Crystal Structure of Argininosuccinate Synthetase from \textit{Thermus thermophilus} HB8

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Argininosuccinate synthetase catalyzes the ATP-dependent condensation of a citrulline with an aspartate to give argininosuccinate. The three-dimensional structures of the enzyme from \textit{Thermus thermophilus} HB8 in its free form, complexed with intact ATP, and complexed with an ATP analogue (adenylyl imidodiphosphate) and substrate analogues (arginine and succinate) have been determined at 2.3-, 2.3-, and 1.95-Å resolution, respectively. The structure is essentially the same as that of the \textit{Escherichia coli} argininosuccinate synthetase. The small domain has the same fold as that of a new family of “N-type” ATP pyrophosphatases with the P-loop specific for the pyrophosphate of ATP. However, the enzyme shows the P-loop specific for the γ-phosphate of ATP. The structure of the complex form is quite similar to that of the native one, indicating that no conformational change occurs upon the binding of ATP and the substrate analogues. ATP and the substrate analogues are bound to the active site with their reaction sites close to one another and in a geometrical orientation favorable to the catalytic action. The reaction mechanism so far proposed seems to be consistent with the locations of ATP and the substrate analogues. The reaction may proceed without the large conformational change of the enzyme proposed for the catalytic process.

Argininosuccinate synthetase (AsS)\footnote{The abbreviations used are: AsS, argininosuccinate synthetase; tAsS, argininosuccinate synthetase from \textit{T. thermophilus} HB8; AMP-PNP, adenylyl imidodiphosphate; r.m.s., root mean square.} catalyzes the second step of the urea cycle where a citrulline, the product of the first step catalyzed by ornithine transcarbamoylase, reacts with an aspartate to give an argininosuccinate, which is cleaved into an arginine and a fumarate by argininosuccinase. AsS reversibly catalyzes the adenosine triphosphate (ATP)-dependent condensation of a citrulline with an aspartate (Scheme 1).

The ureido oxygen atom of a citrulline attacks the α-P atom of the triphosphate in ATP to produce a citrullyl-AMP intermediate and a pyrophosphate as a leaving group. The AMP moiety of the activated citrullyl-AMP intermediate is displaced by an aspartate, thus producing an argininosuccinate (1, 2). Support for the existence of the citrullyl-AMP intermediate comes from experiments using \textsuperscript{32}P-labeled citrulline (Scheme 1).

Based on the structure of the ATP pyrophosphatase domain of the GMP synthetase (3) and the modified version of the P-loop of H-Ser-Gly-Gly-X-Asp-Ser-Thr/Thr-Ser/Thr (where H is any hydrophobic amino acid and X is any one) observed in GMP synthetase, NAD$^+$ synthetase, asparagine synthetase, and AsS (4), these four distinct enzymes, which show ATP pyrophosphatase activity, were proposed to have a common fold specific for nucleotide binding and belong to a new family of “N-type” ATP pyrophosphatases (3, 4). NAD$^+$ synthetase (5, 6) and the C-terminal domain of asparagine synthetase (7) have been proved to be folded into an open αβ structure with a topology similar to that of the ATP pyrophosphate domain of GMP synthetase. In the structures of the GMP and NAD$^+$ synthetases (3, 8), the P-loop specifically interacts with the β- and γ-phosphates of ATP.

AsS from \textit{Thermus thermophilus} HB8 (tAsS), which has been overexpressed in \textit{Escherichia coli}, has 400 residues per subunit, with a subunit molecular weight of 44,815. The sequence alignment of tAsS with other AsSs by the program CLUSTAL W (9) showed that the sequence identities of tAsS with respect to human AsS, yeast AsS, and \textit{E. coli} AsS are 52.7, 46.5, and 29.3%, respectively. Therefore, it can be assumed that these enzymes have the same folding topology with similar three-dimensional structures, and the structure of tAsS can be used as a reliable molecular model for eukaryotic AsS. The 15.3% amino acid sequence identities were observed among these enzymes with the consensus sequence of Tyr/Phe-Ser-Gly-Gly-Leu-Asp-Thr-Ser (P-loop) specific for the pyrophosphate of ATP (4).

