Several bacteria and archaea utilize the amino group-carrier protein, LysW, for lysine biosynthesis, in which an isopeptide bond is formed between the C-terminal Glu of LysW and an amino group of α-aminoadipate (AAA). The resulting LysW-γ-AAA is phosphorylated by LysZ to form LysW-γ-AAA phosphate, which is subsequently reduced to LysW-γ-aminoadipic semialdehyde (LysW-γ-AASA) through a reaction catalyzed by LysY. In this study, we determined the crystal structures of LysY from Thermus thermophilus HB27 (TtLysY) complexed with TtLysW-γ-AASA and TtLysW-γ-AAA, respectively. In both structures, the globular domain of TtLysW was recognized by positively charged residues on helix e9 and the β11-α10 loop of TtLysY through conformational changes. A mutational analysis confirmed that the interactions observed between TtLysY and TtLysW are important for the function of TtLysY. The extended LysW recognition loop and conserved arginine residue were identified as signatures to discriminate LysY from ArgC, which is involved in arginine biosynthesis. Combined with the previously determined TtLysZ-TtLysW complex structure, TtLysW may simultaneously bind TtLysZ and TtLysY. These structural insights suggest the formation of a TtLysWZY ternary complex, in which the flexible C-terminal extension of TtLysW promotes the efficient transfer of the labile intermediate from the active site of TtLysZ to that of TtLysY during the sequential reactions catalyzed by TtLysY.

Bacteria and plants have been suggested to biosynthesize lysine from aspartate via the dianimopimelate pathway (1). We previously reported that the hyperthermophilic bacterium, Thermus thermophilus, biosynthesizes lysine from 2-oxoglutarate via α-aminoadipate (AAA) as an intermediate (2). The first half of this pathway (3–6) is similar to the fungal AAA pathway (7, 8), whereas the latter half utilizes the small acidic protein LysW to protect the α-amino group of AAA (9), unlike the fungal AAA pathway. LysX catalyzes the formation of an isopeptide bond between the C-terminal Glu of LysW and the α-amino group of AAA, and the resulting LysW-γ-AAA is further converted into LysW-γ-lysine through sequential reactions catalyzed by LysZ, LysY, and LysJ, which show homology to the arginine biosynthetic enzymes, ArgB, ArgC, and ArgD, respectively (10). Phylogenetic studies have indicated that a large number of bacteria and archaea utilize LysW homologs for lysine biosynthesis, whereas the hyperthermophilic archaeon, Sulfolobus acidocaldarius, utilizes LysW for the biosynthesis of lysine and arginine (11). Thus, LysW-mediated biosynthesis appears to be one of the common systems involved in prokaryotic amino acid biosynthesis.

LysW is a zinc finger protein consisting of the N-terminal globular domain stabilized by zinc and the C-terminal flexible extension (11, 12). The crystal structures of the ArgX-LysW complex from S. acidocaldarius (11) and the LysZ-LysW-γ-AAA complex from T. thermophilus (12) indicate that in addition to its role in protecting the α-amino group of the intermediates from intramolecular cyclization, LysW acts as a carrier protein to facilitate the recognition of the biosynthetic intermediates by catabolic enzymes through electrostatic interactions between the negatively charged surface of LysW and the positively charged residues surrounding the active site of each biosynthetic enzyme. However, the mechanisms by which the other biosynthetic enzymes, including LysY, LysJ, and LysK, recognize LysW derivatives currently remain unknown. Moreover, the structural determination of each of the enzymes binding LysW derivatives may provide insights into how amino group-carrier proteins are transferred from one enzyme to another.

LysY is the third enzyme involved in LysW-mediated lysine biosynthesis from AAA, which catalyzes the reduction of LysW-γ-AAA phosphate (LysW-γ-AAAP), a reaction product of LysZ, to LysW-γ-aminoadipic semialdehyde (LysW-γ-AASA) (Fig. 1A) (9). As described above, LysY shows high sequence identity with ArgC, catalyzing the conversion of N-acetyl-γ-glutamyl phosphate to N-acetyl-γ-glutamic semialdehyde in the biosynthesis of arginine (13). According to Cherneny et al. (14), conserved Cys, His, and Arg residues have important roles in the function of ArgC as a nucleophile, base catalyst, and phosphate-binding site, respectively. Because corresponding residues are also conserved in LysY homologs, the catalytic chemistry of LysY may be similar to that of ArgC. Because of sequence homologies and functional similarities between LysY and ArgC, they are considered to have evolved from a common ancestor (10). However, the substrate of LysY contains an isopeptide-bonded LysW instead of an N-acetyl group in the ArgC substrate to protect the α-amino group of

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**Crystal Structure of the LysY·LysW Complex from Thermus thermophilus**

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Tetsu Shimizu, Takeo Tomita, Tomohisa Kuzuyama, and Makoto Nishiyama

From the Biotechnology Research Center, University of Tokyo, 1-1-1, Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

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The atomic coordinates and structure factors (codes SE11N and SE1O) have been deposited in the Protein Data Bank ([http://wwpdb.org/](http://wwpdb.org/)).

1 To whom correspondence should be addressed. Tel.: 81-3-5841-3074; Fax: 81-3-5841-8030; E-mail: umanis@mail.ecc.u-tokyo.ac.jp.

