Mechanism of Concerted Inhibition of $\alpha_2\beta_2$-type Hetero-oligomeric Aspartate Kinase from Corynebacterium glutamicum

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Aspartate kinase (AK) is the first and committed enzyme of the biosynthetic pathway producing aspartate family amino acids, lysine, threonine, and methionine. AK from Corynebacterium glutamicum (CgAK), a bacterium used for industrial fermentation of amino acids, including glutamate and lysine, is inhibited by lysine and threonine in a concerted manner. To elucidate the mechanism of this unique regulation in CgAK, we determined the crystal structures in several forms: an inhibitory form complexed with both lysine and threonine, an active form complexed with only threonine, and a feedback inhibition-resistant mutant (S301F) complexed with both lysine and threonine. CgAK has a characteristic $\alpha_2\beta_2$-type heterotetrameric structure made up of two $\alpha$ subunits and two $\beta$ subunits. Comparison of the crystal structures between inhibitory and active forms revealed that binding inhibitors causes a conformational change to a closed inhibitory form, and the interaction between the catalytic domain in the $\alpha$ subunit and $\beta$ subunit (regulatory subunit) is a key event for stabilizing the inhibitory form. This study shows not only the first crystal structures of $\alpha_2\beta_2$-type AK but also the mechanism of concerted inhibition in CgAK.

In microorganisms and plants, lysine, threonine, and methionine are produced from aspartate. The enzyme catalyzing the first step of this pathway is aspartate kinase (AK$^2$; EC 2.7.2.4), which phosphorylates the $\beta$-carboxyl group of aspartate using ATP. As seen in enzymes catalyzing the first reactions involved in other amino acid biosynthetic pathways, AK is regulated through feedback inhibition by end products (1). Most organisms have several AK isozymes that are regulated differently; for example, Escherichia coli possesses three different AKs. AKI, which is fused with homoserine dehydrogenase I, and AKIII are inhibited by threonine and lysine plus leucine, respectively, whereas the expression of AKII, also fused with homoserine dehydrogenase II, is repressed by methionine (2). On the other hand, Corynebacterium glutamicum has only a single AK (CgAK) but is regulated by lysine and threonine in a concerted manner (3). Additionally, Thermus thermophilus also has a single AK, which is inhibited only by threonine (4) because lysine is biosynthesized via $\alpha$-aminoadipate in this bacterium (5–7).

Recently, crystal structures have been determined for AKs from several sources (8–11). As for AKI from Arabidopsis thaliana, which is inhibited by lysine and S-adenosylmethionine synergistically (12), the crystal structure binding these inhibitors has been determined (9). In AKI from A. thaliana, lysine is bound to the region near the aspartate-binding site in the catalytic domain as well as the regulatory domain for inhibition. In AKIII from E. coli, the mechanism of allosteric inhibition by lysine has been well elucidated by comparing the crystal structures of R-state, a complex with aspartate, ADP, and Mg$^{2+}$, and T-state, a complex with lysine and aspartate (10). In E. coli AKIII, lysine binds only to the regulatory domain and triggers conformational change of the catalytic domain to block ATP binding through displacement of the loop in the regulatory domain. Moreover, three crystal structures of threonine-sensitive AK from Methanococcus jannaschii were determined as complexes, each of which binds threonine, aspartate, or both aspartate and MgAMPPNP, respectively (8, 11). Comparison of these three structures revealed a conformational change to open and closed structures upon nucleotide binding and threonine binding and demonstrated that the cooperative binding of threonine to multiple sites stabilizes the inactive open structure in AK from M. jannaschii.

