Structural Insight into Amino Group-carrier Protein-mediated Lysine Biosynthesis

CRYSTAL STRUCTURE OF THE LYSZ-LYSW COMPLEX FROM THERMUS THERMOPHILUS

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Background: The amino group-carrier protein TtLysW is required for the biosynthesis of lysine by T. thermophilus. The crystal structure of the lysine biosynthetic enzyme TtLysZ was determined in a complex with TtLysW.

Results: The two proteins interacted with each other through electrostatic interactions.

Conclusion: The findings of our previous study on the biosynthesis of lysine by T. thermophilus suggested that the catalytic sites of enzymes involved in the conversion of AAA to lysine were surrounded by positively charged residues (12). Because LysW is a highly acidic protein, we expected its function as a carrier protein to be well recognized by a set of enzymes, thereby ensuring the efficient biosynthesis of lysine. The crystal structure of the ArgX-LysW complex actually supports the electrostatic interaction between biosynthetic enzymes and LysW in the LysW-mediated system. To date, the corresponding part of lysine biosynthesis proceeds in a similar manner, whereas LysW, a small acidic protein composed of 54 amino acid residues, is used to protect the amino groups of the biosynthetic intermediates from LysW-Lys, and the last enzyme LysK releases lysine from LysW-$\gamma$-Lys. Since our discovery of the AAA lysine biosynthetic pathway, many organisms, especially thermophilic bacteria and archaea, have been considered to biosynthesize lysine using this pathway. We also recently revealed that the hyperthermophilic and acidophilic archaea, Sulfolobus, utilized the LysW-mediated system not only to biosynthesize lysine, but also arginine (13). In Sulfolobus, LysX and its paralog ArgX catalyze the formation of LysW-$\gamma$-AAA and LysW-$\gamma$-glutamate, respectively, and the subsequent conversion steps are conducted by a single set of enzymes functioning in the biosynthesis of both lysine and arginine.

The atomic coordinates and structure factors (codes 3WWN, 3WWM, and 3WWN) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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tidyl carrier protein (PCP) have been identified as carrier proteins in the biosynthesis of fatty acids or polyketides and peptides, respectively (14, 15). Both carrier proteins function through a common mechanism; their specific serine residues are modified with the phosphopantetheinyl group of holo-ACP or PCP to form a thioester bond, and they are then elongated on ACP or PCP. In the LysW system, the α-amino group of AAA is covalently bound to the γ-carboxyl group of Glu-54 from LysW by the formation of an isopeptide bond. Therefore, LysW is a novel type of carrier protein involved in primary metabolism. The crystal structure of fatty acid synthetase (FAS) revealed specific electrostatic interactions between ACP and each module in FAS, in which ACP is bound in the central chamber surrounded by modules, and ACP also plays a central role in fatty acid biosynthesis by changing partner modules in the FAS complex (16, 17). Although it currently remains unclear whether the five enzymes necessary for the conversion of AAA to lysine form a LysW-mediated giant complex similar to FAS, how these enzymes recognize LysW conjugates is of interest.

In this study, we determined the crystal structures of TtLysW-γ-AAA and TtLysZ, an amino acid kinase family protein that catalyzes the second step, in a complex with TtLysW-γ-AAA. The results obtained confirmed that AAA attached to the C terminus of TtLysW through the formation of an isopeptide bond, and TtLysZ recognized both the globular and C-terminal extension domains of TtLysW by electrostatic interactions. The results of this study provide a structural basis for a newly identified lysine biosynthetic system in *T. thermophiles* that uses TtLysW as a carrier protein.

**EXPERIMENTAL PROCEDURES**

**Preparation of Expression Vectors**—We used the vectors pET26b-lysW, pET26b-lysX, and pACYCDuet-1-lysZ, which were constructed in our previous study (12), for the expression of TtLysW, TtLysX with a C-terminal His6 tag, and TtLysZ with a C-terminal His6 tag, respectively. The *lysZ* gene for the production of the TtLysZ protein without a His tag was amplified by PCR with appropriate primers. (The sequences of these primers are available on request.) The amplified DNA fragment was cloned into pBluescriptII SK(+) to verify the sequence. The DNA fragment was inserted into the multiple cloning sites of pET26b(+) (Novagen) using Ndel and Xhol sites to yield pET26b-lysZ. In the functional analysis of TTC1586, which is another *N*-acylglutamate kinase (NAGK) homolog of *T. thermophiles* HB27, the pET26b-TTC1586His vector was prepared in the same manner as that for the expression vector of TtLysZ using Ndel and EcoRI.

**Preparation of Crystals**—The recombinant proteins of TtLysW and TtLysZ without His6 tags were prepared for crystallization by using pET26b-lysW and pET26b-lysZ, respectively, and *Escherichia coli* BL21-CodonPlus (DE3)-RIL (Agilent Technologies) as the expression host. The transformants were grown in 2× YT broth (18) supplemented with 50 μg/ml kanamycin and 30 μg/ml chloramphenicol at 37 °C. After a 3-h incubation, gene expression was induced by adding final concentrations of 0.1 and 1 mM isopropyl β-D-thiogalactopyranoside, and the culture was continued for an additional 12–14 h at 25 and 37 °C for TtLysW and TtLysZ, respectively.

