Crystal Structure of Argininosuccinate Synthetase from Thermus thermophilus HB8: Structural Basis for the Catalytic Action*

Masaru Goto‡¶, Yoshitaka Nakajima‡¶, and Ken Hirotsu‡¶**

From the ‡Department of Chemistry, Graduate School of Science, Osaka City University, Osaka 558-8585, Japan, ¶Harima Institute/SPring-8, The Institute of Physical and Chemical Research (RIKEN), Sayo-gun, Hyogo, 679-5148, Japan

Running Title: Structure of Argininosuccinate Synthetase
*This study was supported by Grants-in Aid for Scientific Research on Priority Area from the Ministry of Education, Science, Sports, and Culture of Japan (B: 13125207 (K.H.)), a Research Grant from the Japan Society for the Promotion of Science (Category B: 13480196 (K.H.)), and the Sakabe Project of Tsukuba Advanced Research Alliance (K.H.).

The atomic coordinates and structure factors for native enzyme, ATP complex and AMP-PNP-Argininosuccinate complex structures (codes 1KH1, 1KH2, and 1KOR, respectively) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

** To whom correspondence should be addressed. Tel: +81-6-6605-2507; FAX: +81-6605-3131; E-mail: hirotsu@sci.osaka-cu.ac.jp

1 The abbreviations used are: AsS, argininosuccinate synthetase; tAsS, argininosuccinate synthetase from Thermus thermophilus HB8; AMP-PNP, adenylyl imidodiphosphate; rms, root mean square.
Argininosuccinate synthetase catalyzes the ATP-dependent condensation of a citrulline with an aspartate to give argininosuccinate. The three-dimensional structures of the enzyme from *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 *E. 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 located 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) 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).

Scheme 1

The ureido oxygen atom of a citrulline attacks the \( \alpha \)-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 \(^{18}\text{O}\)-labeled citrulline (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-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 shows ATP pyrophosphate activity, were proposed to have a common fold specific for nucleotide binding, and belong to a new family of \(^\prime\)N-type\(^\prime\) 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 \( \alpha/\beta \) 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 \( \beta \)- and \( \gamma \)-phosphates of ATP.

AsS from Thermus thermophilus HB8 (tAsS), which has been overexpressed in E. coli, has 400 residues per subunit, with a subunit molecular weight of 44815. 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 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 of 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 *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 *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 adenylyl imidodiphosphate (ATP analogue, AMP-PNP), arginine (analogue of citrulline), and succinate (analogue of aspartate). Only the γ-phosphate of ATP or AMP-PNP interacts with the P-loop allowing the β-phosphate to be free from interactions with the P-loop, and indicating that the location of triphosphate of AMP-PNP is different from that of ATP in the proposed model of *E. coli* AsS (10). The loop of tAsS does not show the same specificity for the pyrophosphate that was observed in GMP and NAD$^+$ synthetase structures, but shows a specificity for γ-phosphate (monophosphate). ATP, arginine, and succinate bind to the active site in such a way that the putative reaction sites of these analogues are brought into positions favorable to the catalytic action. This result implies that the enzyme does not require the proposed conformational change of the enzyme for the catalytic process. We now report the X-ray crystallographic study of the following three forms of tAsS: the native form at 2.3Å resolution, the complex form with ATP at 2.3 Å resolution, and the complex form with AMP-PNP, arginine, and succinate at 1.95Å resolution.
EXPERIMENTAL PROCEDURES

Crystallization 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 (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, 2.3, 1.95 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 approximately 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, 2.7, and 3.1 Å resolution at 100K on the BL41XU station or on the BL18B station at the Photon Factory (Tsukuba, 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 MIR 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 ethylmercurithiosalicylic acid and K₂Pt(NO₂)₄ data sets allowed
the interpretation of two mercury sites and two platinum sites, respectively. The positions of
another six mercury and platinum sites, and those of twenty-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 MIR 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 (16). The mean figure-of-merit reached 0.75 with
the same resolution range. The map was of good quality and the model of the subunit was
gradually built into the 3.0 Å map through several cycles of model building using the
program O (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 - 3.0 Å resolution to give an $R_{\text{factor}}$ of 32.2% and an $R_{\text{free}}$ of 37.5%. At this
stage, the restraint on the 222 symmetry was removed, and entire molecule was refined. The
resolution was progressively increased to 2.3 Å, and after several rounds of refinement and
manual rebuilding, $R_{\text{factor}}$ and $R_{\text{free}}$ 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 $R_{\text{factor}}$ of
19.9 % and an $R_{\text{free}}$ of 22.9 %, calculated for 122,785 reflections [$F_o > 2\sigma(F_o)$] observed in
a 10.02.3 Å resolution range (Table II).