Quite recently, the structures of \textit{E. coli} AsS and its complex with citrulline and aspartate have been solved (10). The folding of the nucleotide binding domain of the enzyme was shown to be similar to that of the N-type ATP pyrophosphatases, and the binding mode of the substrates (citrulline and aspartate) was clearly determined. ATP was modeled into the active site of \textit{E. coli} AsS by taking advantage of the GMP and NAD$^+$ synthetase structures, in which the P-loop interacts with the pyrophosphate (β- and γ-phosphates) of ATP. On the basis of this model, the relative orientation of ATP and the substrates in the active site and the conformational change of the enzyme during the catalysis were suggested.

We have determined the structure of the native tAsS, tAsS in the complex with intact ATP, and tAsS in the complex with...
Experimental Procedures

Crystallography and Data Collection—The purified protein was supplied by the RIKEN Structural Genomics Initiative (11). Crystallization of the native and complex of tAsS was carried out at 293 K by the hanging-drop vapor-diffusion method (12), using 30 mg/ml protein solution and 2.4 M ammonium sulfate, 30% (v/v) glycerol, 100 mM Tris-HCl, pH 8.5, as the reservoir solution. Native tAsS and selenomethionyl tAsS were crystallized using the hanging-drop composed of 1:1:1 volume ratio of the reservoir solution and the protein solution. Crystals of tAsS-ATP were obtained by the cocrystallization method using the hanging-drop composed of 3:3:1 of the reservoir solution, the protein solution, and the additive solution of 10 mM ATP and 10 mM MgCl2. Crystals of tAsS-AMP-PNP-arginine-succinate were obtained using the drop composed of 1:1:1 of the reservoir solution, the protein solution, and the additive solution of 10 mM AMP-PNP, 10 mM MgCl2, 100 mM L-Arg, and 100 mM succinate. Within 3 days, crystals had grown to dimensions of about 0.4 × 0.4 × 0.2 mm.

The x-ray diffraction data sets for the native crystal, tAsS-ATP crystal, tAsS-AMP-PNP-arginine-succinate crystal, and a selenomethionyl protein crystal were collected to 2.3–2.35, 2.5, and 2.5 Å resolution at 100 K on the BL41XU, BL41XU, BL44B2, and BL45PX stations at SPring-8 (Hyogo, Japan), respectively. These four crystals are isomorphous with the space group of R3 and have the average cell dimensions of a = b = 228.7 and c = 161.6 Å. There are four subunits in the asymmetric unit, and ~72% of the crystal volume is occupied by solvent. The data sets for the tAsS crystals and a selenomethionyl crystal soaked in solutions containing heavy atom reagents were collected to 2.5, 2.5, and 3.1 Å resolution at 100 K on the BL41XU station or on the BL18B station at the Photon Factory (Takauka, Japan). All the data were processed and scaled using the program MOSFLM and SCALA (13) or DENZO and SCALEPACK (14) (Table I).

Structure Determination and Refinement—The structure of the native tAsS was solved by the multiple isomorphous replacement method, using four isomorphous data sets. The scaling of all the data and map calculations were performed with the CCP4 program suite (15). The difference Patterson map calculations for the ethylmercurithiocarbamoyl acid and K2Pt(NO3)4 data sets allowed the interpretation of two mercury sites and two platinum sites, respectively. The positions of another six mercury sites, and those of six selenium sites, were determined with the program SOLVE (16). Refinement of the heavy atom parameters and calculation of the initial phases were performed with the program MLPHARE (15). The resulting multiple isomorphous replacement map has a mean figure-of-merit of 0.62 at a resolution of 10–3.0 Å. The map was significantly improved by the process of solvent flattening and local symmetry averaging with the program RESOLVE (17).

The structure of tAsS was refined by simulated annealing and energy minimization with 222 noncrystallographic symmetry restraints with the program CNS (18) and x-ray data from 10.0- to 3.0-Å resolution to give an R-factor of 32.2% and an Rfree of 37.5%. At this stage, the restraint on the 222 symmetry was removed, and the entire molecule was refined. The refinement was progressively increased to 2.3 Å, and after several rounds of refinement and manual rebuilding, R-factor and Rfree were reduced to 24.2 and 28.5%, respectively. A difference map displayed one large peak per subunit, which was assigned to a sulfate anion by considering the shape, size and peak height, and possible interactions of this peak with the neighboring amino acid residues. Water molecules were picked up from the difference map on the basis of the peak heights and distance criteria, except for those whose thermal factors after refinement were above 80 Å2 (corresponding to the maximum thermal factor of the main-chain atoms). Further model building and refinement cycles resulted in an R-factor of 19.9% and an Rfree of 22.9%, calculated for 122,785 reflections [Fo > 2σ(Fo)] observed in a 10.0–2.3 Å resolution range (Table II).