To produce TtLysW-γ-AAA, which is a substrate of TtLysZ, His6-tagged TtLysX (TtLysXhis) was also expressed in *E. coli* BL21-CodonPlus (DE3)-RIL harboring pET26b-lysX in the same manner as that described for TtLysW.

Cells harboring pET26b-lysW were harvested and washed with buffer A (20 mM MES-NaOH, pH 6.0). The cells were suspended again in buffer A and disrupted by sonication. The supernatant obtained from centrifugation of the cell lysate was heated at 80 °C for 30 min to remove proteins from *E. coli* by centrifugation. The crude enzymes were applied to the DE52 anion exchange column (Whatman) equilibrated with buffer A. After washing with buffer A, followed by buffer A with 150 mM NaCl, TtLysW was eluted with buffer A containing 500 mM NaCl. The purification of TtLysXhis was conducted as described previously (12). The purified TtLysW and TtLysXhis proteins were concentrated with VIVASPIN (molecular weight cutoff 3000) (Sartorius). We prepared TtLysW-γ-AAA using the TtLysXhis reaction with 100 mM HEPES-NaOH, pH 8.0, 10 mM AAA, 10 mM ATP, 1 mM MgSO4, 0.5 mg/ml TtLysW, and 0.05 mg/ml TtLysXhis at 70 °C overnight. The reaction mixture was applied to the DE52 column to purify TtLysW-γ-AAA. TtLysW-γ-AAA was further purified by gel filtration chromatography using HiLoad 26/60 Superdex 75 (GE Healthcare) with buffer B (20 mM HEPES-NaOH, pH 8.0, 150 mM NaCl). The purities of TtLysW and TtLysW-γ-AAA were monitored by 12% Tricine SDS-PAGE. The concentration of TtLysW was estimated as follows. Purified TtLysW, the concentration of which was estimated by a BCA protein assay kit (Pierce), was diazylated against MilliQ water and weighed after lyophilization. The concentration of TtLysW was calibrated using this value.

To purify TtLysZ without a His tag, cells were harvested and washed with buffer C (20 mM HEPES-NaOH, pH 8.0). After sonication and centrifugation, the supernatant was heated at 80 °C for 30 min and applied to the CM52 cation exchange column (Whatman) equilibrated with buffer B. The passing fraction was concentrated with VIVASPIN (molecular weight cutoff 10,000), and then applied to HiLoad 26/60 Superdex 75 equilibrated with buffer B. TtLysZ and mutant enzymes were purified with the same elution profile in gel filtration column chromatography (data not shown), suggesting that the amino acid replacement did not affect the protein oligomerization state. The purity and concentration of TtLysZ were checked with 15% SDS-PAGE and a protein assay kit (Bio-Rad), respectively.

To crystallize TtLysW-γ-AAA and the complex of TtLysZ with TtLysW-γ-AAA, purified TtLysZ and TtLysW-γ-AAA were mixed at molar ratios of 1:1.6 to 1:8 in a total of 10 mg/ml with 10 mM ATP or AMP-PNP and 10 mM MgSO4. Crystallization was performed at 20 °C using the hanging-drop vapor-diffusion method. Crystals of TtLysW-γ-AAA and the TtLysZ-TtLysW-γ-AAA complex subsequently formed in 2 M ammonium sulfate, 0.1 M sodium acetate, pH 4.6, and 20% (v/v) PEG8000, 0.1 M MES, pH 6.0, 0.2 M calcium acetate, respectively. We also crystallized TtLysZ with 10 mM ADP and 10 mM MgSO4 in the presence of 1.1 M tri-sodium citrate and 0.1 M imidazole, pH 8.0.

**Data Collection and Processing**—Before data collection, the crystals were soaked in a cryoprotectant solution prepared by
mixing the reservoir solution with 20% (v/v) PEG400 for TtLysZ-TtLysW-γ-AAA crystals and in a solution prepared by mixing the reservoir solution with 20% (v/v) glycerol for TtLysZ-ADP crystals. These crystals were then frozen with a nitrogen gas stream. We directly froze TtLysW-γ-AAA crystals with nitrogen gas because they melted in a solution supplemented with the cryoprotectants tested. Diffraction data were collected at 1.000 Å with a CCD camera on the beamline NW12 (for TtLysW-γ-AAA and TtLysZ-ADP crystals) and BL-5A (for TtLysZ-TtLysW-γ-AAA crystals) of the Photon Factory, High Energy Accelerator Research organization (KEK) (Tsukuba, Japan). Diffraction data were indexed, integrated, and scaled using the HKL2000 program suite (19). Because TtLysW is a Zn²⁺-binding protein (12), we collected the anomalous diffraction data of Zn²⁺, which came from the host E. coli cells, to determine the crystal structure of TtLysW-γ-AAA.

Structural Determination and Refinement—The structure of TtLysW-γ-AAA was determined using the MAD phasing method. TtLysW-γ-AAA crystals contained one monomer per asymmetric unit and belonged to the space group P4₁2₁2 with unit cell parameters of a = b = 48.9 Å, c = 38.7 Å. We used the program Solve (20) to detect the Zn²⁺ site and for phase determination and Resolve (21) for automated model building. Molecular replacement was performed with MOLREP (22) in the CCP4 program suite (23) for data collected at 1.000 Å using the initial structural model as a template. Subsequent structural refinement was performed using Refmac5 (24) and Coot (25).