The same refinement procedure was applied to tAsS•ATP, but using the coordinates of
the native tAsS as the initial model. When the $R_{\text{factor}}$ value became less than 30 %, the
difference Fourier map showed the residual electron density corresponding to the bound ATP. Water molecules were picked up from the difference map, and further model building and refinement cycles gave $R_{\text{factor}}$ and $R_{\text{free}}$ values of 20.9 and 24.1 %, respectively, calculated for 138,518 reflections [$F_o > 2\sigma(F_o)$] observed in a 10.02.3 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 $R_{\text{factor}}$ 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 $R_{\text{factor}}$ and $R_{\text{free}}$ values of 23.2 and 25.5 %, respectively, calculated for 225,836 reflections [$F_o > 2\sigma(F_o)$] observed in a 10.01.95 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 anion with 458 water molecules with an $R_{\text{factor}}$ 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 Å$^2$. The final model of the tAsS•ATP complex contains 1,538 amino acid residues for all subunits, and four ATPs with 477 water molecules with an $R_{\text{factor}}$ of 20.9 % at 2.3 Å resolution. The model lacks 68 residues. The average thermal factors of the main-chain atoms are 33 Å$^2$. The final model of 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 $R_{\text{factor}}$ of 23.2 % at 1.95 Å resolution. The model lacks 62 residues, and the average thermal factors of the main-chain atoms are 31 Å$^2$. 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).
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 non-crystallographic *D*₂ 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 subunit in the tetramer interacts with the other three subunits and the surface areas of the subunit interfaces are 4,390 for a and b, 1201 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 (21) in Fig. 1B. The Cα 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 rms 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 Cα positions of the native *E. coli* AsS and the complex one with citrulline and aspartate superimpose within rms deviations of 0.6 Å. The subunit superposition of tAsS’”AMP-PNP’”arginine”succinate and *E. coli* AsS”citrulline”aspartate resulted in 284 equivalenced Cα atoms with rms 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 has a typical α/β structure of an open parallel β-sheet, and is folded into the structure similar to that of the ATP pyrophosphate domains of the GMP, NAD+, and asparagine synthetases (3, 5, 7). The five β-strands 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 Ala91 (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, 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 Complex** – The active site structure of the tAsS•ATP 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•ATP complex was crystallized from the solution containing MgCl₂. ATP binds to the C-terminal side of the β-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 Ile95 from α-helix H4 and the backbone between Tyr7 and Ser8 of the P-loop. The N<sup>1</sup> and N<sup>6</sup> amino groups are hydrogen bonded to the main-chain nitrogen and oxygen of Ala33 of β-strand b2, respectively, as were observed in other ATP pyrophosphatase domains (3, 5, 6, 8). The O2’ and O3’ of the ribose moiety makes hydrogen bonds with the main-chain oxygen and nitrogen of Ala6 and Gly114 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 guanidino group of Arg92 from the α-helix H4 weakly 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 four 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 Gly10, Leu11, Asp12, and Thr13, and the hydroxy groups of
Ser8 and Thr13 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 citrulline 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 (Tyr84, Asn120, and Arg124),
and a water molecule, and the amino group makes hydrogen bonds with large domain
residues (Glu184, Glu258, and Tyr270). Arginine directs its side chain toward AMP-PNP
with the guanidino group of arginine forming 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 tAsS). 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 citrulline in *E. coli* AsS, respectively, although
the guanidino group of arginine occupies a position different from its counterpart (ureido
group) of citrulline. The binding mode of two carboxylates of succinate in tAsS are 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 interacts with the active site residues in the same manner as
those of citrulline 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 citrulline ) (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 citrulline in *E. coli* AsS interacts with Ser191
(Ser173 in tAsS), Thr200 (Ser182), and a water molecule (10), implying that the ureido
group of citrulline 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\(^+\) 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 $\alpha$-phosphate of AMP-PNP, the guanidino group of arginine, and the $\alpha$-methylene group of the succinate are close to one another (Fig. 3B). The guanidino group of arginine interacts with the $\alpha$-phosphate of AMP-PNP, and the $C\alpha$ atom of succinate approaches the $\alpha$-phosphate with a distance of 4.1 Å between O1A of $\alpha$-phosphate and C$\alpha$ of succinate (Figs. 3B and 4). The side chain of arginine makes a van der Waals contact with the C$\alpha$-C$\beta$ 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 NH$_2$ groups of the guanidino moiety in arginine were replaced by oxygen atom, and an $\alpha$-amino group was modeled into the succinate to mimic the location of the $\alpha$-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 $\alpha$-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 $\alpha$-phosphate group of ATP is ready to attack the $\alpha$-P atom. The distance between the ureido oxygen and $\alpha$-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 difference 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 $\alpha$-amino group of aspartate in the current model is within hydrogen bonding distance (3.3 Å) with the $\alpha$-phosphate, thus stimulating the $\alpha$-phosphate by an electrostatic interaction to give citrullyl-AMP. Furthermore, the protonated $\alpha$-amino group of aspartate is directed toward the ureido carbon atom, and is positioned suitably to react with citrullyl-AMP, although the $\alpha$-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 $\alpha$-amino group of aspartate must be deprotonated to displace AMP. Possibly, the pyrophosphate of ATP as a leaving group accepts the $\alpha$-proton from the protonated $\alpha$-amino group. Thus, the X-ray structures 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, Mg$^{2+}$, 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 $\alpha$-amino group. The geometrical arrangement of ATP and substrates bound to the active site may be modulated by the interactions among the Mg$^{2+}$-coordinated triphosphate of ATP, the uncharged ureido group of citrulline, and the protonated $\alpha$-amino group of aspartate. Thus, the precise mechanism of AsS must await further crystallographic studies of the enzyme in the complex with ATP, ATP analogues, substrates, and various substrate analogues.
REFERENCES