2 The abbreviations used are: AAA, α-aminoadipate; TtLysW, T. thermophilus HB27 LysW; TtLysY, T. thermophilus HB27 LysY; TtLysYHB8, T. thermophilus HB8 LysY; TtLysZ, T. thermophilus HB27 LysZ; TtLysXHis, His11-His12-tagged T. thermophilus HB27 LysX; LysW-γ-AAA, LysW-γ-aminoadipic semialdehyde; PDB, Protein Data Bank; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; CHES, 2-[cyclo-hexylamino]ethanesulfonic acid.

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each substrate. Hence, the elucidation of residue(s) in LysY responsible for the recognition of LysW will provide a clearer understanding of the molecular evolution of LysY and ArgC, each of which has adapted to different N-modification systems.

We herein presented two crystal structures of LysY from *T. thermophilus* HB27 (TtLysY) bound to TtLysW-γ-AASA and TtLysW-γ-AAA at resolutions of 1.8 and 1.7 Å, respectively. Combined with the apo structure of an ortholog from *T. thermophilus* HB8 (TtLysYHB8) showing 99% amino acid identity with TtLysY, these structures revealed that TtLysY recognizes TtLysW via conformational changes to accommodate the globular domain of TtLysW with the contribution of electrostatic interactions. Moreover, TtLysY recognizes the opposite side of the globular domain of TtLysW recognized by TtLysZ. The results obtained in this study suggest that TtLysY and TtLysZ sequentially convert TtLysW-γ-AAA to TtLysW-γ-AASA by amino group-carrier protein-mediated substrate channeling.

**Experimental Procedures**

**Preparation of Expression Vectors**—The previously constructed vectors (9, 11) pET26-lysX, pET26-lysZ, and pET-lysW were used for the expression of C-terminal His6-tagged TtLysX (TtLysXHis), TtLysZ, and TtLysW, respectively.

To construct a TtLysY expression vector, the *lysY* gene was amplified by a PCR using appropriate primers (The sequences of the primers are available on request.) with pACYCDuet-1 as template DNA. The amplified fragment was cloned into the EcoRI-HindIII sites of pBluescript SKII(+), which was further transformed into BL21-CodonPlus (DE3)-RIL-harboring appropriate plasmid to generate pET26b-(TtLysXHis), TtLysZ, and TtLysW, respectively.

**Preparation of Recombinant Proteins**—TtLysXHis, TtLysZ, and TtLysW were produced and purified according to our previous studies (9, 12). To produce TtLysY and its mutants, an *Escherichia coli* BL21-CodonPlus (DE3)-RIL-harboring appropriate plasmid was precultured in 2×YT medium (15) supplemented with 50 μg ml⁻¹ kanamycin and 30 μg ml⁻¹ chloramphenicol at 37 °C overnight. After the transfer of the preculture into the same fresh medium at 1% followed by a 3-h culture at 37 °C, isopropyl β-D-thiogalactopyranoside was added to culture medium at a final concentration of 0.1 mM, and the culture was then continued at 25 °C for 12 h. Cells were harvested by centrifugation and washed with buffer A (20 mM Tris-HCl, pH 8.0, 1 mM EDTA). The cells resuspended in buffer A were disrupted by sonication, and cell debris was removed by centrifugation. The resulting cell lysate was heated at 80 °C for 30 min, and denatured proteins were removed by centrifugation. The supernatant was loaded onto a Blue-Sepharose CL6B (GE Healthcare, Tokyo, Japan) column equilibrated with buffer A, and the column was washed with 5 bed volumes of buffer A containing 200 mM NaCl. Bound proteins were eluted with 3 bed volumes of buffer A containing 500 mM NaCl without 1 mM EDTA. Eluted proteins were concentrated by Vivaspin (GE Healthcare) and used for enzyme assays. As for TtLysY and TtLysYCl148A for crystallization, the proteins purified by Blue-Sepharose CL6B were further purified by Superdex 200 (GE Healthcare) gel filtration equilibrated with buffer B (20 mM Tris-HCl, 150 mM NaCl). The purity of TtLysY and its mutants, TtLysXHis and TtLysZ, were confirmed by 15% SDS-PAGE. Regarding TtLysW and its derivatives, 12% Tricine-SDS-PAGE was performed. Protein concentrations were determined using a protein assay kit (Bio-Rad) calibrated with bovine serum albumin, and the concentrations of TtLysW and its derivatives were determined by a BCA protein assay kit (Thermo Fisher Scientific, Kanagawa, Japan) calibrated with TtLysW (12).

**Analytical Gel Filtration**—A protein sample (500 μl) containing 2 mg ml⁻¹ of purified TtLysY was applied to a Superdex 200 10/300 GL (GE Healthcare) equilibrated with a buffer containing 20 mM Tris-HCl, pH 8.0, and 150 mM NaCl. Gel filtration was performed at a flow rate of 0.4 ml min⁻¹ at 4 °C. Eluted proteins were monitored by measuring absorbance at 280 nm. Ferritin (440 kDa), aldolase (118 kDa), conalbumin (75 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and ribonuclease A (13.7 kDa) were used as standard proteins for calibration.

