The commitment step to the aspartate pathway of amino acid biosynthesis is the phosphorylation of aspartic acid catalyzed by aspartokinase (AK). Most microorganisms and plants have multiple forms of this enzyme, and many of these isofunctional enzymes are subject to feedback regulation by the end products of the pathway. However, the archaean species Methanococcus jannaschii has only a single, monofunctional form of AK. The enzymes are subject to feedback regulation by the end products of the pathway, resulting in a rearrangement of the catalytic domain that blocks the ATP binding site. The bifunctional threonine-sensitive E. coli AKI-HDHI has been extensively characterized by equilibrium gel permeation (10), ultrafiltration (11), and equilibrium dialysis and fluorescence binding studies (12), which support the hypothesis that this cooperative tetramer also undergoes large conformational changes upon threonine binding. However, the detailed structure of this isoform has not been determined, and attempts to crystallize various complexes of this enzyme have not yet yielded diffraction quality crystals (13).

The genome of the archaean organism Methanococcus jannaschii contains only a single structural gene that encodes an aspartokinase (14). Based on the monofunctional nature of this AK, the gene was designated as lysC, corresponding to the gene encoding the monofunctional AK found in E. coli that is feedback-inhibited by the end product amino acid l-lysine. However, recent studies of this enzyme have shown that, unlike the monofunctional E. coli AK, mjAK is not inhibited by l-lysine but is instead subject to allosteric inhibition by l-threonine (15). Genomic analysis shows that M. jannaschii has only about 40% of the protein-encoding genes as compared with E. coli, and it appears that several amino acid pathways are not present in this archaean species, including those for the biosynthesis of methionine and lysine (16). Because of this simplified set of pathways, the multivalent regulation of AKs seen in other bacterial species is not required in M. jannaschii. The structure of mjAK has now been determined under three different transition states: ternary complex with MgAMP-PNP and l-aspartate, binary complex with l-aspartate, and binary complex in the presence of its allosteric inhibitor l-threonine, which there-
fore allows understanding of the mechanism of regulation by this end product amino acid.

**EXPERIMENTAL PROCEDURES**

**Cloning, Expression, and Purification**—Gene cloning and enzyme expression were carried out as described previously (15), using the pET-41a vector (Novagen) and Rosetta (DE3) *E. coli* cells for protein expression. The resulting cells, harvested after overnight growth following isopropyl 1-thio-β-D-galactopyranoside induction, were suspended in 50 mM Tris buffer, pH 8.0, 50 mM KCl, 1 mM EDTA, and 1 mM dithiothreitol and were lysed by ultrasoundication. The soluble crude protein fraction containing the overexpressed *mj*AK was separated from the cellular debris by centrifugation at 10,000 rpm at 4 °C. Incubation at 80–85 °C for 25 min resulted in significant denaturation of *E. coli* proteins without loss of the hyperthermophilic *mj*AK. The supernatant after centrifugation was treated with ammonium sulfate to 23% saturation for 1 h at 4 °C. The supernatant after centrifugation was then dialyzed overnight against the lysis buffer and then was subjected to two successive column (Superdex 200; GE Healthcare) for the final purification against the lysis buffer and then was subjected to two successive ular weight cut-off Amicon concentrator. The activity of *mj*AK was measured by monitoring the conversion of NADPH to NADP at 340 nm by using an aspartate-hydrogenase-coupled assay.

**Crystallization of a Substrate Complex of *mj*AK**—Crystalliza- tion of a ternary complex of *mj*AK with MgADP and l-aspartate has previously been reported (15). To investigate the initial state of the forward reaction, a new ternary complex was con- structed by incubating *mj*AK (12 mg/ml) with 5 mM magnesium acetate, 5 mM AMP-PNP, and 30 mM l-aspartate overnight. Thick plate-shaped crystals grew at 20 °C from 100 mM Tris, pH 8.0, and 800 mM ammonium formate with 13–15% polyethyl- ene glycol 4000 as the precipitant. The crystals were cryopro- tected by the direct addition of 2-methyl-2,4-pentanediol into the crystallization drop to 25% of the final volume prior to flash- cooling in liquid nitrogen. Unexpectedly, the unharvested crys- tals remaining in the drop dissolved after several weeks, and a new crystal form grew at 4 °C within a week. Cryoprotection of this new crystal form was achieved by using an artificial solution containing 25% ethylene glycol in well solution.

**Crystallization of an Allosteric Inhibitor Complex of *mj*AK**—To examine the effect of the allosteric inhibitor l-threonine on the structure of *mj*AK, a complex was formed by incubating 12 mg/ml *mj*AK with 6 mM l-threonine and 12 mM l-aspartate overnight at 4 °C. Initial crystal hits were obtained from avail- able commercial screen kits, with the best crystals observed in the presence of 0.2 M KI and NH4I with 2.2 M ammonium sulfate as the precipitant from the Nextal AmSO4® screening kit. Optimization around the above starting condition yielded diffraction quality crystals of about 0.5 × 0.3 × 0.2 mm at 20 °C from 0.2 M KI with 1.4–1.6 M ammonium sulfate as the precipitant. These crystals were cryoprotected with an artificial solution consisting of 15% ethylene glycol in well solution prior to flash- cooling in liquid nitrogen.