The same refinement procedure was applied to tAsS-AMP-PNP-arginine-succinate, but using the coordinates of the native tAsS as the initial model. When the R-factor value became less than 30%, the difference Fourier map showed the residual electron density corresponding to the bound AMP. Water molecules were picked up from the difference map, and further model building and refinement cycles gave R-factor and Rfree values of 20.9 and 24.1%, respectively, calculated for 138,518 reflections [Fo > 2σ(Fo)] observed in a 10.0–2.3 Å resolution range (Table II).

The same refinement procedure was applied to tAsS-AMP-PNP-arginine-succinate complex, but using the coordinates of the native tAsS as the initial model. When the R-factor value went below 30%, the difference Fourier map clearly exhibited the residual electron densities corresponding to the bound AMP-PNP, arginine, and succinate to the active site. Water molecules were picked up from the difference map, and further model building and refinement cycles gave R-factor and Rfree values of 23.2 and 25.5%, respectively, calculated for 225,836 reflections [Fo > 2σ(Fo)] observed in a 10.0–1.95 Å resolution range (Table II).

Results and Discussion

Quality of the Structure—The final model of the native tAsS contains 1,532 amino acid residues for all subunits and four sulfate anions with 458 water molecules with an R-factor of 19.9% at 2.3 Å resolution. The model lacks 68 residues, and the average thermal factor of the main-chain atoms are 34 Å2. The final model of the tAsS-AMP-PNP complex contains 1,538 amino acid residues for all subunits and four ATPs with 477 water molecules with an R-factor of 20.9% at 2.3 Å resolution. The model lacks 68 residues. The average thermal factors of the main-chain atoms are 33 Å2. The final model of the tAsS-AMP-PNP-arginine-succinate complex contains 1,538 amino acid residues for all subunits and four ATPs, four arginines, and four succinates with 799 water molecules with an R-factor of 23.2% at 1.95 Å resolution. The model lacks 62 residues, and the average thermal factor of the main-chain atoms are 31 Å2. All models had a good quality with 99.8% of residues falling in the most favorable and additionally allowed region and only 0.2% in the generously allowed region, when the stereochemistry was assessed by PROCHECK (19) (Table II). Structure diagrams were drawn using the programs MOLSCRIPT (20) and BOBSCRIPT (21).

Overall and Subunit Structure—The overall structure of tAsS-ATP is shown in Fig. 1A. The overall and subunit structure of tAsS is quite similar to that of E. coli AsS, although the precise structure is different between them, probably because the polypeptide chain of tAsS is 47 amino acid residues shorter than that of E. coli AsS (10). The tAsS is folded into a tetrameric form with a noncrystallographic D2 symmetry, having the shape of a twisted rectangle with an active site at each of the four corners. In the center of the molecule, there are clusters of α-helices which are surrounded by β-sheets. One sub-
unit in the tetramer interacts with the other three subunits and the surface areas of the subunit interfaces are 4,390 Å² for a and b, 1,201 for a and d, and 923 Å² for a and c subunits (Fig. 1A). The largest of these areas is found between two subunits of a and b or c and d, indicating that the tetramer may be considered to be an assembly of two dimer units (a dimer of dimers) around a 2-fold axis. All the subunit interfaces are distant from the active site and not essential for the catalytic action.

