lys$\rightarrow$E$\cdot$Lys $\cdot$ ATP $\rightarrow$ E$\cdot$Lys $\cdot$ AMP $\cdot$ PPi

Reaction Scheme 2

Estimated activation free energies ($\Delta G^i$) and binding free energies ($\Delta G^0$) are listed in Table III. The deletion of the anticodon-binding domain from B. s. LysRS significantly stabilizes the transition state of CAT-CD ($\Delta G^0$ = 3.79 kcal mol⁻¹) but slightly affects the ground state of the substrate binding ($\Delta G^0_{\text{Lys}}$ = 0.86 kcal mol⁻¹, $\Delta G^0_{\text{ATP}}$ = 0.44 kcal mol⁻¹, $\Delta G^0_{\text{PPi}}$ = −0.62 kcal mol⁻¹). Fig. 5 shows the energy diagrams of B. s. LysRS, CAT-CD, and the TAB-ND−CAT-CD complex in the ATP-PPi exchange reaction. The energy diagram of the TAB-ND-CAT-CD complex can almost be superimposed on that of the intact B. s. LysRS, supporting the idea that these $\Delta G^0$ and $\Delta G^i$ values were derived from the loss of interactions between the anticodon-binding domain and the catalytic domain. The binding of TAB-ND to CAT-CD significantly improves $\Delta G^i$ ($\Delta G^i$ = −3.18 kcal mol⁻¹), but only slightly improves $\Delta G^0$ ($\Delta G^0_{\text{Lys}}$ = −0.90 kcal mol⁻¹, $\Delta G^0_{\text{ATP}}$ = −0.57 kcal mol⁻¹, $\Delta G^0_{\text{PPi}}$ = 0.41 kcal mol⁻¹). These data reconfirm that the interactions between these domains were
FIG. 6. The cross-subunit interface between the anticodon-binding domain and motifs 2 and 3 of the catalytic domain in *E. coli* LysRS(U). Based on the crystallographic structure of *E. coli* LysRS(U) (26), amino acid residues of the catalytic domain of one subunit are shown by ribbons or bold lines in blue for motif 2 and red for motif 3, whereas those of the anticodon-binding domain of the other subunit are shown by thin lines in black or magenta. The substrate L-lysine is shown green. When an amino acid residue of *E. coli* LysRS (U) was not conserved in *B. s*. LysRS, the substituted residue of *B. s*. LysRS is shown in parentheses.

particularly important in the stabilization of the transition state in the L-lysine-dependent ATP-PPi exchange reaction compared with the ground state. Consequently, the effect of the break in the covalent link between the anticodon-binding domain and the catalytic domain in the same subunit may be estimated as: \( \Delta G^\dagger = 0.61 \text{ kcal mol}^{-1} \), \( \Delta G^0_{\text{Lys}} = -0.04 \text{ kcal mol}^{-1} \), \( \Delta G^0_{\text{ATP}} = -0.13 \text{ kcal mol}^{-1} \), \( \Delta G^0_{\text{PPi}} = -0.21 \text{ kcal mol}^{-1} \) (Table III). These results evidently indicate that the direct link between these domains does not play a significant role in the ground state of *B. s*. LysRS, but contributes to some extent to the stabilization of the transition state in the ATP-PPi exchange reaction.

Thus, the present study was the first case for an aaRS in which the binding of the non-catalytic domain restores the reduced activity of the catalytic domain both in the ATP-PPi exchange reaction and the aminoacylation reaction. It was suggested that the conformational change induced by the deletion of the anticodon-binding domain was related to the reformation of the dimeric HisRS (Class IIa), both the non-catalytic domain and the catalytic domain have been separately purified (7). However, in that case, the catalytic domain of HisRS purified as a monomer. Although the deletion of the noncatalytic domain of HisRS resulted in a preferential decrease in \( k_{\text{cat}} \), both in the ATP-PPi exchange and the aminoacylation reactions, the addition of the non-catalytic domain to the catalytic domain did not restore the activity of the catalytic domain in both reactions.