All AKs whose crystal structures have been revealed so far are homo-oligomeric AK composed of $\alpha$ subunits; however, CgAK has a unique $\alpha_2\beta_2$-type heterotetrameric structure, which is composed of equimolar $\alpha$ and $\beta$ subunits encoded by in-frame overlapping genes (13, 14). In this $\alpha_2\beta_2$-type AK, the amino acid sequence of the $\beta$ subunit is the same as that of the C-terminal domain of the $\alpha$ subunit. The N-terminal portion of the $\alpha$ subunit functions as a catalytic domain, whereas the $\beta$ subunit and C-terminal portion of the $\alpha$ subunit serve as regulatory domains. We have previously determined the crystal structure of the regulatory domain (subunit) dimer of $\alpha_2\beta_2$-type AKs, CgAK and AK from T. thermophilus (15, 16). The regulatory domain of AK, regardless of $\alpha_2\beta_2$-type or homo-oligomeric type, has two ACT domain motifs with a $\beta\alpha\beta\beta\alpha\beta$ fold, which is conserved in many allosteric enzymes involved in amino acid and purine biosynthesis (17, 18). Although in most ACT
domain-containing enzymes two ACT domains are aligned in a side-by-side manner to serve as an effector-binding unit for regulating protein functions, they bind a variety of ligands with a variety of domain organizations, as summarized by Grant (19). It should be noted that the organization of ACT domains in AKs differs between homo- and hetero-oligomeric AKs, as reviewed by Curien et al. (20). In \( \alpha_2\beta_2 \)-type AK, an effector-binding unit is composed of ACT1 and ACT2 from different chains (15, 16), whereas in homo-oligomeric AK an effector-binding unit is made up of equivalent ACT domains from different chains, and only two ACT1 domains or two ACT2 domains are involved in effector binding (8–10). The different assembly of ACT domains between \( \alpha_2\beta_2 \)-type and homo-oligomeric AK may suggest an unknown inhibitory mechanism in \( \alpha_2\beta_2 \)-type because inhibition is presumably transferred to the catalytic domain through motion of the regulatory domain by effector binding.

Apart from these scientific interests, CgAK is an attractive target in its application. A large amount of lysine is used as a food additive for livestock, and lysine is produced by a fermentation process using a high lysine-producing \( C. \) glutamicum mutant with feedback-resistant AK, although the mechanism of feedback inhibition is unknown. The aspartate pathway producing lysine, threonine, and methionine is found in microorganisms and plants, whereas mammals lack the pathway; therefore, AK can be a promising target for the development of antibiotics. Moreover, \( Mycobacterium \) tuberculosis, which is a pathogenic bacterium of tuberculosis, is taxonomically related to \( C. \) glutamicum, and its AK has high identity to CgAK in an amino acid sequence (about 72%) (supplemental Fig. S1), sharing a similar \( \alpha_2\beta_2 \)-type structure (21). Elucidation of the regulatory mechanism of CgAK is anticipated to enable the design of new antibiotics and antitubercular drugs.

By previous structural studies coupled with mutational experiments, we showed that threonine binding induces an interaction between the \( \beta \) subunit and the regulatory domain of the \( \alpha \) subunit, which is important for catalytic regulation (15); however, the mechanism of concerted inhibition along with information on the lysine-binding site is unknown. In this study, to elucidate the mechanism of \( \alpha_2\beta_2 \)-type AK in concerted inhibition by lysine and threonine, we determined the crystal structures of \( \alpha_2\beta_2 \)-type CgAK in several forms.