**Data Collection and Processing**—Diffraction data were col- lected at 100 K at the Advanced Photon Source (Argonne National Laboratory). The data set for the ternary complex of *mj*AK with MgAMP-PNP and l-aspartate (crystal form I) was collected at the GM/CA 23ID beam line, whereas the second crystal form out of the same condition was collected at the IMCA 17ID beam line. Unfortunately, crystals of *mj*AK com- plexes with l-threonine diffracted quite weakly, and it was neces- sary to screen more than 100 crystals to find the optimal crystals for data collection. Seven data sets were collected at SER-CAT, of which merging three data sets collected at the 22ID beam line from a single crystal yielded the best data. Although this crystal decayed from a maximum diffraction of 2.7 Å to 3.0 Å after 100 frames, inclusion of the high resolution shell reflections did improve the electron density maps. All of the data sets were indexed, integrated, and scaled with the HKL2000 program suite (17), and the data collection statistics for each complex are reported in Table 1.

**Structure Determination and Refinement**—The ternary com- plex structure with MgAMP-PNP and l-aspartate was solved by rigid body refinement with our published *mj*AK structure (Pro- tein Data Bank code 2hmf) as the model in Refmac5 (18), fol- lowed by restrained refinement with each subdomain as one noncrystallographic symmetry restraint. The structure was then further refined by a combination of TLS and restrained refinement with each subdomain as one TLS group. Manual model correction and ligand building in the Coot software package (19) yielded a complete model with the exception of N-terminal methionine, residues 384–387, and C-terminal res- idues 471–473. In addition to the functional ligands MgAMP- PNP and l-aspartic acid, six formate ions from the crystalliza- tion setup were observed on the surface of the *mj*AK structure.

The structure of the second crystal form was solved by molecular replacement in Phaser (20) by using the first *mj*AK/ MgAMP-PNP/l-aspartate structure as the search model. A molecular replacement search with a complete monomer failed to yield a solution, whereas a search performed with truncation of the C-terminal regulatory domain (residues 309–470) as the model led to a clear solution with a Z-score of 8.6. Next, a search with the N-terminal kinase domain (residues 2–300), omitting the flexible loops (residues 160–169 and 287–296), resulted in a clear solution for the kinase domain with a Z-score of 34.7. In contrast to the tetrameric structure of the *mj*AK complex with MgAMP-PNP and l-aspartate that is observed in the asymmet- ric unit, the initial model of this second crystal form appears as a dimer in one asymmetric unit. Two dimers are related by a 2-fold crystallographic symmetry axis to form a tetramer. The structure was refined with rigid body refinement followed by restrained refinement, and the complete model was manually corrected in Coot. Although the enzyme was crystallized with both MgAMP-PNP and the substrate l-aspartate present in solution, electron density is only observed for l-aspartate in the active site of both monomers. Besides the missing density for MgAMP-PNP, two formate ions are found bound within the dimer interface.
Allosteric Inhibition of Aspartokinase

The structure of the \( \text{m}j\text{AK} \) complex with \( \text{l}-\text{threonine} \) was solved by molecular replacement using a combination of Phaser and Molrep (21). The searches in Phaser with the truncated N-terminal lobe of the kinase domain (residues 2–204) and the C-terminal regulatory domain (residues 310–470) yielded clear solutions for these two portions of the structure. However, a search with the C-terminal lobe of the kinase domain (residues 205–300) failed to lead to a solution in Phaser, whereas a search for this portion of the structure resulted in a clear solution in Molrep. Therefore, the new model was generated by combination of the results from both Phaser and Molrep. The structure refinement was performed with rigid body refinement followed by restrained refinement after molecular replacement in Refmac5 associated with manual model building in Coot, resulting in \( R_{\text{cryst}} \) of 31.3% and \( R_{\text{free}} \) of 38.2%. The restrained refinement was applied by dividing each subdomain into four loose noncrystallographic symmetry restraint regions (residues 2–165, 170–290, 300–389, and 406–469), and this resulted in an improved electron density map. Then TLS motion determination was utilized to identify the optimal structure in each subdomain for TLS refinement. Finally, the structure was further refined by a combination of TLS and restrained refinement associated with loose noncrystallographic symmetry restraints, yielding \( R_{\text{cryst}} \) of 23.6% and \( R_{\text{free}} \) of 29.0% (Table 1). Although \( \text{l}-\text{aspartate} \) was included in the crystallization conditions, no electron density was observed for this substrate in the active site of this newly determined structure. Therefore, this initially constructed ternary complex of \( \text{m}j\text{AK} \) with \( \text{l}-\text{aspartate} \) and \( \text{l}-\text{threonine} \) became a binary \( \text{m}j\text{AK}/\text{l}-\text{threonine} \) complex upon crystallization.