**Preparation of TtLysW Derivatives**—TtLysW-γ-AASA was prepared as described in our previous study (12). To prepare TtLysW-γ-AASA, the reaction mixture (10 ml) containing 100 mM Tris-HCl, pH 9.0, 1 mM MgSO₄, 5 mM NADPH, 5 mM AAA, 3 mM ATP, 400 μM TtLysW, 200 μg/ml TtLysXHis, 200 μg/ml TtLysZ, and 340 μg/ml TtLysY was incubated at 60 °C for 2 h. The reaction mixture was desalted by Vivaspin 3000 molecular weight cutoff (GE Healthcare), then loaded onto Mono Q 5/50 GL (GE Healthcare) equilibrated with buffer C supplemented with 100 mM NaCl. Proteins were eluted with a linear gradient of 100–500 mM NaCl in buffer C. The eluted fractions were resolubilized by Tricine-SDS-PAGE, and fractions containing TtLysW-γ-AASA were loaded onto HiLoad16/60 Superdex 30 pg (GE Healthcare) equilibrated with buffer B. Eluted fractions containing TtLysW-γ-AASA were concentrated and stored at 4 °C.

**LC/ESI(−)/MS Analysis**—Purified TtLysW and TtLysW-γ-AASA were buffer-exchanged to MilliQ by Amicon 3000 (Merck Millipore). The final concentrations of the protein aliquots were set to 340 μM, and absolute methanol was added to each sample at a 1:1 ratio prior to being injected. An LC/ESI(−)/MS analysis was conducted using a Triple TOF 5600 system (ABSciex) equipped with a Nexera UHPLC system (SHIMADZU, Shimadzu, Japan) and CAPCELL PAC C18, 2 μm, 2.0 × 50 mm (SHISEIDO, Tokyo, Japan). Samples were eluted by the gradient mobile phase with 0.1% (v/v) formic acid as solvent A and acetonitrile + 0.1% (v/v) formic acid as solvent B. Scans were performed in the negative electrospray ionization mode (ion source gas1, 50; ion source gas2, 50; curtain gas, 25; temperature 550, ion spray voltage floating, 5500 V). Raw data containing multiple negatively charged protein peaks were deconvoluted using Analyst (ABSciex).

Co-crystallization and Structural Determination of TtLysY Complexed with TtLysW Derivatives—Crystals of TtLysY·TtLysW-γ-AASA-NADP⁺ were obtained in 2 μl of hanging drops of a 1:1 mixture of the protein aliquot (20 mM Tris-HCl, 10 mM NaCl, 30% PEG 3350, 0.1 M citrate, pH 6.5) and well solution (30% PEG 3350, 0.1 M citrate, 0.1 M Tris, pH 6.5). Crystals were flash-cooled in liquid nitrogen after equilibration in 30% PEG 3350, 0.1 M citrate, 0.1 M Tris, pH 6.5. Structure refinement and analysis were performed using the CCP4 suite and |P| program |P| (42). The best crystal structure (R1 = 0.177, wR2 = 0.285) was solved by molecular replacement with Phaser (43) and refined at 1.8 Å resolution using the program REFMAC5 (44). Further refinement and model building were performed using COOT (45) and Phenix (46). The structures were analyzed using PyMOL (47) and RAVE (48).
Crystal Structure of the LysY-LysW Complex

### Table 1
Data collection and refinement statistics

|                          | TtLysY-TtLysW-γ-AASA-NADP⁺ | TtLysYC148A-TtLysW-γ-AAA-NADP⁺ |
|--------------------------|-----------------------------|---------------------------------|
| Data collection*         |                             |                                 |
| X-ray source             | PF-NE3A                     | PF-NE3A                         |
| Wavelength (Å)           | 1.00                        | 1.00                            |
| Space group              | P3₂1₃₁                        | P3₂1₃₁                         |
| Cell dimensions (Å)      | a = b = 83.5, c = 168.3     | a = b = 83.5, c = 167.8         |
| Resolution (Å)           | 1.80 (1.83–1.80)            | 1.70 (1.73–1.70)                |
| Reflections (total/unique)| 692060/64110                | 791507/75384                    |
| Rmerge (%)               | 11.2 (49.0)                 | 7.5 (44.1)                      |
| Rfree (%)                | 3.4 (11.2)                  | 2.9 (13.0)                      |
| CC(1/2) (%)              | 99.8 (96.4)                 | 99.7 (94.2)                     |
| I/σ(I) (%)               | 27.8 (2.8)                  | 38.1 (4.2)                      |
| Completeness (%)         | 99.4 (100)                  | 98.9 (100)                      |

**Refinement**

|                          | TtLysY-TtLysW-γ-AASA-NADP⁺ | TtLysYC148A-TtLysW-γ-AAA-NADP⁺ |
|--------------------------|-----------------------------|---------------------------------|
| Resolution (Å)           | 36.37–1.80                  | 44.25–1.70                      |
| R-factor (work/test) (%) | 16.8/21.3                   | 17.1/21.0                       |
| No. of atoms             | 6,244                       | 6,517                           |
| Protein atoms            | 5,727                       | 5,787                           |
| Zn²⁺                    | 1                           | 1                               |
| NADP⁺                   | 96                          | 96                              |
| AAA                     | 11                          | 11                              |
| Formate                 | 69                          | 40                              |
| Water                   | 400                         | 553                             |
| Acetate                 | 20                          |                                 |
| Average B-factor         |                             |                                 |
| Protein atoms            |                             |                                 |
| Chain A                  | 27.59                       | 23.13                           |
| Chain B                  | 33.95                       | 31.05                           |
| TtLysW                   | 59.14                       | 63.38                           |
| TtLysY                   | 41.17                       | 43.99                           |
| Root mean square deviation values |
| Bond length (Å)          | 0.010                       | 0.010                           |
| Bond angle (°)           | 1.488                       | 1.493                           |
| Ramachandran plot*      |                             |                                 |
| Favored (%)             | 98.2                        | 97.3                            |
| Allowed (%)             | 1.8                         | 2.6                             |
| Outlier (%)             | 0.1*                        |                                 |

*Values in parentheses are data for the highest shell.