**EXPERIMENTAL PROCEDURES**

**Preparation of Crystals**—Wild-type CgAK and CgAK carrying S301(F) (S52(\( \beta \)F in the \( \beta \) subunit) mutation were co-expressed by two plasmid vectors in a form containing a His\(_6\) tag at the C terminus of the \( \beta \) subunit. One is a pET26b\((+)(\text{Novagen})\) derivative containing the structural gene for the \( \alpha \) subunit between Ndel/EcoRI sites, and the structural gene for the \( \beta \) subunit between EcoRI/XhoI sites preceded by a typical ribosome-binding site, and the other carries only the \( \beta \) subunit gene in the multicloning site 2 of pACYC184 (Novagen). In the former plasmid, the internal ribosome-binding sequence for translation of the \( \beta \) subunit, which is located in the middle of the \( \alpha \) subunit gene, was mutated not to function. The S301(F) (S52(\( \beta \)F) mutation was introduced by site-directed mutagenesis to each regulatory domain in two plasmids using the QuikChange site-directed mutagenesis kit (Stratagene). Two plasmids were introduced into \( E. \) coli BL21(DE3) cells, respectively. The cells were grown in 2 \( \times \) YT broth, supplemented with 50 \( \mu \)g/ml kanamycin and 30 \( \mu \)g/ml chloramphenicol at 30 °C. After a 3-h incubation, gene expression was induced by adding the final 1 mM isopropyl-\( \beta \)-D-thiogalactopyranoside, and the culture was continued for an additional 12–14 h. The cells were harvested, washed with buffer A (20 mM Tris-HCl, pH 7.5), and suspended in buffer B (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM threonine, and 10 mM lysine). Suspended cells were disrupted by sonication and centrifuged at 40,000 \( \times \) g. The supernatant was purified by Ni\(_2\)\(^{2+}\) affinity with Ni\(_2\)\(^{2+}\)-nitrilotriacetic acid resin (Novagen) and subsequent gel filtration chromatography with HiLoad 26/60 Superdex 200 pg (GE Healthcare) using buffer B. For the crystallization of CgAK-binding threonine, purified AK was dialyzed against buffer C (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 10 mM threonine). Concentrated to 10 mg/mL, and used for crystallization. Crystallization was performed at 293 K by the hanging-drop vapor-diffusion method. Crystals were formed in 1.2 mM sodium citrate and 0.1 M Tris-HCl, pH 8.5, with lysine and threonine, and 2.2 M ammonium sulfate, 0.05 M HEPES-NaOH, pH 7.5, and 2.1% polyethylene glycol (PEG) 400 with threonine. CgAK-S301F was purified in the same way, and the crystals were obtained in 15% PEG4000, 0.05 M trisodium citrate, pH 5.6, and 0.1 M ammonium acetate with lysine and threonine.

**Data Collection and Processing**—Before data collection, the crystals were soaked briefly in cryoprotectant solution containing 20% (v/v) ethylene glycol, glycerol, and PEG400 for CgAK-lysine-threonine, CgAK-threonine, and CgAK-S301F complexes in the reservoir solution, respectively, and flash-cooled in a nitrogen gas stream at 100 K. Diffraction data were collected with a CCD camera on BL-6A (CgAK-lysine-threonine), BL-5 (CgAK-threonine), and NW12 (CgAK-S301F) stations of the Photon Factory (PF), High Energy Accelerator Research Organization (KEK; Tsukuba, Japan). Diffraction data were indexed, integrated, and scaled using the HKL2000 program suite (22).

**Structure Determination and Refinement**—Crystals of CgAK-T contain one \( \alpha_2\beta_2 \) unit/asymmetric unit and belong to space group \( I4 \) with unit cell parameters of \( a = b = 162.2 \) \( \text{Å} \), \( c = 133.9 \) \( \text{Å} \). The structure of CgAK with lysine and threonine was determined by molecular replacement with MOLREP (32) in the CCP4 program suite (23) using the crystal structure of the regulatory domain of CgAK (Protein Data Bank (PDB) code 2DTJ) and the catalytic domain of MjAK (PDB code 2HMF) with the removal of two \( \alpha \) helices (residues 61–117). Crystals of CgAK-threonine and CgAK-S301F contain four \( \alpha_2\beta_2 \) tetramers/asymmetric unit and belong to space group \( P1 \). Their structures were determined by molecular replacement using the structure of CgAK-threonine-lysine as a model. Subsequent manual model building and refinement were achieved using Coot (24), Refmac5 (25), and CNS1.2 (26). The structures were further refined by a combination of TLS and restrained refinement with each subdomain determined by TLSMD server (27) and Refmac5. The overall geometry of the model according to the program MolProbity (28) is of good quality, with 97.2%, 96.1%, and 95.6% of the residues in favored regions in the Ram-
**Data collection and refinement statistics**

| Parameter                  | CgAK-T  | CgAK-R* | CgAK-S301F |
|----------------------------|---------|---------|------------|
| **Data collection**        |         |         |            |
| X-ray source               | PF-BL6A | PF-BL5  | PF-NW12    |
| Wavelength (Å)             | 0.978   | 1.000   | 1.000      |
| Space group                | 4       | P1      | P1         |
| Unit cell dimensions       |         |         |            |
| a (Å)                      | 162.2   | 101.8   | 99.03      |
| b (Å)                      | 162.2   | 119.1   | 112.9      |
| c (Å)                      | 133.9   | 124.4   | 120.0      |
| α (°)                      | 90      | 71.9    | 76.0       |
| β (°)                      | 90      | 69.5    | 71.1       |
| γ (°)                      | 90      | 72.7    | 74.5       |
| Resolution (Å)*            | 2.50    | 2.59    | 2.47       |
| Reflections (total/unique) | 456,152 | 590,477 | 314,940    |
| Rwork (%)                  | 5.6     | 15.0    | 4.5        |
| Rfree (%)                  | 27.1    | 15.0    | 3.3        |
| Completeness (%)           | 99.8    | 98.2    | 96.3       |