TtLysZ-TtLysW-γ-AAA crystals belonged to the space group P4₁2₁2 with unit cell parameters of a = b = 69.8 Å, c = 148.0 Å. Structural determination was conducted by molecular replacement with Phaser (26) in the CCP4 program suite using the structures of TtLysW-γ-AAA and LysZ from T. thermophillus HB8 (PDB code 3U6U). The space group of TtLysZ-ADP was also P4₁2₁2, and its unit cell parameters were a = b = 80.5 Å, c = 152.3 Å. The structure of these crystals was also determined by the molecular replacement method using LysZ from T. thermophilus HB8 as a template. The subsequent structural refinement step was carried out in the same way as that for TtLysW-γ-AAA. The overall geometry of each structural model was checked by MolProbity (27), and the figures were prepared using PyMOL (28). Data collection and the refinement statistics of these crystal structures are summarized in Table 1. CAVER (29) in PyMOL was used for the cavity calculation. The atomic coordinates of the crystal structures for TtLysW-γ-AAA, TtLysZ-ADP, and the TtLysZ-LysW-γ-AAA complex have been deposited in the RCSB PDB with the accession numbers 3WWI, 3WWM, and 3WVN, respectively.

Analysis of the Structure of the TtLysZ Reaction Product—Because our previous experiment revealed that the phosphate group of the expected LysZ product, LysW-γ-AAA phosphate, was unreactive and easily converted to LysW-γ-AAA during the MS analysis, we added hydroxylamine to convert the unstable phosphate group of the product to the hydroxamate group. The TtLysZ reaction was conducted with 200 mM HEPES-NaOH, pH 8.0, 1 mM MgSO₄, 160 mM NH₂OH-HCl, 10 mM ATP, and 0.1 mM (0.6 mg/ml) TtLysW-γ-AAA (neutralized with 160 mM potassium hydroxide), and the reaction mixture was incubated with 25 µg/ml TtLysZ at 60°C for 1 h. The reaction product was applied to Tricine SDS-PAGE, and the gel containing the TtLysW derivative was sliced. The chemical structure of the reaction product was determined by in-gel digestion followed by LC-MS/MS and MALDI-TOF-MS analyses as described previously (12).

Preparation of TtLysZ Mutants—The plasmids for the expression of the TtLysZ mutants, which were used in the activity assay and isothermal titration calorimetry (ITC) experiments, were constructed by site-directed mutagenesis using QuickChange site-directed mutagenesis kit (Agilent Technology) for pET26b-LysZ. The partially purified proteins of these mutants prepared by the heat treatment (70°C, 30 min) were used in the activity assay. These mutants were purified in the same way as wild-type TtLysZ. Because R111E and R112E variants were not thermostable, site-directed mutagenesis was performed with pACYCDuet-1-LysZ as a template. The Ni²⁺-nitritolactiic acid column was used to purify these two mutant proteins. The oligonucleotides used for site-directed mutagenesis are available on request.

ITC Analysis of the Interaction between TtLysZ and TtLysW-γ-AAA—To elucidate the mechanism underlying the interaction between TtLysZ and TtLysW-γ-AAA, the caloric change was monitored during binding by ITC. This measurement was performed with the ITC200 microcalorimeter (GE Healthcare). We prepared 0.1 mM TtLysZ and 0.8 mM TtLysW-γ-AAA in buffer B for the cell and syringe, respectively. TtLysW-γ-AAA was injected through the computer-controlled 40-μl microsyringe at an interval of 2 min into the TtLysZ solution (cell volume = 200 μl) while stirring at 1000 rpm. All of the experiment was conducted at 60°C. Enthalpy changes (ΔH) and affinities (Kₐ) upon binding were measured, and the values obtained were used to calculate Gibbs free energy (ΔG) and entropy changes (ΔS) according to the equations

\[ K_a = e^{-\frac{\Delta G}{RT}} \]

and

\[ \Delta G = \Delta H - T\Delta S \]

Data were analyzed using a single binding site model implemented in the ORIGIN software package provided by GE Healthcare.

We also calculated the surface charges of TtLysZ, TtLysW-γ-AAA, and the TtLysZ-TtLysW-γ-AAA complex using the Delphi module (30) of Discovery Studio 4.0 (Accelrys, K.K., Japan).

Activity Assay—TtLysZ catalyzes the formation of TtLysW-γ-AAA-phosphate. In the activity assay, unstable TtLysW-γ-AAA-phosphate was converted to the stable hydroxamate derivative in the presence of hydroxylamine. Because the derivative of TtLysW-γ-AAA-phosphate migrated slower than that of TtLysW-γ-AAA on Tricine SDS-PAGE, we evaluated the activities of the wild-type and mutant TtLysZ enzymes by analyzing the densitometry of the bands derived from TtLysW-γ-AAA (substrate) and TtLysW-γ-AAA-hydroxamate (product). The reaction was performed in the same way as that to determine the product structure described above. The concentrations of TtLysW-γ-AAA and TtLysZ were changed to 0.05 mM (0.3 mg/ml) and 10 µg/ml, respectively, and the reaction time was set to 15, 30, and 60 min to follow the reaction. The reaction was stopped by adding the SDS-PAGE sample buffer (250 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 20% (v/v) glycerol, 5% β-mercaptoethanol, and 0.2% (w/v) bromophenol blue) and immediate boiling. After the reaction, the samples were applied to Super Sep Ace (Tricine gel) 15–20% (Wako Pure Chemical, Japan).
To examine activity as NAGK, the reaction was performed with 200 mM Tris-HCl, pH 7.5, 20 mM N-acetylglutamate, 10 mM ATP, 10 mM MgSO4, 160 mM KOH, 160 mM NH2OH-HCl, and 4 μg/ml TTC1586 or TtLysZ. The reaction mixture was incubated for 15, 30, and 60 min at 60 °C and stopped with 5% FeCl3. Absorbance was then measured at 540 nm.