### pH and Reactions
- **pH 8.0, 150 mm NaCl, 10 mg ml⁻¹ TtLysY, 3 mg ml⁻¹ LysW-γ-AASA, and 10 mm NADP⁺**: Crystals of TtLysY and its mutants were obtained in 3 μl of a 1:1 mixture of the protein aliquot (20% (w/v) PEG2000 monomethyl ester and 200 mm calcium acetate) at 20 °C over a 1-ml reservoir. Crystals of TtLysY and TtLysW were obtained over a 1-ml reservoir.

### Enzyme Assays
- **TtLysW-γ-AAA phosphate reductase activity**: TtLysY was assayed in 1 ml of a reaction mixture containing 100 mm HEPES-NaOH, pH 8.0, 5 mm MgSO₄, 1 mm ATP, 40 μM TtLysW-γ-AAA, 0.2 mm NADPH, 170 nm TtLysZ, and 26 nm TtLysY at 60 °C. Reactions were initiated after 5 min of pre-heating at 60 °C by the addition of TtLysW, and the oxidation of NADPH was monitored for 30 s using a UV2600-UV-visible spectrophotometer (SHIMADZU, Kyoto, Japan) to measure decreases in absorbance at 340 nm.

The TtLysW-γ-AASA dehydrogenase activities (reverse reaction) of TtLysY and its mutants were assayed in 800 μl of a solution containing 50 mm HEPES-NaOH, pH 8.0, 5 mm MgSO₄, 1 mm ATP, 40 μM TtLysW-γ-AAA, 0.2 mm NADPH, 200 mm calcium acetate (for TtLysY), and 0.2 mm NADPH, 200 mm calcium acetate (for TtLysW), and the oxidation of NADPH was monitored for 30 s using a UV2600-UV-visible spectrophotometer (SHIMADZU, Kyoto, Japan) to measure decreases in absorbance at 340 nm.
reaction mixture containing 100 mM CHES-NaOH, pH 9.5, 1 mM NADP⁺, 25 mM sodium phosphate, 25 μM TtLysW-γ-AASA, and 65 nM TtLysY or an appropriate amount of its mutants at 60 °C. After 5 min of pre-heating at 60 °C, reactions were initiated by the addition of the enzyme solution, and the reduction of NADP⁺ was monitored for 1 min. The inhibition of the TtLysW-γ-AASA dehydrogenase activity of TtLysY by TtLysW was examined as described above, and the concentration of TtLysW-γ-AASA was set to 20 μM, and 250–1000 μM TtLysW was added to the reaction mixture. To determine the kinetic parameters of TtLysY and its mutants for TtLysW-γ-AASA, the concentration of TtLysW-γ-AASA was varied between 3 and 400 μM at a fixed concentration of 1 mM NADP⁺ and 25 mM sodium phosphate. The data obtained were fit to the Michaelis-Menten equation using the non-linear regression tool of Origin 6.0 (Light Stone, Tokyo, Japan).

**Results**

**TtLysY Catalyzes the NADPH-dependent Reduction of TtLysW-γ-AAAP**—Our previous findings (9) indicated that TtLysY catalyzes the NADPH-dependent reduction of TtLysW-γ-AAAP, a reaction product of TtLysZ (12), to produce TtLysW-γ-AAASA (Fig. 1A). Because the preparation of purified TtLysW-γ-AAAP containing a reactive carbonyl phosphate group was impractical because of its low stability, we constructed a coupled reaction system containing TtLysZ and TtTtLysW-γ-AAAP to detect TtLysY activity in the forward reaction. As expected, a high rate of NADPH consumption with a specific activity of 10.1 ± 0.3 μmol min⁻¹ mg⁻¹ was detected under the assay conditions used. In contrast, no activity was detected when NADH was used in the assay. These results confirmed that TtLysY catalyzes the NADPH-dependent reduction of the TtLysW-γ-AAAP.

Our previous studies revealed that TtLysW showed the same migration as that of TtLysW-γ-AAAP and TtLysW-γ-AAAP on Tricine-SDS-PAGE (9, 12). The reaction product of TtLysY showed slower migration than other TtLysW derivatives, including TtLysW, TtLysW-γ-AAAP, and TtLysW-γ-AASA (Fig. 1B). Extra bands appeared in Fig. 1B, lane 6, at molecular mass of 35, 37, and 45 kDa. The extra band at 45 kDa (a band above TtLysY) turned out to contain fragments of TtLysY and TtLysW; therefore, this might be formed through a non-enzymatic reaction between the carbonyl phosphate group of LysW-γ-AAAP and the side-chain amino groups located on the surface of TtLysY. Two other bands with molecular mass of 35 and 37 kDa might be formed in a similar manner between LysW-γ-AAAP and TtLysZ and TtLysX, respectively. The TtLysY reaction product was purified to homogeneity on Tricine-SDS-PAGE through anion exchange chromatography and gel filtration (Fig. 1C). An LC/ESI/MS analysis revealed that the purified TtLysY reaction product possessed a deconvoluted mass of 5,939.2 Da (Fig. 2A), which is 127 Da larger than that of TtLysW (5,812.1 Da) (Fig. 2B), and the observed values agreed with the theoretical masses of TtLysW-γ-AAAS (5,939.5 Da) and TtLysW (5,812.5 Da). These observations indicated that TtLysY catalyzes the NADPH-dependent reduction of TtLysW-γ-AAAP to yield TtLysW-γ-AASA.