| **Refinement**             |         |         |            |
| Resolution (Å)             | 32.1-2.50 | 42.0-2.59 | 27.1-2.50 |
| Rwork/Rfree (%)            | 21.0/25.1 | 23.2/28.0 | 22.3/28.8 |
| No. of atoms               | 8,191   | 31,624  | 30,400     |
| Protein atoms              | 30      | 30      | 30         |
| Threonine molecules        | 32      | 120     | 128        |
| Lysine molecules           | 30      | 246     | 387        |
| Water molecules            | 222     | 246     | 387        |
| Average B-factor           | 36.5    | 44.7    | 38.2       |
| Protein atoms              | 36.5    | 44.7    | 38.2       |
| Threonine                  | 38.2    | 41.8    | 30.6       |
| Lysine                     | 48.6    | 48.6    | 27.3       |
| Water                      | 34.7    | 34.7    | 30.6       |
| Root mean square deviations|         |         |            |
| Bond length (Å)            | 0.008   | 0.006   | 0.009      |
| Bond angle (°)             | 1.1     | 0.9     | 1.2        |
| Ramachandran plot*         | 97.6    | 96.3    | 95.7       |
| Favored region             | 1.8     | 3.0     | 3.4        |
| Allowed region             | 0.6     | 0.7     | 0.9        |
| Outlier region             | 2       | 8       | 8          |
| No. of NCS molecules*      | 8       | 8       | 8          |

*Values in parentheses are data for the highest resolution shell.
*Calculated using MolProbity (7).
*No. calculated b as a single unit.

Overall Structure of αβ₂₂'-type CgAK—We determined the crystal structure of CgAK in three forms: (i) an inhibitory form complexed with lysine and threonine, (ii) an active form with only threonine, and (iii) a feedback inhibition-resistant mutant carrying S301F mutation binding both lysine and threonine (CgAK-S301F) at 2.50 Å, 2.59 Å, and 2.47 Å resolution, respectively (Fig. 1A and supplemental Fig. S2). Data collection and refinement statistics are shown in Table 1. The crystal structure of the complex binding lysine and threonine contains an αβ₂₂' heterotetramer, four threonine molecules, three lysine molecules, and 234 water molecules per asymmetric unit. A and C chains represent α subunits, whereas B and D chains correspond to β subunits in the structure. The catalytic domain of the α subunit has the typical topology of the amino acid kinase family fold (Fig. 1B) (30) as seen in that of homo-oligomeric AK. The most striking difference between homo-oligomeric AK and αβ₂₂'-type AK is the presence and absence of two α helices (α3 and α4 in homo-oligomeric AK) of about 50 amino acid residues between helices α2 and α3 in CgAK.
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FIGURE 1. Overall structure of \(\alpha_2\beta_2\)-type CgAK. A, overall structure of CgAK-T. Green and magenta are \(\alpha\) subunits (chains A and C), and cyan and yellow show \(\beta\) subunits (chains B and D). Threonine and lysine molecules are presented in the CPK model in orange and blue, respectively. B, topology diagram of catalytic domain in the \(\alpha\) subunit of CgAK. Circles and triangles indicate helices and strands, respectively. C, interface between \(\alpha\) subunits. Green and magenta are \(\alpha\) subunits (chains A and C), \(\alpha_2\), \(\alpha_3\), \(\alpha_4\), and \(\beta_3\) are shown.