**Isothermal Titrati** -On a direct measure of the binding affinity of \( \text{l}-\text{threonine} \) to \( \text{m}j\text{AK} \) the enzyme was titrated with the allosteric inhibitor in an ITC experiment carried out on a VP-ITC titration calorimeter (Microcal Inc., Northampton, MA). Purified \( \text{m}j\text{AK} \) enzyme was thoroughly dialyzed against 25 mM potassium phosphate, pH 7.0, and 0.5 mM \( \beta \)-mercaptoethanol. The ITC experiments were performed at 30 °C with enzyme concentrations ranging from 20 \( \mu \text{M} \) for titration of the tight binding site to 100 \( \mu \text{M} \) for measurement of weaker binding, whereas the \( \text{l}-\text{aspartate} \) concentration was increased from 0.3 \( \text{mM} \) for the tight site to 5 \( \text{mM} \) for the weaker binding interactions. The ligand was titrated into the \( \text{m}j\text{AK} \) solution, and each condition was repeated at least twice to ensure reproducible results. The data were fitted with the Origin software package.

**RESULTS AND DISCUSSION**

**Overall Structures**—To investigate the effect of \( \text{l}-\text{threonine} \) binding on the structure of \( \text{m}j\text{AK} \), three different enzyme-ligand complexes were prepared. The ternary complex with the ATP analog AMP-PNP and \( \text{l}-\text{aspartate} \) was structurally determined with diffraction data collected at the highest resolution to date among the full-length aspartokinase structures. In addition, two binary complex structures, \( \text{m}j\text{AK}/\text{l}-\text{aspartate} \) and \( \text{m}j\text{AK}/\text{l}-\text{threonine} \), were determined by molecular replacement using the ternary complex structure as the model. Identifying the appropriate search model ensemble was critical for the successful structure determination of the complex with \( \text{l}-\text{threonine} \). The model structure was divided into three individual search components by hypothesizing that any domain movements are likely to be accommodated by bending hinges located within the long loops connecting each relatively rigid subdomain. This approach produced a good starting model for structural refinement. Determination of these three complex structures yielded excellent refined models, with the majority of the residues (greater than 88%) residing in favorable regions of the Ramachandran plot. Only one residue, Ile\(^{298} \) in monomer A of the ternary complex, falls into a disallowed region, but the modeled conformation of this residue is supported by clear electron density.

An examination of the overall structures of these complexes of \( \text{m}j\text{AK} \) reveals that each are assembled into a dimer of dimers. Each monomer folds into an N-terminal kinase domain and a C-terminal regulatory domain that consists of two ACT domains (22) connected by a 10-residue flexible loop (Fig. 1). This overall structure is similar to our previous determined \( \text{m}j\text{AK} \) structure of an abortive ternary complex with \( \text{l}-\text{aspartate} \) and the product ADP (15). The interface within each dimer is quite extensive, comprising about 14–15% of the overall monomer surface area, whereas the interface between the two dimers involves less than 3% of the surface area (Table 2). Despite this small interdimeric interface, solution measurements on each enzyme complex indicate that this binding affinity is strong enough to sustain a stable dimer of dimers.

**Substrate Binding Sites**—The ATP analog, AMP-PNP in the ternary complex, is bound in the C-terminal lobe of the kinase domain at same site as was previously observed with ADP in an extended conformation with binding interactions between the enzyme and each structural component of the nucleotide. In contrast to the low resolution abortive ternary complex structure, the \( \gamma \)-phosphate of AMP-PNP provides complete information on nucleotide binding, and the higher resolution of this new complex structure allows more detailed insights into the substrate binding sites. The adenine ring is positioned in a hydrophobic pocket composed of Val\(^{235} \), Tyr\(^{236} \), Pro\(^{249} \), Ala\(^{265} \),
and Val267 adjacent to a β-strand of the ACT2 domain. The ribose position is fixed through a hydrogen bond to the side-chain guanidino N atoms of Arg241 that is part of a 20-residue long flexible loop adjacent to an α-helix of the ACT2 domain. The phosphate groups coordinate the divalent cation, with the Mg$^{2+}$ bridging across terminal O atoms from each phosphate group in a tridentate interaction in monomers A and D. Three water molecules complete the distorted octahedral geometry around the metal ion in these monomers (Fig. 2A). In monomer B, the terminal oxygen of the α-phosphate and the bridging oxygen between the α- and β-phosphate groups coordinate the Mg$^{2+}$. In monomer C, the bound Mg$^{2+}$ bridges between the α-
Allosteric Inhibition of Aspartokinase