The subunit structure of tAsS·AMP-PNP-arginine·succinate is shown with secondary structure assignments by the program DSSP (22) in Fig. 1B. The Ca carbon atoms of the subunit in the native tAsS can be superimposed onto the corresponding ones in tAsS·ATP and tAsS·AMP-PNP-arginine·succinate within r.m.s. deviations of 0.14 and 0.19 Å with the maximum displacement of 0.88 and 0.73 Å, respectively. Thus, tAsS does not significantly change its conformation upon binding of the ATP (AMP-PNP) and substrate analogues. Similarly, E. coli AsS does not change its overall conformation on substrate binding, although the enzyme shows a few localized conformational changes (10). The Ca positions of the native E. coli AsS and the complex one with citrulline and aspartate superimpose within r.m.s. deviations of 0.6 Å. The subunit superposition of tAsS·AMP-PNP-arginine·succinate and E. coli AsS·citrulline·aspartate resulted in 284 equivalenced Ca atoms with r.m.s. deviations of 0.54 Å with the maximum displacement of 2.98 Å, indicating that the overall structures of both subunits are essentially the same (Fig. 2).

The subunit is divided into a small domain (ATP binding domain, N-terminal to Pro165), a large domain (Val166 to Arg359), and a C-terminal arm (Gln360 to C-terminal). The small domain, N-terminal to Pro165), a large domain (Val166 to Arg359), and a C-terminal arm (Gln360 to C-terminal). The small domain consists of four stranded antiparallel β-sheets designated as b3, b2, b1, b4, and b5 (all parallel) form a twisted β-sheet structure as a central core surrounded by two α-helices (H1 and H2) from the convex surface side of the sheet and two α-helices (H4 and H5) from the concave side. The residues from Phe69 to Ala81 (α-helix H3) a loop α-helix H4) go through the large hole formed at the center of the C-terminal domain, and the loop reaches the exit of the hole to interact with the other subunit of the dimeric unit. Thus, this region of the N-terminal domain behaves like a part of the C-terminal one. The α-helix H7 covers the α-helix H1, and its C-terminal loop goes to the C-terminal domain.

The large domain consists of four stranded antiparallel β-sheets (b6, b7, b12, and b11), five stranded antiparallel β-sheets (b10, b9, b8, b13, and b14), and four α-helices. Two β-sheets of the large domain are connected through the α-helix H8 between the β-strands of b10 and b11 and a succession of three α-helices of H9, H10, and H11 between the β-strands of b12 and b13. The four-stranded β-sheet adjacent to the small domain

### Table I

| Data set | EMTS* | K₂Pt(NO₃)₄ | Se-Met | Se-Met + EMTS |
|----------|-------|------------|--------|--------------|
| Resolution (Å) | 2.5   | 2.7        | 2.5    | 3.1          |
| No. of reflections |        |            |        |              |
| Unique | 103,686 | 85,404     | 99,171 | 56,846       |
| Observed | 329,500 | 303,285    | 251,330| 705,720      |
| Completeness (%) | 95.4  | 98.6       | 90.7   | 99.7         |
| Rmerge (%) | 7.1   | 6.6        | 6.4    | 6.5          |
| MIR |        |            |        |              |
| Rdiff (%) | 15.6  | 12.0       | 14.9   | 18.1         |
| Phasing power | 1.63 | 0.76       | 1.07   | 1.85         |
| No. of sites | 8     | 8          | 26     | 26 + 8       |

* a Ethylmercurithiosalicylic acid.

* b Rmerge = \( \sum_{i} I_{hkli} - \langle I_{hkli} \rangle \) showing deviations from the native structure.

* c Rdiff = \( \langle |F_{o}\rangle \rangle - |F_{c}| / |F_{o}| \) showing deviations from the native structure.

* d Phasing power is the ratio of the r.m.s. of the heavy atom scattering amplitude and the lack of closure error.