(supplemental Fig. S1). The absence of the two helices allows \(\alpha\) subunit-\(\alpha\) subunit interaction mainly by \(\alpha_2\) and \(\alpha_3\) helices, forming two 3-helix bundle-like structures in CgAK (Fig. 1C). In homo-oligomeric AK, \(\alpha\) subunit-\(\alpha\) subunit interaction is prevented because of two additional \(\alpha\) helices covering the interacting surface. As a result, homo-oligomeric AK forms a dimer only by the interaction between regulatory domains (supplemental Fig. S3). On the other hand, in CgAK, subunit interfaces are formed between two \(\alpha\) subunits and between the \(\beta\) subunit and the regulatory domain in the \(\alpha\) subunit. CgAK, which binds both lysine and threonine, is inactive; therefore, the crystal structure of the complex binding lysine and threonine represents an inactive (T-state) form of CgAK (CgAK-T).

An asymmetric unit of the crystal of the CgAK-threonine structure contains four \(\alpha_2\beta_2\) heterotetramers, 15 threonine molecules, and 246 water molecules (supplemental Fig. S2A). Chains A, C, E, G, I, K, M, and O stand for \(\alpha\) subunits, and chains B, D, F, H, J, L, N, and P correspond to \(\beta\) subunits. In chain I, a threonine molecule at site 1 in the \(\alpha\) subunit was not observed. In the present structure, the complex bound threonine but not the substrates, aspartate and ATP. By definition, the R-state should represent a form of enzyme-binding substrates. CgAK remains active in the presence of threonine, suggesting that this form is easily convertible to the substrate-binding form. Hereafter, we refer to the threonine-bound form as R*-state of CgAK (CgAK-R*). Similarly, CgAK-S301F was crystallized as a space group P1 containing four \(\alpha_2\beta_2\) heterotetramers, 16 threonine molecules, 7 lysine molecules, and 389 water molecules per asymmetric unit (supplemental Fig. S2B). The lysine-binding site in chain I was vacant. The subunit organizations of CgAK-R* and CgAK-S301F are principally the same as that of CgAK-T.

In the CgAK-R* and CgAK-S301F structures, there are four \(\alpha_2\beta_2\) units per asymmetric unit. On the other hand, in CgAK-T, an asymmetric unit contains a single \(\alpha_2\beta_2\) unit. In this point, by applying 4-fold crystallographic symmetry it can be considered that the T-state structure is also composed of four \(\alpha_2\beta_2\) units; however, gel filtration experiments suggested that CgAK was eluted at the volume corresponding to \(\alpha_2\beta_2\) heterotetramer in the presence of threonine, irrespective of lysine (data not shown). This observation indicates that the biological unit of CgAK is \(\alpha_2\beta_2\) heterotetramer.