**Kinetic Analysis of TtLysY**—TtLysY exhibited NADP⁺-dependent activity for purified TtLysW-γ-AASA in the presence of the TtLysY reaction product. **FIGURE 1. Enzymatic synthesis of the TtLysY reaction product.** A, scheme of the TtLysZ and TtLysY reaction. B, Tricine-SDS-PAGE analysis of the TtLysY reaction product. Lane 1, low molecular mass marker; lane 2, peptide molecular weight marker; lane 3, reaction mixture containing TtLysW; lane 4, reaction mixture containing TtLysW and TtLysX; lane 5, reaction mixture containing TtLysW, TtLysY, and TtLysZ; lane 6, reaction mixture containing TtLysW, TtLysX, TtLysZ, and TtLysY; lane 7, reaction mixture containing TtLysW, TtLysX, TtLysZ, TtLysY, and 5 mM NADPH. C, Tricine-SDS-PAGE of the purified TtLysY reaction product. Lane 1, peptide molecular mass marker; lane 2, TtLysW; lane 3, TtLysY reaction product.
Crystal Structure of the LysY-LysW Complex

![Graph showing the deconvoluted LC/ESI(−)/MS spectra of the TtLysY reaction product (A) and TtLysW (B).]

**FIGURE 2.** Deconvoluted LC/ESI(−)/MS spectra of the TtLysY reaction product (A) and TtLysW (B).

**TABLE 2**

| Kinetic parameters of TtLysY | $k_{cat}$ | $k_m$ | $k_{cat}/k_m$ |
|-----------------------------|----------|-------|---------------|
| TtLysY                      | $9.6$    | $9.8$ | $0.98$        |
| TtLysW-γ-AASA               | $1.2$    | $14$  | $0.09$        |
| NADP+                       | $1.1$    | $12,000$ | $0.9$     |

| TtLysYC148A                 | $14$    | $400$ | $3.6$        |

**Effects of TtLysW addition on the TtLysW-γ-AASA dehydrogenase activity of TtLysY.** Raw data of six replicates in a total of three technical replicates for each of two biological replicates are shown as open circles, and their average values ± S.D. are shown as closed circles.

**FIGURE 3.** Effects of TtLysW addition on the TtLysW-γ-AASA dehydrogenase activity of TtLysY. Raw data of six replicates in a total of three technical replicates for each of two biological replicates are shown as open circles, and their average values ± S.D. are shown as closed circles.

of sodium phosphate, indicating that its catalytic activity is reversible. A kinetic analysis for this reverse reaction revealed that TtLysY possessed lower $k_{cat}$ values and a higher affinity for TtLysW-γ-AASA (Table 2) than those of EcArgC for N-acetyl-γ-glutamic semialdehyde, as reported by McLoughlin et al. (24), suggesting that the amino group modification by TtLysW contributes to the better recognition of the substrate but not to enhancing the turnover number. Furthermore, the $k_{cat}$ value for the reverse reaction was ~6-fold lower than $k_{obs}$ (6.4 ± 0.3 s$^{-1}$) for the forward reaction in the TtLysZY-coupled reaction, indicating that TtLysY prefers the forward reaction to produce TtLysW-γ-AASA.

We also examined the inhibition of the TtLysW-γ-AASA dehydrogenase activity of TtLysY by an excessive amount of TtLysW. Even in the presence of a 50 mM excess of TtLysW (1 mM) against TtLysW-γ-AASA (20 μM), activity was only inhibited by 47% (Fig. 3). This result indicates that TtLysY discriminates the structural difference between TtLysW-γ-AASA and TtLysW.

**Overall Structure of the TtLysYW-TtLysW-γ-AASA-NADP+ Complex**—To capture the TtLysY-TtLysW complex with the C-terminal extension of TtLysW inserted into the active site of TtLysY, we chose to use an inactive mutant of TtLysY to stabilize the isopeptide-bonded substrate within the active site of TtLysY. Based on a structural comparison between TtLysY and ArgC from M. tuberculosis (14), Cys-148 of TtLysY was expected to be a catalytic nucleophile. Thus, the prepared TtLysYC148A exhibited no detectable LysW-γ-AASA dehydrogenase activity. We subsequently determined the crystal struc-
ture of TtLysYC148A complexed with TtLysW-γ-AAA as a substrate analog and NADP\(^+\) at a resolution of 1.7 Å. We noted that no crystal was obtained when wild-type TtLysY and TtLysW-γ-AAA were used for crystallization. The organization of the asymmetric unit and assembly of the crystal structure was essentially the same as that of the TtLysY-TtLysW-γ-AASA-NADP\(^+\) complex structure (Fig. 4, C and D). In this crystal structure, the electron density for the C-terminal extension of TtLysW-γ-AAA was clearly observed, and its AAA moiety was bound to the active site of TtLysYC148A. Interestingly, a monomer of TtLysW-γ-AAA was recognized by two subunits of TtLysY forming a dimer, one subunit of which recognized the globular domain of TtLysW, whereas the other subunit accommodated the C-terminal extension, including the AAA moiety.