### Table II

| Data set | Native | ATP | AMP-PNP · arginine · succinate |
|----------|--------|-----|--------------------------------|
| Resolution (Å) | 2.30 | 2.30 | 1.95 |
| No. of reflections |        |        |      |
| Unique | 124,435 | 143,708 | 227,309 |
| Observed | 281,918 | 685,640 | 979,957 |
| Completeness (%) | 89.2 (78.3)* | 99.9 (99.9)* | 99.9 (99.9)* |
| Rmerge (%) | 6.5 (12.7)* | 7.4 (27.0)* | 4.4 (28.4)* |
| Refinement |        |        |      |
| Resolution limits (Å) | 48.8–2.30 | 19.9–2.30 | 20.0–1.95 |
| Rfactor (%) | 19.94 (25.25) | 20.87 (26.76) | 23.19 (31.27) |
| Rfree (%) | 22.91 (29.07) | 24.10 (31.49) | 25.53 (34.02) |
| Deviations |        |        |      |
| Bond lengths (Å) | 0.007 | 0.007 | 0.007 |
| Bond angles (Å) | 1.31 | 1.43 | 1.35 |
| Mean B factors |        |        |      |
| Main chain atoms (Å²) | 33.78 | 33.45 | 30.79 |
| Side chain atoms (Å²) | 25.33 | 35.46 | 34.51 |
| Hetero atoms (Å²) | 47.52 | 48.25 | 45.26 |
| Water atoms (Å²) | 12.37 | 11.27 | 35.57 |
| Ramachandran plot |        |        |      |
| Favoured | 91.9 | 91.5 | 92.8 |
| Additional allowed | 8.0 | 8.2 | 7.1 |
| Generously allowed | 0.2 | 0.2 | 0.2 |
| Disallowed | 0.0 | 0.0 | 0.0 |

* a The values in the parentheses are for highest resolution shells.

* b Rmerge = \( \sum_{i} I_{hkli} - \langle I_{hkli} \rangle \) showing deviations from the native structure.
and α-helices H9, H10, and H11 make a large hole going through the center of the large domain. The long C-terminal arm region reaches the small domains of the other subunits with its C-terminal α-helix interacting with them. When the arm is neglected, the surface areas of the subunit interfaces for a and b, a and d, and a and c subunits are reduced to 2,750, 11, and 654 Å², respectively, indicating that the arm is extensively involved in the subunit interactions as the joint for the formation of a dimer or a tetramer.

**ATP-Protein Interaction in tAsS**

ATP-Protein Interaction in tAsS-AMP-PNP Complex—The active site structure of the tAsS-AMP-PNP complex is shown in Fig. 3A. The difference Fourier map indicated a positive electron density peak in the vicinity of the P-loop, to which ATP could be assigned. The Mg ion was not detected near the triphosphate of ATP, although the tAsS-AMP-PNP complex was crystallized from the solution containing MgCl₂. ATP binds to the C-terminal side of the α-sheet of the small domain at the topological switch point between b1 and b4, including the P-loop which has the consensus sequence of H-Ser⁸-Gly⁹-Gly¹⁰-X-Asp¹²-Thr¹³-Ser¹⁴ and is assumed to be specific for the pyrophosphate binding (3). The ATP binding site is surrounded by the C-terminal sides of β-strands b2, b1, and b4, the P-loop, the N-terminal loops of β-strands b2 and b4, and the α-helices H4 and H5 (Fig. 3A). The
adenine ring of ATP is sandwiched by the side chain of Ile$_{95}$ from α-helix H4 and the backbone between Tyr$_7$ and Ser$_8$ of the P-loop. The N$^\alpha$ and N$^\beta$ amino groups are hydrogen bonded to the main-chain nitrogen and oxygen of Ala$_{95}$ of β-strand b2, respectively, as were observed in other ATP pyrophosphatase domains (3, 5, 6, 8). The O$_3$ and O$_3'$ of the ribose moiety makes hydrogen bonds with the main-chain oxygen and nitrogen of Ala$_6$ and Gly$_{114}$ belonging to the P-loop and the loop between β-strand b4 and α-helix H5, respectively. The γ-phosphate of ATP approaches the P-loop, making hydrogen bonds with the residues of the P-loop with its negative charge partially compensated by the access of the N-terminal side of α-helix H1. The α- and β-phosphates are free from direct hydrogen bonds with the active site residues, although the guanido group of Arg$_{92}$ from the α-helix H4 interacts with the α-phosphate.