RESULTS

LysZ Catalyzed the Phosphorylation of LysW-γ-AAA—Although LysZ may phosphorylate the δ-carboxyl group of the AAA moiety of LysW-γ-AAA using ATP to yield LysW-γ-AAA phosphate (Fig. 1A), this activity has not yet been measured directly. The conventional enzyme assay used to monitor the hydroxamate converted by the addition of hydroxylamine from carboxyl phosphate, which was formed by an enzyme reaction (31, 32), was not applicable in this study because the substrate, LysW-γ-AAA, was precipitated by the addition of FeCl3, which disabled the photometric detection of the hydroxamate formed. In our previous study (12), we demonstrated that TtLysW-γ-AAA phosphate migrated on Tricine SDS-PAGE in a similar manner to that of TtLysW-γ-AAA, whereas the hydroxamate derivative that reacted with hydroxylamine
We also confirmed that TtLysZ could not catalyze the phosphorylation of N-acetylglutamate, caused by the reaction of NAGK in arginine biosynthesis (Fig. 1E); however, the ortholog from \textit{T. thermophilus} HB8 was annotated as NAGK, and its crystal structure was described as NAGK in a previous study (33). Another homolog of LysZ, TTC1586, exhibited distinct NAGK activity (Fig. 1E), suggesting that TTC1586 functioned as NAGK in the biosynthesis of arginine by \textit{T. thermophilus} HB27.

\textbf{Crystal Structure of TtLysW-γ-AAA—} We determined the crystal structure of TtLysW-γ-AAA prior to examining how TtLysZ recognized its proteinaceous substrate. The multiple wavelength anomalous diffraction method using the anomalous dispersion data of zinc, which was bound to TtLysW-γ-AAA, was conducted to determine this structure at a resolution of 1.20 Å. Data collection and refinement statistics are summarized in Table 1. The structure contained one TtLysW-γ-AAA, one zinc atom, and 90 water molecules per asymmetric unit. The crystal structure of TtLysW-γ-AAA consisted of a globular domain, which was composed of five β-strands, and C-terminal extension containing 10 residues (Fig. 2A). In the crystal structure, AAA was bound to the side chain of the C-terminal Glu-54 of TtLysW by an isopeptide bond (Fig. 2B). The zinc atom was stabilized by four cysteine residues from loop β1-β2 and loop β3-β4. The structure was similar to LysW from \textit{Sulfobolus toko-daii} (StLysW) with 36% identity in the amino acid sequence. Except for the C-terminal extension, the root mean square deviation between these homologs was 0.79 Å (1.10 Å for the whole molecule). Because we did not discuss the structural features of StLysW in our previous study, we described the LysW structure in detail. A DALI search (34) indicated that the globular domain of TtLysW took the form of the zinc ribbon superfamily (35), with the second highest Z score of 2.9 to the zinc finger domain of ESCRT (endosomal sorting complex required for transport)-II (PDB code 2J9U) after StLysW (36). However, strand β5 formed an antiparallel β-sheet with strands β3-β4 in...
Crystal Structure of the LysZ-LysW Complex

TtLysW, whereas the corresponding strand $\beta 5$ was associated with strand $\beta 1$ in ESCRT. Thus, the fold in TtLysW was novel for a zinc finger protein (Fig. 2, D and E).

Crystal Structure of the TtLysZ Complex with ADP—We determined the crystal structure of the TtLysZ-ADP complex at a resolution of 2.8 Å (Fig. 3A). The TtLysZ-ADP complex contained one TtLysZ monomer binding one ADP molecule and 27 water molecules in an asymmetric unit. TtLysZ had a typical amino acid kinase family structure, called the $\alpha\beta\alpha$-sandwich fold, similar to NAGK (37). In this structure, ADP was bound at the C-lobe (Fig. 3B), similar to other amino acid kinase family proteins, and the $\alpha$- and $\beta$-phosphate groups of ADP were stabilized by Lys-5 (Fig. 3C), suggesting that Lys-5 served as a catalytic residue in TtLysZ. In addition to Lys-5, six amino acid residues, Gly-8, Gly-37, Ser-38, Tyr-78, Tyr-200, and Arg-230, interacted with ADP. The main chain amide atoms of Gly-8 and Gly-37 bound the $\alpha$- and $\beta$-phosphate groups of ADP, respectively. The hydroxyl groups of Ser-38 and Tyr-78 were hydrogen-bonded to the phosphate groups, and the 2'-OH group of ribose was stabilized by Arg-230. The amino group of the adenine ring was recognized by the hydroxyl group of Tyr-200 through a hydrogen bond. However, only Gly-8 and Gly-37 were conserved among the ADP-binding residues. In the amino acid kinase family proteins, the binding of ATP and substrate was previously reported to cause domain closure, thereby forming a closed complex (38). The TtLysZ-ADP complex, the crystal structure of which was determined in this study, took a half-closed form.
Crystal Structure of the LysZ-LysW Complex