TABLE 1
Data collection and refinement statistics

| Parameter | MgAMP-PNP/L-aspartate | L-Aspartate | L-Threonine |
|-----------|-----------------------|-------------|-------------|
| Data collection statistics | | | |
| Temperature (K) | 100 | 100 | 100 |
| Space group | P2₁,2,2₁ | C2,2,2 | P2₁,2,2₁ |
| Unit cell dimensions | | | |
| a, b, c (Å) | 101.4, 104.5, 192.0 | 107.5, 199.4, 95.7 | 110.0, 144.3, 155.4 |
| α, β, γ (degrees) | 90.0, 90.0, 90.0 | 90.0, 90.0, 90.0 | 90.0, 90.0, 90.0 |
| Wavelength (Å) | 1.00 | 1.00 | 1.00 |
| Resolution (Å) | 50.00–2.30 (2.38–2.30) | 50.00–2.70 (2.77–2.70) | 50.00–2.70 (2.80–2.70) |
| Total no. of reflections | 305988 | 114452 | 403684 |
| No. of unique reflections | 86820 | 30794 | 56798 |
| Rmerge | 0.058 (0.305) | 0.076 (0.395) | 0.091 (0.236) |
| Output (I/σ(I)) | 18.5 (2.9) | 28.3 (3.0) | 18.1 (3.4) |
| % completeness | 95.7 (79.8) | 95.6 (96.6) | 83.6 (21.2) |
| Redundancy | 3.5 (2.9) | 3.7 (3.4) | 7.2 (2.0) |
| Refinement statistics | | | |
| Resolution range (Å) | 50.00–2.30 | 50.00–2.70 | 50.00–2.75 |
| Wilson B-factor (Å²) | 46.2 | 64.2 | 69.1 |
| No. of molecules/ASU | 4 | 2 | 4 |
| Rwork/Rfree (%) | 19.4/24.4 | 23.8/27.3 | 23.6/29.0 |
| No. of atoms | 14221/164/18/622 | 7080/18/6/13 | 13771/56/0/71 |
| Protein/ligands/formate/water (B-factors) | 26.8/34.4/47.3 | 33.8/63.5/40.9 | 63.7/63.7/70.4 |
| Root mean square deviations | 0.010/1.336 | 0.009/1.183 | 0.008/1.124 |
| Bond length (Å)/Bond angle (degrees) | 92.0/7.7/0.3/0.1 | 88.6/9.0/1.5/0 | 88.2/10.9/0.9/0 |
| Ramachandran plot | | | |

*Values in parentheses are for the highest resolution shell.

and γ-phosphate groups in a bidentate interaction forming a planar coordination geometry along with two water molecules (Fig. 2A). In addition to the metal ion coordination, these phosphate groups are positioned in each active site through interactions with several side-chain functional groups. The β-phosphate is hydrogen-bonded to the side-chain hydroxyl of Thr²⁰⁸ in each monomer and to the side-chain amino group of Lys⁶ in monomers B and C. The γ-phosphate forms additional hydrogen bonds to the side-chain hydroxyl of Ser⁴⁰ and the backbone amide N of Gly⁹ in each monomer and to the side-chain amino group of Lys⁶ in monomers B and C.

In the ternary substrate complex structure, L-aspartate binds in the N-terminal lobe of the kinase domain but with two different orientations in the dimer of dimers structure. In monomers A, B, and D, the α-carboxyl group of the amino acid substrate is positioned through an electrostatic interaction with the side-chain guanidino group of Arg⁴⁰ and a hydrogen bond with a side-chain O atom from the carboxyl of Glu¹³⁰, whereas the position of the α-amino group of L-aspartate is fixed through hydrogen bonds with Glu¹³⁰ and Thr⁴⁶. The β-carboxyl group of L-aspartate is positioned in a productive orientation toward the γ-phosphate that will be transferred from ATP in these monomers, which is further stabilized through two hydrogen bonds with the main-chain amide N of Ser²¹⁰ and the main-chain carboxyl O of Gly²⁰⁸. L-Aspartate is found in the same substrate binding site in monomer C but, unexpectedly, binds with the opposite orientation from that present in the other active sites. Here it is the α-carboxyl group that points toward the γ-phosphate of ATP, whereas the β-carboxyl group is oriented away from the nucleotide. The carboxyl groups in this alternative substrate binding orientation are each stabilized by the same interactions that are accessible for L-aspartate in its normal, productive orientation (Fig. 2B). However, because of the one-carbon shift in the position of the α-amino group, it can no longer interact with enzyme through the same hydrogen bonds as in the other monomers but instead forms a relatively long and distorted hydrogen bond with the side-chain hydroxyl group of Ser²¹⁰. The absence of a strong, compensating interaction with the α-amino group in this alternative orientation to replace the hydrogen bonding found in the “normal” substrate binding mode means that this orientation should have lower affinity and be less likely to occur. Attempts to model L-aspartate bound in the “normal” orientation in monomer C result in a much poorer fit to the Fₐ – F₀ density generated from an omit map in this region (Fig. 2B). This incorrect model also caused an increase of 0.2% in the overall Rwork and Rfree values from those reported for this final structure in Table 1.