Effector-binding Sites—The T-state form contains two threonine and a single lysine molecules in regulatory domains per \(\alpha\beta\) dimer. Threonine molecules are bound at the sites named site 1 in two effector-binding units, which are composed of ACT1 (residues 253–342) in the \(\alpha\) subunit and ACT2 (1–13, 94–160) in the \(\beta\) subunit, and ACT1 (14–93) from the \(\beta\) subunit and ACT2 (250–262, 343–409) from the \(\alpha\) subunit, as seen in the crystal structure of the regulatory domain dimer binding threonine (\(\beta_2\) dimer) (15). Threonine is recognized by ionic bonds between the hydroxyl group of threonine and Glu\(^{298(49)}\), N\(^{\epsilon2}\), and the amino group of threonine and Asp\(^{274(25)}\).O\(^{\delta2}\) (residue numbers in parentheses represent the residue number for the \(\beta\) subunit). Moreover, the hydrogen bond network, including two water molecules and hydrophobic interactions, stabilizes threonine binding, as is the case in \(\beta_2\) dimer (Fig. 2, A and B). Although both site 1s in two effector-binding units per \(\alpha\beta\) dimer are fully occupied by threonine, a lysine molecule is bound at one of two site 2s in two effector-binding units of \(\alpha\beta\) dimer, which is composed of ACT1 from the \(\beta\) subunit and ACT2 from the \(\alpha\) subunit (Fig. 2A). Lysine is bound between two subunits in a manner similar to that for the threonine binding at site 1 (Fig. 2C). The carboxyl group of lysine is stabilized by hydrogen bonds with Ile\(^{44}(\beta\)-N, Val\(^{160}(\alpha\)-N, Thr\(^{361}(\alpha\)-N, and Thr\(^{361}(\alpha\)-O\(^{\gamma1}\) (\(\alpha\) or \(\beta\) in parentheses after the residue number represents the residue from either \(\alpha\) or \(\beta\) subunit). Furthermore, it also forms hydrogen bonds with Gly\(^{355}(\alpha\)-N and Ile\(^{42}(\beta\)-O via two water molecules. The \(\alpha\)-amino group is recognized by Ile\(^{44}(\beta\)-O and Met\(^{554}(\alpha\)-O. The recognition is further stabilized by a bridging water molecule which forms a hydrogen bond network with Ile\(^{44}(\beta\)-O, Met\(^{554}(\alpha\)-O, Glu\(^{382}(\alpha\)-O, and N\(^{\epsilon4}\) atom of bound lysine, which is fixed by an
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The crystal structures of CgAK with fixed α3(α) (residues 63–87) at the α-α dimer interface. Superposition between α subunits from CgAK-T and CgAK-R* revealed that CgAK-T structures of two NCS-related enzymes, domain opening/closing of CgAK, Asp45(β), which recognizes the N² atom of lysine, was replaced with alanine. The inhibition of CgAK by lysine was substantially reduced in mutants carrying D45(β)A (Fig. 3). On the other hand, when Asp294(α), which corresponds to Asp45(β) and forms a vacant lysine-binding site, was replaced with alanine, the resulting mutant exhibited no reduction in the sensitivity to lysine. These results demonstrate that lysine is bound to site 2 apart from the catalytic center, shown by the crystal structure to exert inhibitory activity on CgAK. In site 2 where lysine is bound, loop β15–α10(α) is positioned to be suitable for lysine recognition by the interacting residues, Met354(β)–Thr361(α), whereas in the vacant site 2, loop β6–α3(β) corresponding to β15–α10(α) is displaced inside maximally 3 Å (Lys355(α)) (Fig. 2D). The structural difference between both site 2s indicates that additional lysine cannot bind to another site 2.

Comparison of Three Structures of CgAK—In AKs and related enzymes, domain opening/closing is shown to be a key event regulating enzyme activity (10). We compared the crystal structures of CgAK with fixed α3(α) (residues 63–87) at the α-α dimer interface. Superposition between α subunits from CgAK-T and CgAK-R* revealed that CgAK-T has a closed conformation (Fig. 4A), suggesting that the binding of both threonine and lysine stabilizes the inactive compact conformation. When the structures of two NCS-related α subunits in CgAK-T are superimposed, the root mean square devi-
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CgAK indicate that Ser301 is close to the threonine-binding site of both lysine and threonine (15). The crystal structures of CgAK-T (chain A in T. thermophilus, which is equivalent to the loop in CgAK, has a certain role in regulation because the loop is disordered in the crystal structure of the regulatory domain of AK from T. thermophilus without threonine, which is an inhibitor of this enzyme (16). As the loop is distant from the catalytic site, it is unlikely that the loop conformation directly functions to regulate catalysis (supplemental Fig. S4). Because of the substitution of Phe for Ser301(α)/Ser52(β), hydrogen bonds between Ser301(α)/Ser32(β) and Asp274(α)/Asp25(β) are lost; however, no marked change in the hydrogen bond network and the structure of the regulatory domain was observed except for the loops containing Phe301(α)/Phe25(β). We cannot interpret the effect of S301F mutation on the regulation in detail, based on the crystal structures. It should be noted that the structures of regulatory domains (β subunits) are more flexible in CgAK-S301F than in CgAK-R* (Fig. 5); the average root mean square deviation value between regulatory domains in CgAK-S301F than in CgAK-R*, both with the same space group, is 0.68 Å and 0.43 Å, respectively. We speculate that it is related to the high flexibility in overall structure of CgAK-S301F.