The structure of the TtLysZ-TtLysW-γ-AAA complex was also determined at a resolution of 1.85 Å. It contained one TtLysZ monomer, one TtLysW-γ-AAA, and 255 water molecules per asymmetric unit (Fig. 4, A and B). The amino acid residues 220–225 of TtLysZ were not assigned due to weak electron densities, although these residues were observed in the TtLysZ-ADP complex. Furthermore, the AAA molecule, which was attached to the C terminus of TtLysW, was not visible in the structure due to low electron densities. In contrast to the TtLysZ-ADP structure, the TtLysZ-TtLysW-γ-AAA structure was in an open state, and this may have been due to the lack of ATP. Accompanied by domain closure, tighter and new interactions may have been generated between TtLysZ and TtLysW.

We compared TtLysZ structures between the crystal structures of TtLysZ-TtLysW-γ-AAA, TtLysZ-ADP, and apo-LysZ from T. thermophilus HB8 (PDB code 3U6U) (33), and we detected structural changes in the following two regions: the β-sheet region composed of strands β3, β4, β7, and β8, and the helical region containing α7 and α8 in TtLysZ of the TtLysZ-ADP complex (Fig. 4C). Accompanying these structural changes in two regions, ADP-bound TtLysZ took a more closed conformation than that of TtLysZ in the apo-form, whereas no structural change was principally observed between TtLysZ-TtLysW-γ-AAA and apo-TtLysZ (root mean square deviation of 0.822 Å) (Fig. 4D). These results indicated that conformational changes in the β-sheet region and helical region were induced by ADP binding. In the TtLysZ-ADP structure, ADP did not interact with the β-sheet region but with the residues from β1, α2, α3, α8, and adjacent turn regions. The mechanism underlying the displacement of the β-sheet region by ADP binding currently remains unclear.

By applying crystal symmetry, TtLysZ could bind TtLysW-γ-AAA molecules at two different surfaces (Fig. 4, A and B). These potential interaction sites were tentatively named interacting region 1 (IR1) and interacting region 2 (IR2) and were located close to strand β8 and the N-terminal region of helix α8, respectively. The C-terminal extension binding AAA via an isopeptide linkage in both TtLysW molecules at either IR1 or IR2 did not enter the active site of TtLysZ but was found on the surface of TtLysZ. Therefore, we could not conclude which interaction was necessary for the reaction based only on the crystal structure. The functional interaction site of TtLysZ was examined biochemically using mutants coupled with a modeling study as described below.

Interaction between TtLysZ and TtLysW-γ-AAA—When the electrostatic potential was mapped to the surface of TtLysZ, two surface regions were occupied by positively charged residues (Fig. 5, A and B). Very negatively charged TtLysW interacted with the oppositely charged surfaces corresponding to IR1 and IR2 (Fig. 5, A and B). In the interaction at IR1, Lys-117, Lys-123, Lys-125, and Arg-128 of TtLysZ formed ionic bonds with Glu-31, Glu-21, Glu-18, and Glu-33 of TtLysW, whereas in the interaction at IR2, Glu-26 of TtLysW formed an electrostatic interaction with Lys-227 and Lys-231 of TtLysZ and Asp-27 of TtLysW electrostatically interacted with Arg-227 and Arg-230 from TtLysZ (Fig. 5, C and D). The interacting surface area was slightly larger in IR1 (4729 Å2) than in IR2 (4484 Å2).

To confirm the electrostatic interaction between TtLysZ and TtLysW, we examined changes in thermodynamic parameters upon binding of TtLysW-γ-AAA to TtLysZ by ITC (Fig. 6A). The results obtained showed that TtLysZ bound TtLysW-γ-AAA with a $K_d$ value of 6.62 ± 0.56 μM, and the stoichiometry of the interaction was 0.834 ± 0.015, which corresponded to the 1:1 complex observed in the crystal structure of the TtLysZ-TtLysW-γ-AAA complex. This binding was mainly stabilized by enthalpic forces ($ΔH = -63.6 ± 0.16$ kcal/mol, $-TDΔS = -1.34$ kcal/mol). The enthalpic contribution coincided well with TtLysZ binding TtLysW by electrostatic interactions, as observed in the crystal structure.

Mutation Analysis for the Interaction between TtLysZ and TtLysW-γ-AAA—Two possible interaction sites have been identified in the crystal structure of the TtLysZ-TtLysW-γ-AAA complex as follows: IR1 around strand β8 and IR2 located on the N-terminal region of helix α8 (Fig. 4, A and B). To determine which site was responsible for the TtLysZ function in lysine biosynthesis, we constructed mutants of TtLysZ by replacing the basic amino acid residues that interacted by ionic bonds with TtLysW through glutamate, and we measured their activities to yield TtLysW-γ-AAA phosphate using the Tricine SDS-PAGE assay system. We also analyzed their abilities to interact with LysW-γ-AAA by ITC. The data obtained from
Although all the variants with amino acid replacements on IR2 had reduced activities, they still retained the ability to bind TtLysW-γ-AAA. IR2 was near the ATP-binding site of TtLysW. These results may explain the changes in activity and interaction profiles in IR2 mutants.