Although this structure indicates that L-aspartate is bound in one subunit with the α-carboxyl group in position to be phosphorylated, there is no evidence showing that any α-phosphorylated aspartic acid is produced during the physiological reaction catalyzed by AK. However, in earlier kinetic studies, β-derivatized aspartic acids were found to be substrates of both the threonine-sensitive bifunctional AK (23) and the monofunctional AK of E. coli (24), and phosphorus-31 NMR spectra of the reaction mixtures showed that these alternative substrates were phosphorylated on the α-carboxyl group (23). The α-acyl phosphates produced from these alternative substrates are much less stable than β-aspartyl phosphates. It is possible that α-aspartyl phosphate is produced as a minor product during the physiological reaction, but this product is not stable enough to be easily detected in the presence of much higher levels of the β-phosphorylated product. Importantly, this new complex provides the first structural evidence showing how the relaxed substrate specificity previously observed with this enzyme family can actually occur through reversal of the regiospecificity of binding to synthesize an α-phosphorylated product.
Effect of Nucleotide Binding—Structural comparisons between the binary L-aspartate complex and the ternary complex that includes L-aspartate and a nucleotide analog indicate that mjAK undergoes a significant conformation change upon nucleotide binding. As a consequence of this conformational rearrangement, the total surface area of the ternary complex decreases by about 3% compared with the mjAK/L-aspartate binary complex to yield a more compact structure (Table 2). To further quantitate this structural change, the distance along the 2-fold axis in the central cavity between the dimers was measured between the Cα atoms of corresponding surface lysine residues in monomers B and D. This distance decreases by 35% upon binding of the nucleotide analog to produce the ternary complex (see Fig. 7).

To investigate the domain movements induced by nucleotide binding, the DynDom program (25) was used to identify the mobile regions and the hinge bending residues that allow these conformational rearrangements. The assigned fixed region consists of the kinase domain, whereas the mobile region contains the entire regulatory domain and two latch loops (labeled I and II) that are critical for nucleotide binding (Fig. 3). The hinge bending regions lie along an axis that consists of three sets of residues: Val235-Tyr236 and Pro236-Ile237 that comprise hinge latch loop I, Ser253-Glu259 and Ala275-Pro275 that make up hinge latch loop II, and a Thr296-Thr299 hinged regulatory domain (highlighted in green in Fig. 3A). The binding of MgAMP-PNP induces latch loop I to move toward the nucleotide, forming hydrogen bonding interactions between the sidechain functional groups of Asp239 and Arg241 and the ribose ring (Fig. 3B). The movement of this intact regulatory domain is facilitated by a combination of electrostatic forces, hydrogen bonds, van der Waals forces, and hydrophobic interactions between the latch loop I and the regulatory domain, resulting in 12.5° rotation of the regulatory domain toward the kinase domain around this hinge axis. In concert with this domain rotation, latch loop II moves toward the nucleotide, forming interactions through the side chains of Ala265 and Val267, thus completing the hydrophobic adenine binding pocket. A facile equilibrium must exist in solution between the "closed" form of the ternary enzyme complex and the "relaxed" form of the binary complex with L-aspartate that allowed crystals of these two complexes to grow in different crystal forms in the same crystallization drop.

The Allosteric Binding Sites—Functional studies on threone-nine-sensitive AKs have identified the cooperative nature of allosteric inhibition (26, 27), and the location of putative L-threonine binding sites has been suggested from sequence alignment studies (28). Previous studies have shown the presence of two independent sets of threonine sites in the bifunctional

| Enzyme complex            | Total surface area | Monomer surface area | Interface area within the dimer | Interface area between two dimers |
|---------------------------|--------------------|----------------------|-------------------------------|----------------------------------|
|                            | ΔÅ²                | ΔÅ²                  | ΔÅ² %                         | ΔÅ² %                            |
| MgAMP-PNP/L-aspartate     | 67590             | 20546                | 2857 13.9                     | 518 2.5                          |
| L-Aspartate               | 69500             | 21152                | 3184 15.1                     | 494 2.3                          |
| L-Threonine               | 71040             | 21156                | 2886 13.6                     | 510 2.4                          |

FIGURE 3. Nucleotide-induced domain closure in mjAK. A, an overlay of the ternary complex (yellow ribbons) on the binary complex (blue ribbons) shows that binding of the ATP analog AMP-PNP induces a 12.5° rotation around the hinge bending region (green) of the regulatory domain (light blue) toward the kinase domain (dark blue). B, an expansion of the active site showing the movement of latch loop I into position to form binding interactions with the ribose ring of AMP-PNP and the closing of latch loop II to complete the hydrophobic pocket of adenine ring binding.

E. coli AKI-HDHI, with the $K_{d}$ for the second set of sites 5–10-fold weaker than the high affinity sites. Binding of threonine to the high affinity sites causes 80–90% inhibition of the AK activity, with additional binding at the low affinity sites required to completely inhibit the enzyme (11, 29). Our newly determined mjAK structure with L-threonine provides the first direct structural evidence illustrating the complexity of threonine regula-
Allosteric Inhibition of Aspartokinase

Each L-threonine is positioned in the binding site by interactions between its functional groups and the enzyme. Inset A, the binding modes of two threonines at the A-B dimer interface site. This inset is rotated by 90° to provide a clearer view of the two bound threonines. Inset B, the binding of a single threonine with lower occupancy at the C-D dimer interface site. This inset is rotated by 180° to show the binding interactions at this site. Inset C, representative binding of threonine at the weaker secondary sites in each monomer, located adjacent to the active site.