Following the above discussions, we would like to tentatively propose that the cross-subunit interaction of 2,100 Å² between the anticodon-binding domain and the catalytic domain contributes significantly to the stabilization of the transition state. The following lines of evidence support this proposition. First, the amino acid residues at the cross-subunit interface of *E. coli* LysRS(U) are well conserved in *B. s*. LysRS (Fig. 6). Of 17 amino acid residues, 15 residues were conserved in *B. s*. LysRS, and only Asp⁷⁷ and Arg⁴⁰, which interact with each other, were converted to Lys and Glu residues, respectively. This strict conservation may support the significance of the cross-subunit interface. Second, six residues (Glu⁴⁵², Tyr²⁸³, Tyr⁴⁶⁶, Gly⁴⁶⁷, Pro⁴⁶⁸, and Pro⁴⁷⁰), which belong to either motif 2 or 3, were located in the cross-subunit interface. Because motifs 2 and 3 constitute most of the active site of Class II aaRS, it seems likely that the signal from the cross-subunit interface was transmitted through these residues to other residues that participate in the stabilization of the transition state. Although no residues that specifically stabilize the transition state have yet been identified in LysRS, it has been revealed that a motif 2 loop in *E. coli* LysRS(U) (260EGISVRHN271), which was close to the active site and disordered in the complex with L-lysine, became ordered in the presence of adenine molecules (30). Because the loop was strictly conserved in *B. s*. LysRS except that Val⁶⁸ was substituted by a Thr residue (25), such a conformation of the loop may be held in the complex of *B. s*. LysRS with an adenine molecule. The unfavorable changes in \( \Delta G^\dagger \) and \( \Delta G^0_{\text{ATP}} \) for CAT-CD in the energy diagrams (Fig. 5) may be because of a failure in the formation of the ordered structure of the motif 2 loop because of the loss of the cross-subunit interaction between the catalytic domain and the anticodon-binding domain. Amino acid residues that contribute to the stabilization of the transition state but not the ground state have been reported for MetRS (31, 32) and TyrRS (33, 34). The energy diagrams (Fig. 5) also indicate that CAT-CD suffers from a disturbance in \( \Delta G^0_{\text{Lys}} \) as well as in \( \Delta G^\dagger \) and \( \Delta G^0_{\text{ATP}} \). The loss of the cross-subunit interaction may also have disturbed the conformational changes that may be induced by the addition of L-lysine to *B. s*. LysRS as was observed with *E. coli* LysRS(S) (35). Overall, it was considered that the cross-subunit interaction between the catalytic domain and the anticodon-binding domain in *B. s*. LysRS contributes more significantly to the activation free energy than to the binding free energy, suggesting a fine mechanism for the transition state stabilization. In the process of biological evolution where the anticodon-binding domain was appended to the catalytic domain and the cross-subunit interaction between them has been finely tuned, the original molecular mechanism of the transition state stabilization in the primordial LysRS may have been lost and changed to require a cross-subunit interaction in the modern *B. s*. LysRS.

It has been reported that the binding of eukaryotic aaRSs to tRNA was reinforced through an additional RNA-binding
was a cross-subunit interaction or not. Between the anticodon-binding domain and the catalytic domain.

The hamster LysRS aminoacylated a microhelix that mimics the amino acid acceptor minihelix at a very low level as compared with an intact tRNA (40-fold increase in $K_m$ for tRNA and 65,000-fold decrease in $k_{cat}$). These data indicate that the interaction between the tRNA anticodon and the anticodon-binding domain of the enzyme was very important in the transition state stabilization of the aminoacylation reaction. Is the communication between the region participating in the recognition of anticodon and the catalytic site achieved by a direct interaction between the anticodon-binding domain and the catalytic domain or an indirect interaction that was mediated through the "elongated" N-terminal polypeptide chain extension? The finding that the effect of the deletion of the N-terminal polypeptide chain extension on the aminoacylation of intact tRNA was limited to a change in the $K_m$ for tRNA and no observed change in $k_{cat}$ (40) may support the importance of a direct interaction in the transition state stabilization. In mammalian LysRS, it remains to be uncovered whether the direct interaction between the anticodon-binding domain and the catalytic domain was a cross-subunit interaction or not.

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