**Binding Site of the C-terminal Extension of TtLysW**—In the TtLysZ-TtLysW-γ-AAA structure, the location of the C terminus of TtLysW was distant from the catalytic center of TtLysZ. As described above, TtLysZ took an open structure when binding to LysW-γ-AAA. Because ADP-bound TtLysZ took a more closed conformation, ATP binding was necessary to form the Michaelis complex that bound the AAA moiety of TtLysW-γ-AAA at the catalytic site as the closed form. When we examined the cavity leading the substrate to the catalytic site of TtLysZ, the cavity opened the entrance to accept the C-terminal extension of LysW-γ-AAA bound at IR1 (Fig. 8, A and B), which also supported IR1 being the functional binding site of TtLysZ for TtLysW. To more precisely elucidate the recognition of the C-terminal extension of TtLysW by TtLysZ, we constructed a model with the C-terminal extension bound at the catalytic site of TtLysZ. We recently determined the crystal structure of a complex of ArgX from *S. tokodaii* (StArgX) with LysW from *S. tokodaii* (StLysW), glutamate, and ADP, which is involved in arginine biosynthesis using the LysW system (13). StLysW is a homolog of TtLysW with a similar structure. The N-terminal globular domains in particular were very similar to each other (root mean square deviation = 0.729 Å). When the globular domains were superimposed between StLysW and TtLysW-γ-AAA in the TtLysZ complex bound at IR1, the C-terminal extension region of StLysW was oriented toward the catalytic center of TtLysZ (Fig. 8A). We generated a model structure of the C-terminal extension of TtLysW based on the structure of StLysW bound to StArgX (Fig. 8B). In the model structure, there were several ionic bonds between residues from TtLysZ and TtLysW as follows: Arg-128–Glu-33*, Lys-117–Glu-31*, Lys-117–Glu-45*, Arg-128–Glu-48*, Lys-113–Glu-50*, and Lys-113–Asp-51* (the asterisk means residues from TtLysW). Glu-50* and Asp-51* were in the conserved C-terminal sequence$^{50EDWGE}^{54}$ of TtLysW. In addition to these residues, there were also other basic amino acid residues from TtLysZ, such as His-57, Arg-111, and Arg-112, in the route to the catalytic center in the cavity of TtLysZ. To examine the validity of the model, we constructed TtLysZ mutant enzymes carrying an Ala or Glu residue at each basic site, and we measured their activities using Tricine SDS-PAGE. The result obtained revealed that all the activities of all the mutant TtLysZ proteins were reduced. The complete loss of activity was observed in mutant Lys-113, which was modeled to interact with Glu-50* and Asp-51* of TtLysW by ionic bonds (Figs. 7C and 8B). Other mutants at His-57, Arg-111, and Arg-112 also showed reduced but apparent activities, suggesting some contribution of these residues in the catalytic function and/or TtLysW recognition of TtLysZ; however, they did not have specific interactions with residues from TtLysW in the modeled structure. These results suggested that the constructed model structure was appropriate and indicated that the recognition of both the globular domain and C-terminal extension of TtLysW-γ-AAA was important for the function of TtLysZ.

**Catalytic Mechanism of TtLysZ**—TtLysZ is a protein member of the amino acid kinase family sharing an overall structure composed of an N-lobe and C-lobe, which bind a phosphate...
acceptor and ATP, respectively. The reaction mechanism in amino acid kinase has been extensively studied for NAGK from *E. coli* (EcNAGK) based on the crystal structures mimicking several stages, including the transition state in the phosphoryl transfer process (39). In EcNAGK, Lys-217 interacted with the β-phosphate group to develop the negative charge of the β-γ bridging oxygen atom of ATP. The ε-amino group of Lys-8 interacted with the leaving γ-phosphate group to stabilize the transition state. Furthermore, the carboxyl group of Asp-162 interacted with the ε-amino groups of Lys-8 and Lys-217.
There were two possible LysW-interacting sites (IR1 and IR2) and the TtLysZ crystal structure of the homolog of TtLysX, StArgX, binding LysW-γ-AAA confirmed that LysX catalyzed the formation of the isopeptide bond between the α-amino group of AAA and the γ-carboxyl group of Glu-54 from LysW. In addition, the crystal structure of the complex of TtLysZ with TtLysW-γ-AAA revealed that the globular domain of TtLysW-γ-AAA interacted with TtLysZ by electrostatic forces. The crystal structure of the homolog of TtLysX, StArgX, binding StLysW, as determined in our previous study, demonstrated that StArgX recognized StLysW via electrostatic interactions. The results of this study revealed that the subsequent biosynthetic enzyme in the second step also recognized LysW electrostatically. The biochemical analyses using mutants and the modeling study suggested that the C-terminal extension of TtLysW-γ-AAA bound to IR1. The cavity calculated by CAVER (29) is shown in pink. TtLysZ and TtLysW-γ-AAA are drawn in cyan and green, respectively. StLysW is shown in magenta. B, recognition model of the C-terminal extension domain of TtLysW-γ-AAA bound at IR1. The model of TtLysW-γ-AAA is colored yellow. Amino acid residues from TtLysW-γ-AAA are shown in the numbers with asterisks. The residues in TtLysZ used for the mutant analysis are shown in the stick models. Conserved residues responsible for catalysis and substrate binding (Lys-5 and Arg-64) are also shown.