Comparison of Structures between CgAK and Homo-oligomeric AK—Several crystal structures have been determined for homo-oligomeric AKs. In particular, the crystal structures of both T-state and R-state have been determined for AKIII from E. coli and AK from M. jannaschii (8, 10). In AKIII from E. coli, the binding of lysine causes the enzyme to take a more open conformation to form a tetramer. In that form, a loop from the regulatory domain covering the catalytic site is displaced to destabilize ATP binding. As for AK from M. jannaschii, three crystal structures, T-state binding threonine, R-state binding both AMPPNP and aspartate, and R-state binding only aspartate, have been determined. From these structures, it was found that inhibitor (threonine) binding induces the enzyme to form a open conformation. Thus, it is common for inhibitor binding to stabilize the open conformation in these homo-oligomeric enzymes (10) (Fig. 6A). In contrast, the comparison of CgAK-T and CgAK-R* structures indicates that the binding of inhibitors to the regulatory

FIGURE 3. Inhibition profile of wild-type and mutated CgAK by lysine. Filled circles, wild-type CgAK; open circles, CgAK carrying two amino acid replacements, D294(α)A in the α subunit and D45(β)A in the β subunit; squares, CgAK with D294(α)A only in the α subunit; triangles, CgAK with D45(β)A only in the β subunit.

FIGURE 4. Conformational change between CgAK-T and CgAK-R* or CgAK-S301F. A, superposition of α subunits in CgAK-T and CgAK-R* with helix α3(α) fixed. Green molecule indicates chain A in CgAK-T, and magenta molecules are chain l in CgAK-R*. Helix α3(α) is indicated as α3. B, superposition of α subunits in CgAK-T (chain A in green) and CgAK-S301F (chains G, I, and K) with helix α3(α) fixed. a, b, and c, α subunit in CgAK-S301F in more open (chain l), the same (chain K), and a slightly more compact (chain G) conformation compared with that in CgAK-T, respectively.
Although the structure of the regulatory domain (site by lysine binding. We previously showed the crystal structure of either ACT1 or ACT2 from a different chain; therefore, we assume that the difference in conformational change between Κ and chain M, both of which take T-state conformation. Similar to the above case, the conformational change triggered by lysine binding—In addition to the wide ranging changes in conformation, local changes are induced in the region away from the lysine-binding site by lysine binding. We previously showed the crystal structure of the regulatory domain (β subunit) dimer of CgAK binding only threonine (β, dimer) (15). Because CgAK is not inhibited by threonine alone, this structure is considered to represent the R-like structure of the regulatory domain dimer. Although the β subunit (regulatory domain in the α subunit) is mostly composed of two ACT domains, ACT1 and ACT2, there is one extra β strand (strand β9) at the C terminus, which does not belong to the ACT domain motif (Fig. 7A). Strand β9 is associated with the N-terminal portion of β1 strand in β2 dimer. Meanwhile, in CgAK-T, which binds lysine and threonine, extrashort β strands at the C terminus of both α and β subunits are not observed, suggesting that these regions are disordered in the crystal structure (Fig. 7, B and D). Interestingly, the vacant space for strand β9 in the β subunit is occupied by another β strand (strand β5(α)) from the catalytic domain of the α subunit, forming a β sheet with strand β1(β) in CgAK-T (Fig. 7B). The C-terminal flanking region of strand β5(α) contains Arg151(α), Gly152(α), and Ser154(α), whose corresponding residues are involved in aspartate binding in homo-oligomeric AK (Fig. 7E). In CgAK-T, Arg151(α) forms bidentate ionic bonds with Glu74(α) (Fig. 7F), which is also responsible for aspartate binding. In CgAK-R*, both strand β9(β) and β5(α) are disordered, and no ionic bond between Arg151(α) and Glu74(α) is formed (Fig. 7G). These results indicate that lysine binding stabilizes T-state by the association of β5(α) with β1(β), in which the Arg151(α)-Glu74(α) interaction prevents aspartate binding (Fig. 7, C and F). It should be noted that strand β5(α) and Arg151(α)-Glu74(α) interactions are not observed in any α subunit chains of CgAK-S301F, except for chain Κ and chain M, both of which take T-state conformation. Thus, lysine binding induces wide ranging and local changes in conformation; however, the direct effect by lysine binding on the conformation is not obvious. Actually, there are no meaningful differences in the hydrogen bond network or ion pairing in the regulatory domain between CgAK-T and CgAK-R*. By lysine binding, the regulatory domain moves slightly as a rigid body to allow strand β5(α) to extend the β sheet by
interaction with $\beta 1(\beta)$. We assume that the conformational change induced by lysine is achieved with a subtle energetic gain.