**Binding of NADP\(^+\)** — In both structures, NADP\(^+\) bound at the cleft formed between the nucleotide-binding domain and dimerization domain (Fig. 5A). Interdomain movement upon the binding of NADP\(^+\) was reported in aspartate-β-semialdehyde dehydrogenases (28, 29); however, this was not the case for TtLysY. A comparison of the present TtLysY structure with the apo-TtLysYHB8 structure revealed that both structures were essentially the same (root mean square deviation = 0.4 Å). The pyrophosphate moiety of NADP\(^+\) was recognized by the typical glycine-rich phosphate-binding loop, \(^{10}\)GASGYAG\(^{16}\). TtLysY specifically recognized the 2′-phosphate group of NADP\(^+\) by hydrogen bonds with the side chain of Ser-36 and the main chain nitrogens of Arg-37 and Arg-38 and ionic interactions with the side chain of Arg-37 (Fig. 5B). In addition, Arg-37 was also involved in the recognition of the adenine ring of NADP\(^+\) by the cation-π interaction. Therefore, the presence of the 2′-phosphate group in NADP\(^+\) may be important for the proper positioning of Arg-37 to stabilize the adenine ring of the co-factor.
These structural features agreed with the observed co-factor preference of TtLysY in the activity assay.

**Recognition of the Globular Domain of TtLysW**—In both complex structures, the globular domain of TtLysW was recognized by a patch consisting of helix α9 and the β11-α10 loop of TtLysY chain B. Mapping of the surface electrostatic potential of TtLysY revealed that the corresponding region possessed a positively charged surface (Fig. 6), which is suitable for recognizing the negatively charged surface of TtLysW. This indicated that electrostatic forces contribute to the interactions between TtLysY and the globular domain of TtLysW. TtLysY recognized one side of the TtLysW globular domain, in which the zinc atom was coordinated by Cys-5W (Cys-5 from TtLysW), Cys-8W, Cys-25W, and Cys-28W (Fig. 7A). The ionic bond with Arg-258B (Arg-258 from chain B) plays important roles in the function of TtLysY. Arg-258B formed an ionic bond with Asp-27W and Cys-8W of which the thiolate anion was stabilized by the zinc atom. Other interactions between TtLysY and the globular domain of TtLysW were mediated by hydrogen bonds and water-mediated hydrogen bonds (Fig. 7A). Notably, interactions between the globular domain of TtLysW and the β11-α10 loop of TtLysY were mediated by hydrogen bonds involving main chain atoms but not side chain atoms.

**Recognition of the C-terminal Extension of TtLysW**—Because the active site of TtLysY was surrounded by a positively charged surface (Fig. 6), electrostatic forces may have guided the negatively charged C-terminal extension of TtLysW to the active site of TtLysY. Residues located on the β11-α10 loop of TtLysY were also involved in recognizing the C-terminal extension of TtLysW-γ-AAA through which the side chain Arg-278B formed ionic bonds with the side chains of Glu-50W (Fig. 7B). Arg-278B also stabilized the indole ring of Trp-52W via the cation-π interaction, and the main chain carbonyl oxygen of Val-276B formed a hydrogen bond to the side chain nitrogen of Trp-52W. The last two residues of the C-terminal extension of TtLysW were recognized by two arginine residues in chain A; Arg-200A formed a hydrogen bond with the main chain carbonyl oxygen of Gly-53W, and Arg-195B stabilized the α-carboxyl group of C-terminal Glu-54W.

**Active Site Architecture**—Because TtLysW-γ-AAA lacking the phosphate group is not a substrate of TtLysY, a water molecule instead of phosphate occupied the vacant space of the phosphate-binding site, forming hydrogen bonds with the conserved Arg-102, in the active site of the TtLysYC148A·TtLysW-γ-AAA·NADP⁺ structure (Fig. 7C). However, the side chain methyl group of mutated C148A was located 3.7 Å from C6 of the AAA moiety. Moreover, C4 of the nicotinamide moiety of NADP⁺ was located 3.8 Å from C6 of the AAA moiety, a distance that is suitable for hydride transfer. Thus, the structure may be a good mimic for the Michaelis complex of TtLysY·TtLysW-γ-AAA·NADP⁺, which represents an arrangement just before the nucleophilic attack by Cys-148 on the carbonyl group of the AASA moiety. The active site architecture of TtLysY was similar to that of ArgC from *M. tuberculosis* (Fig. 7D). Accordingly, His-209 of TtLysW was predicted to be the base catalyst responsible for the deprotonation of Cys-148. In contrast, in ArgC, Asn-149 and Ile-177 of TtLysY were replaced with Tyr-159 and Ser-186, respectively, and the corresponding residues were conserved in ArgC (Fig. 8), suggesting that these residues play a role in recognizing the N-acetyl group of the substrate in ArgC.