**DISCUSSION**

LysW is a novel family of carrier proteins in that it binds the amino group of the ligand, which differs from ACP and PCP, which bind the carboxyl group with the aid of a phospho-pantetheinyl group attached to a specific serine residue. In this study, we determined the crystal structures of TtLysW-γ-AAA and the TtLysZ:TtLysW-γ-AAA complex. The crystal structure of TtLysW-γ-AAA confirmed that LysX catalyzed the formation of the isopeptide bond between the α-amino group of AAA and the γ-carboxyl group of Glu-54 from LysW. In addition, the crystal structure of the complex of TtLysZ with TtLysW-γ-AAA revealed that the globular domain of TtLysW-γ-AAA interacted with TtLysZ by electrostatic forces. The crystal structure of the homolog of TtLysX, StArgX, binding StLysW, as determined in our previous study, demonstrated that StArgX recognized StLysW via electrostatic interactions. The results of this study revealed that the subsequent biosynthetic enzyme in the second step also recognized LysW electrostatically. The biochemical analyses using mutants and the modeling study suggested that the C-terminal extension of TtLysW was also recognized by TtLysZ. These results clearly indicated that LysW functioned not only as a proteinaceous protecting group of the amino group of the substrate, but also as a novel carrier protein that interacted with each biosynthetic enzyme for the efficient biosynthesis of arginine and/or lysine. There were two possible LysW-interacting sites (IR1 and IR2) on TtLysZ in the structure of the TtLysZ:TtLysW-γ-AAA complex, both of which contained basic residues. Detailed analyses of the TtLysZ mutants suggested that IR1 was responsible for

**Figure 8. Modeled structure of the C-terminal extension of TtLysW-γ-AAA bound in the active site of TtLysZ.** A cavity toward the catalytic center of TtLysZ and superposition of StLysW from the StArgX:LysW complex on TtLysW-γ-AAA at IR1. The cavity calculated by CAVER (29) is shown in pink. TtLysZ and TtLysW-γ-AAA are drawn in cyan and green, respectively. StLysW is shown in magenta. B, recognition model of the C-terminal extension domain of TtLysW-γ-AAA bound at IR1. The model of TtLysW-γ-AAA is colored yellow. Amino acid residues from TtLysW-γ-AAA are shown in the numbers with asterisks. The residues in TtLysZ used for the mutant analysis are shown in the stick models. Conserved residues responsible for catalysis and substrate binding (Lys-5 and Arg-64) are also shown.

**Figure 7. Activity assay of TtLysZ mutants using Tricine SDS-PAGE.** Tricine SDS-PAGE after the reaction between TtLysZ and mutants with TtLysW-γ-AAA for 15, 30, and 60 min is shown. The fast-migrating band (blue arrow) indicates the substrate, TtLysW-γ-AAA, and the slow one (red arrow) represents the product, TtLysW-γ-AAA-hydroxamate. Each mutant carries a mutation at the residue in IR1 (A), in IR2 (B), at the putative residue interacting with the C-terminal extension of TtLysW (C), and at a functionally important site (D).

**Table 2. Thermodynamic parameters obtained from ITC experiments**

|        | N  | $K_a$ (μM) | ΔG (kcal/mol) | ΔH (kcal/mol) | ΔS (kcal/mol) |
|--------|----|------------|----------------|----------------|---------------|
| Wild   | 0.834 ± 0.015 | 6.62 ± 0.56 | -7.70          | -6.36 ± 0.16   | -1.34          |
| R227E  | 0.566 ± 0.027 | 3.75 ± 0.85 | -8.08          | -6.03 ± 0.38   | -2.05          |
| R230E  | 0.493 ± 0.037 | 58.8 ± 21.8 | -7.16          | -6.70 ± 5.22   | -0.46          |
| K231E  | 0.930 ± 0.035 | 8.62 ± 1.40 | -7.55          | -5.46 ± 0.28   | -2.09          |

Therefore, this aspartic residue played an important role in the correct positions of these lysine residues. In addition to these charged residues, the main chain amides of Gly-11, Gly-44, and Gly-45 interacted with the β- and γ-phosphate groups of ATP, in which Gly-11 and Gly-44 were involved in withdrawing electrons from the pentavalent γ-phosphate in the transition state, and Gly-45 fixed the oxygen atom of the carboxyl group of N-acetylatedamino acid for attacking the phosphorus atom of the γ-phosphate group of ATP (39). Lys-8, Lys-217, Asp-162, Gly-11, and Gly-44 in EcNAGK were conserved as Lys-5, Lys-231, Asp-172, Gly-8, and Gly-37, in TtLysZ, and Gly-45 was replaced with a similar Ser-38 (Fig. 9A). All these residues, except for Lys-231, occupied similar spatial positions even in TtLysZ. The side chain of Lys-231 was not located close enough to interact with the β-phosphate group of ADP in TtLysZ (Fig. 3C). We suspected that the dislocation of Lys-231 in the TtLysZ:ADP complex may be related to the complex taking a half-closed form. The indispensable role of Lys-231 in the catalytic mechanism was shown by the complete loss of activity in the mutant at Lys-231 (Fig. 7, B and C). Furthermore, the partial positive charges (helix dipoles) at the N-terminal ends of helices α2 and α5 were positioned close to stabilize the β-phosphate group of ADP in the TtLysZ:ADP complex. The similar helix dipole-mediated stabilization of the transition state was proposed in EcNAGK. These results led us to conclude that similar catalytic mechanisms function in both EcNAGK and TtLysZ.
FIGURE 9. Comparison of TtLysZ with other enzymes in the amino acid kinase family. A, amino acid sequence alignment of TtLysZ and EcNAGK based on crystal structures. The sequences were aligned using ClustalW (44) and depicted using ESPript (45). The regions for structural differences observed are surrounded by a dashed box. B–F, structural comparison among amino acid kinase family proteins. α-Helices and β-strands are shown in cyan and magenta, respectively. The yellow regions represent the regions corresponding to the β-sheet region in TtLysZ. B, TtLysZ; C, NAGK from E. coli (PDB code 1GS5); D, aspartate kinase from Corynebacterium glutamicum (PDB code 3AAW); E, carbamate kinase from Enterococcus faecalis (PDB code 1B7B); F, carbamate kinase-like carbamoyl phosphate synthetase from Pyrococcus furiosus (PDB code 1E19).
the function of TtLysZ in catalyzing the reaction. The interacting area of IR1 being slightly larger than that of IR2 may also support this conclusion.