**FIGURE 4.** The mjAK/L-threonine structure reveals two sets of threonine binding sites. Each L-threonine is positioned in the binding site by interactions between its functional groups and the enzyme. Inset A, the binding modes of two threonines at the A-B dimer interface site. This inset is rotated by 90° to provide a clearer view of the two bound threonines. Inset B, the binding of a single threonine with lower occupancy at the C-D dimer interface site. This inset is rotated by 180° to show the binding interactions at this site. Inset C, representative binding of threonine at the weaker secondary sites in each monomer, located adjacent to the active site.

**TABLE 3**

Thermodynamic parameters for L-threonine binding

| Binding sites | n/dimer | $K_d$ (μM) | $\Delta G$ (kcal/mol) | $\Delta H$ (kcal/mol) | $\Delta S$ (cal/mol °C) |
|---------------|---------|------------|------------------------|------------------------|------------------------|
| Primary       | 0.96 ± 0.01 | 3.13 ± 0.16 | $-7.65 ± 0.07$ | $-26.9 ± 0.4$ | $19.2 ± 0.3$ |
| Secondary     | 5.07 ± 0.12 | 30.5 ± 0.33 | $-6.27 ± 0.19$ | $1.08 ± 0.03$ | $-7.35 ± 0.22$ |

L-threonine binds at two different sites in each dimer, one in the ACT2 domain located at the dimer interface and a second site located in the kinase domain (Fig. 4). As was predicted, L-threonine binds at the interface of the two ACT domains in slightly different orientation in each subunit, positioned mainly by hydrogen bonding interactions between the functional groups of L-threonine and the hydrophilic side-chains of Asn434 from one monomer and Gln440 and Glu444 from the adjacent monomer. Additional binding interactions in different subunits come from different combinations of the adjacent main-chain carbonyl O and amide N of Ala417, Gly419, Ile420, Ala421, Val435, and Met444 (Fig. 4). This structure also provides evidence in support of the cooperative nature of threonine regulation. In addition, two threonines are bound in the A-B dimer interface with binding interactions coming from backbone amide and carbonyl groups (Fig. 4, inset A). However, only a single threonine is found in the corresponding position in the C-D dimer interface, and the weaker electron density for this threonine is best modeled at 50% occupancy (Fig. 4, inset B). This asymmetric binding is consistent with the results from ITC studies which show a single tight binding site per dimer for L-threonine (Table 3). However, rather than the expected single bound threonine within each dimer interface, this structure shows the presence of two tightly bound molecules of the allosteric regulator at the interface site in only one of the dimers, leading to an overall stoichiometry of one threonine per dimer. The exothermic nature of this binding mode (Fig. 5A, inset) is dominated by the large negative enthalpy, which indicates that a number of favorable noncovalent bonds, mainly hydrogen bonds and van der Waals interactions, must be formed between the enzyme and ligand, a conclusion supported by our structural results. The unfavorable entropy (Table 3) is consistent with the expected conformational rearrangements that must occur in mjAK during threonine binding. These structural and binding results suggest that L-threonine binds in this regulatory site of mjAK by a “half-sites” binding mode. To further complicate this regulatory picture, each of the bound threonines at the dimer interface sites is bound in a different orientation and forms binding interactions that incorporate somewhat different set of enzyme functional groups (Fig. 4, insets).

An additional level of complexity is introduced through the presence of a second set of L-threonine binding sites in the kinase domain, located less than 10 Å from the L-aspartate binding site. Earlier NMR relaxation studies measured a distance of 4.4 Å between the metal ion bound to the nucleotide and the threonine carboxyl carbon in the bifunctional E. coli AK1-HDHI (30). Our measured distance of 6.1 Å between bound Mg$^{2+}$ and the threonine carboxyl group in this newly determined structure indicates that a similar orientation exists between the nucleotide and allosteric binding sites in solution and in the crystal. Similar to the varied orientations observed in the tight threonine binding sites, the threonines in these additional sites are also oriented differently in each monomer. These orientations are stabilized by hydrogen bonding interactions with residues in these second sites that includes the side chains of Lys5, Thr10, Ser11, Thr230, and Asp231 and the backbone Tyr229 carbonyl O and Gly8 amide N. The occupancy of
the threonines in these secondary sites in the C and D monomers are lower and have been modeled at 50%, whereas the corresponding threonines in the secondary sites in the A and B monomers are fully occupied, further supporting a cooperative allosteric mechanism for threonine inhibition. After saturation of the primary binding sites, these secondary weaker sites were detected by ITC titration using a higher concentration of L-threonine and mjAK. The endothermic nature of binding in this weaker site (Fig. 5B, inset) is mainly a consequence of a positive entropy term, which signifies that this binding is dominated by solvent rearrangement and hydrophobic forces (Table 3). The best fit to this ITC data suggests five additional weak sites for threonine binding per dimer. The structure of the mjAK/L-threonine complex contains only two additional bound threonines per dimer beyond the primary threonine sites already described in the dimer interface region. These binding data probably include a weighted average of these observed secondary sites, binding of the additional threonines at the second dimer interface site, and some nonspecific binding of threonine on the surface of the protein. Since the enzyme complex with threonine was crystallized from high concentrations of ammonium sulfate as the precipitant, these ions would probably compete with threonine for surface binding sites, making it less likely to observe additional bound threonines under the crystallization conditions.