**Conformational Changes upon Binding of the Globular Domain of TtLysW**—Because only one of the two TtLysY subunits interacts with the globular domain of TtLysW in complex structures, the two subunits may take different conformations from each other. Therefore, we superposed two subunits of TtLysYC148A in the asymmetric unit of the TtLysYC148A·TtLysW-γ-AAA·NADP⁺ structure. This superposition revealed conformational changes upon the binding of TtLysW as follows. The side chain of Lys-271B flipped to form ionic bonds with the side chain of Glu-250B, and the side chain of Trp-254B rotated 26° toward the outside. Consequently, the conformation of the β11-α10 loop, including Lys-273B and Lys-274B, changed to accommodate the glob-
ular domain of TtLysW without steric hindrance (Fig. 9A), and the side chain conformation of Arg-278B also changed to recognize Glu-50W and Trp-52W (Fig. 7D). Moreover, the /H9252 11-/H9251 10 loop of the apo structure of TtLysYHB8 took a similar conformation to that of the chain A structure (Fig. 9B) but different from that of the chain B structure (Fig. 9C). These results indicated that conformational changes in chain B were induced by binding of the globular domain; however, the side chain conformation of Arg-278 in chain B was similar to that of the apo structure, suggesting that Arg-278 takes both conformations in the absence of TtLysW.

Mutational Analysis—To evaluate the contribution of the observed interactions between TtLysY and TtLysW to TtLysY activity, we determined the kinetic parameters of TtLysY mutants for TtLysW-/H9253 -AAA in the reverse reaction (Table 3). The Ala substitution of Arg-258 at helix /H9251 9, which is involved in electrostatic interactions with TtLysW, resulted in a slight decrease in $k_{cat}$ and a 17-fold increase in $K_m$. This result indicated that the observed electrostatic interactions between TtLysY and the globular domain of TtLysW are important for the efficient recognition of TtLysW by TtLysY. A 4.6-fold decrease in $k_{cat}$ and a 70-fold increase in $K_m$ were observed in the K271A mutant. Lys-271 at the /H9252 11-/H9251 10 loop plays an important role in conformational changes upon binding of the globular domain of TtLysW, whereas the side chain of Lys-271 is not directly involved in interactions between TtLysY and TtLysW. Therefore, the marked increase in $K_m$ for the
K271A mutant supported the observed conformational changes being important for the function of TtLysY. The Ala substitution of Arg-195, which interacted with the α-carboxyl group of Glu-54\textsuperscript{W} of TtLysW, resulted in a 5-fold decrease in $k_{\text{cat}}$ and a 40-fold increase in $K_{m}$. Moreover, no detectable activity was observed for the R278A mutant. These results indicate that the recognition of the C-terminal extension of TtLysW by Arg-278 is essential for the function of TtLysY. A mutation on the putative catalytic residues, Cys-148 and Arg-102, either abolished or significantly decreased activity, indicating that these residues are involved in the catalytic reaction of TtLysY. However, the H209A mutant exhibited similar activity to that of the wild-type enzyme. Because His-209 is predicted to be involved in the deprotonation of Cys-148 in the catalytic cycle of TtLysY, we assumed that activity was detected under the basic conditions (pH 9.5) used in our standard assay, which was higher than the thiol pK$_{a}$ value of most Cys residues in proteins in the range of 8 to 9. Therefore, we determined the kinetic parameters of the wild-type enzyme and the H209A mutant for TtLysW-γ-AAA under neutral conditions (pH 7.5). Although the $k_{\text{cat}}$ of the wild-type enzyme at pH 7.5 was 5.6-fold lower than that at pH 9.5, a more critical effect was observed for the H209A mutant by lowering the
pH, in which the $k_{cat}$ of the H209A mutant was 61-fold lower than that at pH 9.5. These results supported the previous assumption that His-209 plays a role in the deprotonation of the thiol of Cys-148.

Discussion

In this study, we determined the crystal structures of TtLysY complexed with TtLysW derivatives. The structures obtained revealed dynamic interactions between TtLysY and TtLysW, in which one molecule of TtLysW was recognized by two subunits of TtLysY forming the unit of a dimer. Among the dynamic interactions between TtLysY and TtLysW, the $\beta$11-$\alpha$10 loop of TtLysY was involved in recognizing the globular domain and C-terminal extension of TtLysW. Therefore, we suspect that recognition of the two distant regions of TtLysW by TtLysY is cooperative. Conformational changes involving helix $\alpha$9 and the $\beta$11-$\alpha$10 loop of TtLysY observed in our crystal structures were presumably induced by binding of the globular domain but were also accelerated by stabilization of the C-terminal extension of TtLysW, including an isopeptide-bonded substrate. Therefore, TtLysY may discriminate its proper substrate from TtLysW derivatives containing a common carrier protein because the excessive amount of TtLysW exerted a limited inhibitory effect on the LysW-$\gamma$-AASA dehydrogenase activity of TtLysY.