In this study, we demonstrated that LysZ recognized LysW-γ-AAA but could not utilize N-acetylated glutamate or AAA³ as the substrate for the reaction. This indicated that the recognition of both the AAA moiety and globular domain of LysW-γ-AAA was required for the function of TtLysZ. An IR1-forming β-sheet region composed of strands β3-β4 and β7-β8 is characteristic of TtLysZ. The structure of TtLysZ was similar to that of NAGK, which is involved in arginine biosynthesis recognizing N-acetylated glutamate as the substrate; however, the β-sheet region, especially strands β7 and β8, differed between TtLysZ and NAGK. In TtLysZ, the β7-β8 region following the β6-random coil region contained four positively charged residues, whereas the corresponding region was made of β6-η3 in NAGK and lacked all positively charged residues (Fig. 9, A–C). In addition, the regions corresponding to the β-sheet region in TtLysZ exhibited structural diversity in the amino acid kinase family enzymes (Fig. 9, B–F) and contained amino acid residues for substrate recognition (37). Taken together, these results supported IR1 being a functional LysW-binding region of TtLysZ. Because the negatively charged surface of LysW was distributed over the whole molecule, LysW may be able to interact with each biosynthetic enzyme carrying a positively charged region surrounding the catalytic site through any surfaces. This result reminds us of a metabolon-like super complex in the FAS system with ACP. We currently do not know whether these enzymes form such a complex. Elucidating the interaction manner between other biosynthetic enzymes and LysW will be necessary for a better understanding of this metabolic system.

The interaction between TtLysZ and TtLysW-γ-AAA at IR2 may have been caused by crystal packing. However, the highly positively charged surface at IR2 suggests a function of IR2. IR2 may be involved in LysW binding after (or before) the TtLysZ reaction or the stabilization of complex formation between TtLysW derivatives and other lysine biosynthetic enzymes in the metabolon.

In the biosynthesis of fatty acids and secondary metabolites, such as polyketides and peptidyl compounds, substrates are known to be conjugated with ACP and PCP and used for metabolic enzymes. On the other hand, there are few examples of protein-protein interactions being employed in amino acid biosynthetic pathways. In plants, cysteine synthase forms a protein complex with serine O-acetyltransferase and O-acetylserine sulphydrylase. This enzymatic complex functions as a regulatory complex by reducing inhibitory effects on serine O-acetyltransferase of cysteine (40, 41). In Mycobacterium tuberculosis, a sulfur carrier protein named CysO was shown to be involved in the biosynthesis of cysteine, interacting with cysteine synthase CysM. This complex did not have a regulatory role but functioned as a substrate for CysM (42, 43). This system enables the rapid transfer of sulfur for biosynthesis by protecting the unstable aminoacylate intermediate from solvents. These inter-protein interactions in the biosynthesis of cysteine play an important role in efficient biosynthesis by controlling enzymatic activity and preventing the loss of unstable intermediates. The LysW system for the biosynthesis of lysine and arginine is also considered to enable the efficient synthesis of these amino acids by using interactions between enzymes and proteinaceous substrates. Because protein-protein interactions in metabolic pathways are more widespread than expected, biosynthetic mechanisms using LysW need to be elucidated in more detail to more clearly understand other metabolic pathways.

Using genomic databases, many organisms, mainly thermophilic bacteria and archaea, were found to possess genes encoding lysine biosynthetic enzyme homologs. We recently demonstrated that this LysW-mediated biosynthetic system was also used in arginine biosynthesis by the hyperthermophilic archaeon Sulfolobus. Sulfolobus uses a single set of enzymes (LysW, LysZ, LysY, LysL, and LysK), except for LysX, which is involved in the lysine AAA biosynthetic pathway, to biosynthesize arginine. In this archaeon, LysZ is a bifunctional enzyme that can catalyze the phosphorylation of both LysW-γ-AAA and LysW-γ-Glu, which are biosynthetic intermediates produced by ArgX in arginine biosynthesis, as substrates. A study to investigate differences in substrate specificity between LysZ homologs from T. thermophilus and Sulfolobus based on pathway evolution is warranted. By elucidating the structure of other enzymes involved in the LysW-mediated system, the evolution of this type of amino acid biosynthetic system using LysW may be more clearly understood.

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