**Mechanism of Threonine Inhibition**—The structure of mjAK undergoes additional conformational changes upon L-threonine binding, to a structure with a 2% overall increase in surface area compared with the binary L-aspartate complex and 5% larger than the ternary complex (Table 2). There is a corresponding increase in the central cavity of the threonine binary complex, with the distance between corresponding lysine Cα atoms in monomers B and D increasing by about 20% compared with the L-aspartate binary complex (Fig. 7). DynDom analysis of mjAK/L-threonine, comparing with mjAK/L-aspartate complexes, identified the same mobile region and hinge bending residues that were found in the nucleotide-induced conformational changes. However, unlike what was observed with nucleotide binding, L-threonine binding causes the entire regulatory
domain to rotate by 6.5° away from the fixed kinase domain. Latch loop I follows the regulatory domain rotation by moving about 2 Å, driven by the interactions between this loop and the ACT2 domain. As a consequence, the side chain of one of the critical cofactor binding residues, Arg241, located on latch loop I, becomes disordered and is no longer oriented in a favorable position on average to interact with the ribose ring of the nucleotide. Although latch loop II moves toward the nucleotide binding pocket in the L-threonine binary complex, it does not approach close enough to complete the hydrophobic adenine binding pocket that was observed in the ternary complex. Thus, the nucleotide is less likely to bind to this allosterically inhibited form of mjAK.

The crystals of the L-threonine complex were grown from a solution containing saturating levels of L-aspartate, but no substrate molecules are found bound in this structure. Strikingly, in this inhibited complex, a loop movement is observed that leads to the displacement of Arg207, a critical L-aspartate-binding residue. An examination of each monomer in the threonine complex structure shows slightly different conformations for the Arg207 side chain, but, with an average distance with of more than 6 Å between the guanidine N and carboxyl O of the substrate, it is not possible to form the stabilizing electrostatic interaction that plays an important role in substrate binding (Fig. 6B). In the L-aspartate complex crystallized in the presence of high ammonium formate, a formate ion is found in the allosteric site in the absence of L-threonine. However, the binding of formate at this site does not trigger the same conformational change as L-threonine binding, and the amino acid substrate remains bound in this complex.

Based on the structural information discussed above, we can propose an allosteric inhibition mechanism for the control of mjAK activity. The apoenzyme binds the amino acid substrate L-aspartate in a “relaxed” conformation. When ATP binds, latch loop I moves toward the active site to assist in nucleotide binding through several hydrogen bonding interactions. As a consequence, this loop reorientation induces movement of the regulatory domain to form the “closed” active conformation (Fig. 7). As amino acid biosynthesis proceeds, the accumulation of the end products of this pathway allows L-threonine to bind at the tight allosteric sites in the dimer interface. This binding induces a conformational change that moves the regulatory domain away from the catalytic domain and opens the nucleotide binding site into a relaxed state with a lower affinity for ATP. Additional threonine binding at secondary sites leads to further conformational changes that decrease the affinity for L-aspartate and effectively render the enzyme inactive (Fig. 7).
tional AK (lysC), our earlier studies demonstrated that this enzyme is inhibited by L-threonine rather than L-lysine. This confusion originated because of the overall sequence similarity within the AK enzyme family. Lysine-sensitive E. coli AKIII and Arabidopsis AK share about 30% sequence identity with each other (8, 9) and have about the same level of sequence identity with the threonine-sensitive mjAK. However, these enzyme families can be distinguished through a comparison of their respective regulatory domain sequences, with the lysine-sensitive AKs having greater than 35% identity with each other and less than 25% identity with the threonine-sensitive mjAK. A detailed structural comparison provides further distinctions between the lysine-sensitive and threonine-sensitive AKs. The overall structures of the AKs that have been determined are all quite similar, with each monomer composed of a similar kinase and a regulatory domain (8, 15). Each of these regulatory domains is composed of two signature ACT subdomains that are the identifying structural feature of this enzyme superfamily that includes aspartokinases (A), chorismate mutases (C), prephenate dehydrogenases (TyrA, T), and a number of other dehydrogenases, dehydratases, and oxygenases (22). Although there is also high overall structural similarity within the regulatory domains of the three different AKs, the critical latch loop that undergoes rotational rearrangements leading to tetramer formation and the transition from the R- to T-state in the lysine-sensitive AKs consists of a dozen mainly hydrophilic residues (8, 9). In mjAK, the same loop is shortened to only four residues and does not appear to play any role in conformational rearrangements. Therefore, beyond the sequence differences in their regulatory domains, these two classes of end product-sensitive AKs can be further distinguished by their differences in latch loops and also by differences in ACT subdomain orientation between the different groups of AKs. These structural differences allow a differential response to the respective end product amino acids that are the key functional differences between the different classes of aspartokinases.