Active Site of tAsS in the Complex with AMP-PNP, Arginine, and Succinate—The stereo structure and hydrogen-bonding scheme of the active site are shown in Figs. 3B and 4, respectively. Three positive electron density peaks were revealed in the difference Fourier map based on the diffraction data collected using the fresh crystal obtained 4 days after the hanging drop was set up. AMP-PNP and the substrate analogues (arginine and succinate) could be modeled into these peaks (Fig. 3B). When the old crystal was used, the residual electron density corresponding to AMP appeared, showing that the ATP pyrophosphatase activity of the enzyme hydrolyzed AMP-PNP into AMP and imidodiphosphate (data not shown). The binding mode of AMP-PNP as the ATP analogue to the active site is quite similar to that of ATP in the tAsS-ATP complex. The γ-phosphate of AMP-PNP interacts with the P-loop, while the β-phosphate is free from hydrogen bonds with the P-loop. Although the small domain of tAsS belongs to a family of N-type ATP pyrophosphatases with the consensus P-loop (3, 4), the P-loop of tAsS behaves different from those of the GMP- and NAD$^+$ synthetases (3, 8), in which the α- and β-phosphates of ATP were found to interact with the P-loop. The γ-phosphate of tAsS is involved in the hydrogen bonds with the main-chain amide groups of Gly$_{10}$, Leu$_{12}$, Asp$_{12}$, and Thr$_{13}$, and the hydroxy groups of Ser$_8$ and Thr$_{18}$ in the P-loop.

The substrate binding site is located at the interface between the small and large domains and is formed by the loop between α-helices 3 and 4, the N-terminal loop and N-terminal part of
α-helix 5, the C-terminal part of β-strand b7, and the loop between β-strands b11 and b12 (Fig. 1B). Succinate and arginine are substrate analogues in which the α-amino group of the aspartate and the ureido group of citruline are replaced by a hydrogen atom and a guanidino group, respectively. Two carboxylates of succinate interact with the small domain residues (Thr116, Asn120, Asp121, and Gln122) and two water molecules. The carboxylate of arginine interacts with small domain residues (Tyr184, Asn219, and Arg224), and a water molecule, and the amino group makes hydrogen bonds with large domain residues (Glu194, Glu258, and Tyr270). Arginine directs its side chain toward AMP-PNP with the guanidino group of arginine making hydrogen bonds with the γ-phosphate, Asp121, and a water molecule.

Comparison of the Active Site Structure of tAsS-AMP-PNP-Arginine-Succinate with That of E. coli AsS-Citrulline-Aspartate—Almost all the active site residues of tAsS are conserved in E. coli AsS, human AsS, and yeast AsS. When the active site of tAsS is compared with that of E. coli AsS, all the active site residues directly interacting with AMP-PNP and substrates (or analogues) are conserved between the two enzymes except for one residue, Ser182 in tAsS (Thr200 in E. coli AsS). The subunit superposition of tAsS-AMP-PNP-arginine-succinate onto E. coli AsS-citrulline-aspartate by least squares fitting of the corresponding Cα atoms shows that not only the main chain folding of the active site but also the side chain location of the active site residues are well conserved between two enzymes (Fig. 3C), suggesting that ATP may be coordinated to the active site of E. coli AsS in an analogous manner to that of tAsS. The location of arginine and succinate in tAsS is essentially the same as that of aspartate and citruline in E. coli AsS, respectively, although the guanidino group of arginine occupies a position different from its counterpart (ureido group) of citruline. The binding mode of two carboxylates of succinate in tAsS is almost the same as that of the aspartate in E. coli AsS. The carboxylate and the protonated α-amino group at one end of the arginine interact with the active site residues in the same manner as those of citruline bound to E. coli AsS. The sequence of Thr116-X-Lys118-Gly119-Asn120-Asp121-X-X-Arg124-Phe125 conserved in the small domain of human AsS, yeast AsS, E. coli AsS, and tAsS supplies a firm base to bind the carboxylates of succinate (or aspartate) and arginine (or citruline) (Fig. 4). The guanidino group of arginine approaches AMP-PNP and forms hydrogen bonds with the γ-phosphate, Asp121, and a water molecule, while the ureido group of citruline in E. coli AsS interacts with Ser191 (Ser173 in tAsS), Thr200 (Ser182), and a water molecule (10), implying that the ureido group of citruline may move toward the γ-phosphate on binding of ATP.