In our complex structures, a water molecule instead of phosphate was present at the phosphate-binding site in the active site of TtLysY. In contrast, a sulfate ion, a better mimic of phosphate, was present at the corresponding site of the apo-
TtLysYHB8 structure, which was stabilized by Arg-102 and Arg-208 (Fig. 10). Notably, Arg-208 resides next to His-209, which deprotonates Cys-148 in the catalytic cycle of TtLysY. In the TtLysY-TtLysW-γ-AASA-NADP⁺ structure, the N° atom of His-209 was located 4.6 Å from the S’ atom of Cys-148, which appears to be slightly long for the efficient deprotonation of Cys-148 by His-209 in catalysis. In the apo-TtLysYHB8 structure, the distance between corresponding atoms was shortened to 4.0 Å through the formation of ionic bonds between the sulfate ion and Arg-208, suggesting that the proper positioning of catalytic His is accompanied by the binding of phosphate to the active site of TtLysY. Because the residue corresponding to Arg-208 in TtLysY is conserved in the LysY and ArgC homologs (Fig. 8), similar mechanisms induced by the binding of a phosphate ion or phosphate group may be present in this protein family.

A comparison with ArgC revealed that helix α9 of TtLysY was slightly more abundant in basic residues than in the corresponding helix in ArgC (Fig. 8). Moreover, the β11-α10 loop of TtLysY was 4–6 residues longer than the corresponding loop in ArgC, and the loop extension relative to ArgC was also detected in other LysY homologs (Fig. 8). Therefore, the recognition of LysW by the extended loop may be a common feature among LysY homologs. However, these “LysW-recognition loops” in LysY homologs showed low sequence identity with each other. This may reflect the sequence diversity of LysW homologs, except for zinc-binding residues and the C-terminal EDWGE motif sequence (9). We noted that despite low sequence identity in the LysW-recognition loop, Arg-278 of TtLysY, which recognizes conserved Glu-50 and Trp-52 in TtLysW, was highly conserved among LysY homologs, except those from Crenarchaeota such as *S. acidocaldarius*. In addition to helix α9 and the β11-α10 loop, Arg-195 of TtLysY, which stabilizes the C-terminal carboxyl group of Glu-54 of TtLysW, was strictly conserved in LysY but not in ArgC (Fig. 8). Based on these results, we propose an extended LysW-recognition loop and conserved Arg residue recognizing the C-terminal carboxyl group of LysW as signatures to discriminate LysY from ArgC.

Although TtLysY and TtLysZ both recognize the globular domain of TtLysW, two enzymes recognize the different sides of the globular domain of TtLysW (Fig. 11A), suggesting that TtLysY binds the TtLysZ-TtLysW complex before the dissociation of TtLysZ and TtLysW-γ-AAAP. Moreover, when the TtLysZ-TtLysW-γ-AAA complex and TtLysYC148A-TtLysW-γ-AAA complex were superposed for each of the globular domains of TtLysW, TtLysZ and TtLysY were both spatially positioned in such a manner to form a putative TtLysWZY complex (Fig. 11B), in which the active sites of TtLysZ and TtLysY were located 42 Å from each other. Unfortunately, in the crystal structure of the TtLysZ-TtLysW-γ-AAA complex, the C-terminal extension was not found in the active site of TtLysZ; therefore, direct superposition between TtLysW is useless. However, a comparison between the two structures of TtLysW-γ-AAA in the TtLysY and TtLysZ complexes and the structure of LysW in the ArgX-LysW complex from *S. tokodaii* showed the structural flexibility of the C-terminal extension of LysW (Fig. 11C). Swinging of the C-terminal extension (Fig. 11C) of TtLysW appears to be sufficient to transfer the intermediate from the active site of TtLysZ to that of TtLysY without any rigid body movement of the globular domain of TtLysW. The intermediate of the LysZY reaction, LysW-γ-AAAP, is known to contain a reactive and unstable carboxyl phosphate group (31, 32), and, thus, the transfer of this intermediate from TtLysZ to TtLysY must occur immediately after the TtLysZ reaction to protect the labile intermediate. Similar labile intermediates have also been found in other amino acid biosynthetic pathways such as β-aspartyl phosphate in methionine, threonine, and lysine biosynthesis (33), γ-glutamyl phosphate in proline biosynthesis (34), and N-acetyl-γ-glutamyl phosphate in arginine biosynthesis (13). In these cases, substrate channeling (35) between kinases and reductases has been proposed as a mechanism to implement the efficient transfer of the labile intermediates (36–38); however, the detailed structural basis of these channeling complexes has not yet been determined. Furthermore, Δ-pyrroline-5-carboxylate synthase found in the proline biosynthesis of plants (39) and humans (40) is known as a bifunctional enzyme containing a glutamate kinase domain and γ-glutamyl phosphate reductase domain in a single polypeptide, whereas the full length of the Δ-pyrroline-5-carboxylate synthase structure still remains elusive. Our model of the TtLysWZY complex structure suggested a novel substrate-channeling mechanism between sequential reactions catalyzed by TtLysZ and TtLysY mediated by an amino group-carrier protein. Because we did not observe complex formation between TtLysZ and TtLysY in the pulldown assay and gel filtration (data not shown), they may only form a complex in the presence of TtLysW. Further biochemical and structural studies of these two enzymes and amino group-carrier proteins will provide detailed information on the substrate transfer mechanism between them and will be helpful for understanding the putative channeling complexes composed of kinases and reductases found in other amino acid biosynthetic pathways.

**Author Contributions**—T. S. performed all the experiments and wrote the manuscript; T. T. performed crystallographic analysis; T. K. planned experiments; M. N. planned experiments, analyzed data, and wrote the manuscript. All the authors reviewed the results and approved the final version of the manuscript.

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