Acknowledgments—We thank Dr. Christopher Faehnle for helpful suggestions on ternary complex crystallization, Dr. Lirong Chen (University of Georgia) for advice on data collection on the L-threonine binary complex, and Dr. Jeffrey Ohren for data collection on the L-aspartate binary complex. Use of the APS at the Argonne National Laboratory was supported by the US Department of Energy, Office of Energy Research under Contract W-31-109-ENG-38. We thank the staff at beamlines GM/CA-CAT, IMCA-CAT, and SER-CAT for technical support during data collection.

REFERENCES

1. Viola, R. E. (2001) Acc. Chem. Res. 34, 339–349
2. Roberts, C. J., and Selker, E. U. (1995) Nucleic Acids Res. 23, 4818–4826
3. Ragkousi, C., Eichenberger, P., van Ooj, C., and Setlow, P. (2003) J. Bacteriol. 185, 2315–2329
4. Van Heijenoort, J. (2001) Nat. Prod. Rep. 18, 503–519
5. Lyon, G. L., and Novick, R. P. (2004) Peptides 25, 1389–1403
6. Chen, X., Schauder, S., Potier, N., Van Dorsselaer, A., Pelczer, I., Bassler, B., L., and Highson, F. M. (2002) Nature 415, 545–549
7. Cohen, G. N. (1983) in Amino Acids: Biosynthesis and Genetic Regulation (Herrmann, K. M., and Somerville, R. L., eds) pp. 147–171, Addison-Wesley, Reading, MA
8. Mas-Droux, C., Curien, G., Robert-Genthon, M., Laurencin, M., Ferrer, J. L., and Dumas, R. (2006) Plant Cell 18, 1681–1692
9. Kotaka, M., Ren, J., Lockyer, M., Hawkins, A. R., and Stammers, D. K. (2006) J. Biol. Chem. 281, 31544–31562
10. Vickers, L. P., Ackers, G. K., and Ogilvie, J. W. (1978) J. Biol. Chem. 253, 2155–2160
11. Bearer, C. F., and Neet, K. E. (1978) Biochemistry 17, 3512–3516
12. Heck, H. D. (1972) Biochemistry 11, 4421–4427
13. Janin, J. (1974) FEBS Lett. 45, 318–319
14. Bult, C. J., White, O., Olsen, G. J., Zhou, L., Fleischmann, R. D., Sutton, G. G., Blake, J. A., Fitzgerald, L. M., Clayton, R. A., Gocayne, J. D., Kerlavage, A. R., Dougherty, B. A., Tomb, J.-F., Adams, M. D., Reich, C. L., Overbeek, R., Kirkness, E. F., Weinstock, K. G., Merrick, J. I., Glodek, A., Scott, J. L., Geoghagen, N. S. M., Weidman, J. F., Fuhrmann, J. L., Nguyen, D., Utterback, T. R., Kelley, J. M., Peterson, J. D., Sadow, P. W., Hanna, M. C., Cotton, M. D., Roberts, K. M., Hurst, M. A., Kaine, B. P., Borodovsky, M., Klenk, H.-P., Fraser, C. M., Smith, H. O., Woese, C. R., and Venter, J. C. (1996) Science 273, 1058–1073
15. Faehnle, C. R., Liu, X., Pavlovsky, A., and Viola, R. E. (2006) Acta Crystallogr. F Struct. Biol. Crystalliz. Comm. 62, 962–966
16. Bono, H., Ogata, H., Goto, S., and Kanehisa, M. (1998) Genome Res. 8, 203–210
17. Orwinowski, Z., and Minor, W. (1997) Methods Enzymol. 276, 307–326
18. Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) Acta Crystallogr. Sect. D Biol. Crystallogr. 53, 240–255
19. Emsley, P., and Cowtan, K. (2004) Acta Crystallogr. Sect. D Biol. Crystallogr. 60, 2126–2132
20. McCoy, A. J., Grosse-Kunstleve, R. W., Storoni, L. C., and Read, R. J. (2005) Acta Crystallogr. Sect. D Biol. Crystallogr. 61, 458–464
21. Vagin, A. A., and Teplyakov, A. (1997) J. Appl. Crystallogr. 30, 1022–1025
22. Aravind, L., and Koonin, E. V. (1999) J. Mol. Biol. 287, 1023–1040
23. Angeles, T. S., Hunsley, J. R., and Viola, R. E. (1992) Biochemistry 31, 799–805
24. Keng, Y. F., and Viola, R. E. (1996) Arch. Biochem. Biophys. 335, 73–81
25. Hayward, S., and Lee, R. A. (2002) J. Mol. Graph. Model. 21, 181–183
26. Janin, J., and Cohen, G. N. (1969) Eur. J. Biochem. 11, 520–529
27. Janin, J., and Iwatsubo, M. (1969) Eur. J. Biochem. 11, 530–540
28. Paris, S., Viemon, C., Curien, G., and Dumas, R. (2003) J. Biol. Chem. 278, 5361–5366
29. Bearer, C. F., and Neet, K. E. (1978) Biochemistry 17, 3523–3530
30. Tilak, A., Wright, K., Damle, S., and Takahashi, M. (1976) Eur. J. Biochem. 69, 249–255