ATP was modeled into the active site of E. coli AsS-citrulline-aspartate by utilizing the x-ray structures of homologous proteins (10): NAD+/NADH synthetase complex with intact ATP (8) and asparagine and GMP synthetases (3, 7). In the NAD+ and GMP synthetase structures, the β- and γ-phosphates of ATP interact with the P-loop. There is a difference in the orientation of the triphosphate group of the bound ATP between the homology model of E. coli AsS and the x-ray structure of tAsS complex, although the adenine moiety of ATP binds similarly. In the homology model, the β- and γ-phosphate groups (pyrophosphate group) of ATP coordinate to the P-loop, while in the x-ray structure of tAsS, only the γ-phosphate group interacts with the P-loop, and the β-phosphate group does not interact.

Mechanistic Implications—The catalytic reaction of AsS has been proposed to be initiated by the nucleophilic attack of the citrulline ureido oxygen on the α-P atom of ATP, followed by the formation of a citrullyl-AMP intermediate (Scheme 1) (1, 2, 23). The bound aspartate stimulates this step by a factor of 600 (24). The α-amino group of l-aspartate then attacks the imino carbon atom of the citrullyl-AMP intermediate, producing AMP and argininosuccinate. AMP-PNP and the substrate analogues (arginine and succinate) in tAsS complex are arranged in such a way that the α-phosphate of AMP-PNP, the guanidino group of arginine, and the α-methylene group of the succinate are close to one another (Fig. 3B). The guanidino group of arginine interacts with the α-phosphate of AMP-PNP, and the Cα atom of succinate approaches the α-phosphate with a distance of 4.1 Å between O1A of α-phosphate and Cα of succinate (Figs. 3B and 4). The side chain of arginine makes a van der Waals
contact with the Ca-Cβ portion of the succinate with the contact
distance of 3.6–3.9 Å.

The NH group of PNP in AMP-PNP and one of NH2 groups of
the guanidino moiety in arginine were replaced by oxygen
atom, and an α-amino group was modeled into the succinate to
mimic the location of the α-amino group in the bound aspartate
of E. coli AsS (10), giving an enzyme-substrate complex model
(Fig. 5). The citrulline ureido amino group was positioned to
interact with O1A of the α-phosphate of ATP. The arrangement
of ATP, citrulline, and aspartate in the model seems to be
consistent with the proposed mechanism (1, 2, 23, 24). The ureido
group close to the α-phosphate group of ATP is ready to
attack the α-P atom. The distance between the ureido oxygen
and α-P atom of ATP is 4.2 Å, which is much shorter than the
corresponding value of 7.9 Å observed in the homology model
complex with ATP of E. coli AsS (10). This is due to the differ-
cence in the coordination behavior of the triphosphate group of
ATP toward the P-loop and the location of the ureido group
between the homology model of E. coli AsS and the current
model of tAsS. The protonated α-amino group of aspartate in
the current model is within hydrogen bonding distance (3.3 Å)
with the α-phosphate, thus stimulating the α-phosphate by an
electrostatic interaction to give citrullyl-AMP. Furthermore,
the protonated α-amino group of aspartate is directed toward
the ureido carbon atom and is positioned suitably to react with
citrullyl-AMP, although the α-amino group is too close (2.5 Å)
to the ureido carbon in the current model, and the citrulline
moiety of citrullyl-AMP should be in a somewhat different
location from that of citrulline. The protonated α-amino group of
aspartate must be deprotonated to displace AMP. Possibly,
the pyrophosphate of ATP as a leaving group accepts the α-pro-
ton from the protonated α-amino group. Thus, the x-ray struc-
tures and the current model of tAsS imply that the proposed
conformational change of AsS (10) is not necessary for the
catalytic process, and the enzyme essentially maintains the
native conformation during the catalysis.

This x-ray structure is not in conflict with the proposed
mechanism, but there still remain problems to be solved. The
divalent cation, Mg2+, which is essential for catalysis, could not
be located on the difference Fourier map. Arginine is similar in
structure to citrulline, but different in charge. Succinate lacks
the protonated α-amino group. The geometrical arrangement of
ATP and substrates bound to the active site may be modulated
by the interactions among the Mg2+-coordinated triphosphate
of ATP, the uncharged ureido group of citrulline, and the pro-
tonated α-amino group of aspartate. Thus, the precise mecha-
nism of AsS must await further crystallographic studies of the
enzyme in the complex with ATP, ATP analogues, substrates,
and various substrate analogues.

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