### Table I

**Data Collection for Heavy Atom Derivatives and MIR Statistics**

<table>
<thead>
<tr>
<th>Data set</th>
<th>EMTS (^d)</th>
<th>(K_2\text{Pt(NO}_2\text{)}_4)</th>
<th>Se-Met</th>
<th>Se-Met+EMTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>resolution (Å)</td>
<td>2.5</td>
<td>2.7</td>
<td>2.5</td>
<td>3.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>no. of reflections</th>
<th>unique</th>
<th>observed</th>
<th>completeness (%)</th>
<th>(R_{\text{merge}}) (^a)</th>
<th>(R_{\text{diff}}) (^b)</th>
<th>phasing power (^c)</th>
<th>no. of sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>103686</td>
<td>329500</td>
<td>95.4</td>
<td>7.1</td>
<td>15.6</td>
<td>1.63</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>85404</td>
<td>303295</td>
<td>98.6</td>
<td>6.6</td>
<td>12.0</td>
<td>0.76</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>99171</td>
<td>251330</td>
<td>90.7</td>
<td>6.4</td>
<td>14.9</td>
<td>1.07</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>56846</td>
<td>705720</td>
<td>99.7</td>
<td>6.5</td>
<td>18.1</td>
<td>1.85</td>
<td>26+8</td>
</tr>
</tbody>
</table>

\(^a\) \(R_{\text{merge}} = \frac{|I_{hlk,i} - <I_{hlk}|/I_{hlk}|}{|I_{hlk}|},\) where \(I = \) observed intensity and \(<I> = \) average intensity for multiple measurements.

\(^b\) \(R_{\text{diff}} = \frac{|F_{PH}|-|F_{P}|}{|F_{P}|},\) where \(|F_{PH}|\) and \(|F_{P}|\) are the derivative and native structure-factor amplitudes, respectively.

\(^c\) Phasing power is the ratio of the root-mean-square (rms) of the heavy atom scattering amplitude and the lack of closure error.

\(^d\) Ethylmercurithiosalicylic acid.
### Data Collection and Refinement Statistics

<table>
<thead>
<tr>
<th>Data set</th>
<th>native</th>
<th>ATP</th>
<th>AMP-PNP•arginine•succinate</th>
</tr>
</thead>
<tbody>
<tr>
<td>resolution (Å)</td>
<td>2.30</td>
<td>2.30</td>
<td>1.95</td>
</tr>
<tr>
<td>no. of reflections</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>unique</td>
<td>124435</td>
<td>143708</td>
<td>227309</td>
</tr>
<tr>
<td>observed</td>
<td>281918</td>
<td>685640</td>
<td>979957</td>
</tr>
<tr>
<td>completeness (%)</td>
<td>89.2 (78.3) b</td>
<td>99.9 (99.9) b</td>
<td>99.9 (99.9) b</td>
</tr>
<tr>
<td>$R_{\text{merge}}$ (%) a</td>
<td>6.5 (12.7) b</td>
<td>7.4 (27.0) b</td>
<td>4.4 (26.4) b</td>
</tr>
</tbody>
</table>