Additional Lysine Binding at Active Site—In the CgAK-T structure, a large additional electron density that could be assigned as a lysine molecule is observed in the region binding
aspartate in chain A (Fig. 7E). Such a density is not found in the CgAK-R* structure. This observation implies that lysine is a potential inhibitor competing with aspartate. This hypothesis may be supported by the observation that the D45(β)A mutation in the β subunit does not induce complete resistance to inhibition by lysine (Fig. 3). In the CgAK-T structure, lysine binding to the active site is stabilized by Glu³(β) via a water molecule. The N-terminal region, including Glu³(β), is seen in the CgAK-T structure, not in the CgAK-R structure. This observation suggests that the β1(β) strand is stabilized by the interaction with β5(α) in the CgAK-T structure. To examine the contribution of Glu³(β) in feedback inhibition by lysine, we constructed three mutants: a mutant carrying D45(β)A, a mutant carrying Q(β)A, and a mutant carrying both, and examined the inhibitory response to lysine in the presence of 10 mM threonine. As a result, the mutant with both D45(β)A and Q(β)A mutations was not inhibited by lysine completely, and the mutant with only Q(β)A mutation escaped inhibition by lysine to some extent (Fig. 8). From these results, we conclude that in T-state, the ionic bond between Arg¹⁵¹(α) and Glu⁷⁴(α) makes the active site unfavorable for aspartate binding. Lysine can bind to the aspartate-binding site as an inhibitor competing with aspartate molecule and stabilizes Arg¹⁵¹(α)-Glu⁷⁴(α) interaction by forming a hydrogen bond network with Ser⁴¹(ε), Ser¹⁵⁴(α), and Glu³(β) (via a water molecule). These results indicate that the interaction between β5(α) strand and β1(β) strand plays a key role in concerted inhibition by lysine and threonine in CgAK. It should be noted that inhibitor binding to the aspartate-binding site has been reported in the T-state crystal structures of AK from A. thaliana and AK from M. jannaschii (8, 9).

Conclusion—Based on available information on the structure and function of CgAK, we propose a two-step mechanism of concerted inhibition by lysine and threonine in CgAK as follows. The first step is the interaction of the β subunit with the regulatory domain from the α subunit triggered by threonine binding, and the second step is the alteration of the interacting partner of the β1(β) strand from the β9(β) strand to the β5(α) strand, which is provoked by lysine binding. CgAK binding both effectors has a closed conformation. In the closed conformation, interaction between the β5(α) strand from the catalytic domain of the α subunit and the β1(β) strand from the β subunit is enabled, and subsequently the inactive closed conformation is stabilized by an ionic bond between Arg¹⁵¹(α) and Glu⁷⁴(α), which can be further stabilized by binding lysine to the aspartate-binding site in T-state. Although we have not yet determined the crystal structure with binding substrates, the outline of the concerted inhibition by lysine and threonine was elucidated in this study. Determination of the crystal structure with both substrates will lead to a better understanding of catalytic and inhibitory mechanisms and will also provide solid information for developing a new specific antitubercular drug.

During revision of this manuscript, the crystal structure of AK from Cyanobacterium, Synechocystis, complexed with lysine and threonine, was published (32). AK from Cyanobacterium is a dimer of two equivalent subunits that associate with each other by interaction between the catalytic domains. The most striking feature of AK from Cyanobacterium is that it has C-terminal extension forming two additional ACT domains, different from the AKs so far investigated. ACT domains in the extension are arranged in a manner similar to the β subunit in CgAK and form two effector-binding units with two “normal” ACT domains that are also included in the same chain. As a result, the domain arrangement is quite similar to that of CgAK-T, although the atomic coordinate for the structure of AK from Cyanobacterium (PDB code 1YBD) is not yet available, and therefore we cannot compare these structures in detail. The complex of AK from Cyanobacterium with lysine and threonine contains electron density that can be assigned as lysine at the putative aspartate-binding site. Similar occupation of lysine at the active site is seen in AK from A. thaliana and AK from M. jannaschii as well as CgAK-T. We assume that inhibitor binding to the aspartate-binding site is an inhibitory mechanism commonly used for the regulation of AK.

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