**Refinement**

<table>
<thead>
<tr>
<th>resolution limits (Å)</th>
<th>48.8 – 2.30</th>
<th>19.9 – 2.30</th>
<th>20.0 – 1.95</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R_{\text{factor}}$ (%)</td>
<td>19.94 (25.25)</td>
<td>20.87 (26.76)</td>
<td>23.19 (31.27)</td>
</tr>
<tr>
<td>$R_{\text{free}}$ (%)</td>
<td>22.91 (29.07)</td>
<td>24.10 (31.49)</td>
<td>25.53 (34.02)</td>
</tr>
</tbody>
</table>

| deviations | bond lengths (Å) | 0.007 | 0.007 | 0.007 |
|            | bond angles (deg) | 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**

| favored   | 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  |

---

**Notes:**

*a* $R_{\text{merge}} = \frac{\sum |I_{hkl,i} - \langle I_{hkl} \rangle|}{\sum I_{hkl,i}}$, where $I$ = observed intensity and $\langle I \rangle$ = average intensity for multiple measurements.

*b* The values in the parentheses are for highest resolution shells.
FIGURE LEGENDS

**Fig. 1.** Ribbon structural drawings of tAsS in the complex with AMP-PNP•arginine•succinate. *A*, Stereoview of tetrameric tAsS viewed down the molecular 2-fold axis. The subunits, a, b, c and d are represented by blue, green, olive, and purple ribbon. AMP-PNP, arginine, and succinate, shown by the ball-and-stick model (yellow), are bound to the crevice formed at four corners of the tetramer. The dimeric unit comprises subunit a and b (or d and c) and its 2-fold axis is parallel to the horizontal axis. *B*, Stereoview of the subunit with the secondary structure assignments. The small domain (ATP binding domain), the large domain, the C-terminal arm and the P-loop of the small domain are shown in cyan, blue, purple, and red, respectively. α-Helices are denoted as H1 - H7 in the small domain, H8 - H11 in the large domain, and H12 in the arm. β-Strands are denoted as b1 - b5 in the small domain, and b6 - b14 in the large domain. AMP-PNP and substrate analogues are drawn by the ball-and-stick model (yellow). The active site is formed at the interface between the small and large domains.

**Fig. 2.** The superimposition of the subunit of tAsS•AMP-PNP•arginine•succinate (red) onto that of *E. coli* AsS•citrulline•aspartate (green). The N and C termini (residues 1 and 395, 1 and 440, respectively) are indicated. Both structures match well, although the regions of helices 6 and 7 (see Fig. 1B), and the C-terminal arm show small deviations.

**Fig. 3.** Stereo diagrams of the active site in tAsS and *E. coli* AsS. *A*, A close-up view of the active site of tAsS•ATP. ATP binds to the small domain with its γ-phosphate interacting with the P-loop and its β-phosphate free from hydrogen bonds. ATP is drawn by the ball-and-stick model. *B*, A close up view of the active site of tAsS•AMP-PNP•arginine•succinate. AMP-PNP binds to the small domain in the same manner as ATP in tAsS•ATP. The conserved loop of T116-X-K118-G119-N120-D121-X-X-R124-F125 interacts with the substrate analogues (arginine and succinate). AMP-PNP and substrate analogues drawn by the ball-and-stick models are neighbored. Water molecules are shown by green circles. *AB*, The secondary structures of small and large domains are
displayed by cyan and blue violet ribbons, respectively. Red and dark magenta bonds indicate the consensus P-loop of H-S8-G9-G10-X-D12-T13-Ser14 and the conserved loop, respectively. Acidic, basic, hydrophilic, and hydrophobic side chains of the active site residues are shown by purple, blue, light blue and green bonds, respectively. Omit electron density maps are contoured at 1.0σ level. C, The superposition of the active site structure of tAsS"AMP-PNP"arginine"succinate onto that of E. coli AsS"citrulline"aspartate. The tAsS and E.coli AsS are drawn by green and gray, respectively, with the P-loop, AMP-PNP, and substrate analogues in tAsS colored orange AMP-PNP and substrates (or analogues) are shown by the ball-and-stick models.

Fig. 4. Schematic diagram showing hydrogen bond and salt bridge interactions of the active site residues in tAsS•AMP-PNP•arginine•succinate. Putative interactions are shown by dotted lines if the acceptor and donor are less than 3.5 Å apart. W indicates a water molecule. AMP-PNP, arginine and succinate bound to the active site are drawn by thick bonds.

Fig. 5. Model of ATP, citrulline, and aspartate binding to tAsS. Important short contacts are shown by dotted lines.
Scheme 1.

ATP

\[ AMP + PP_i \rightarrow \text{Citrulline} \]

\[ \text{Citrulline} + \text{Aspartate} \rightarrow \text{Citrullyl-AMP} \]

\[ \text{Citrullyl-AMP} \rightarrow \text{Argininosuccinate} \]