Argininosuccinate synthetase (AS) is the rate-limiting enzyme of both the urea and arginine-citrulline cycles. In mammals, deficiency of AS leads to citrullinemia, a debilitating and often fatal autosomal recessive urea cycle disorder, whereas its overexpression for sustained nitric oxide production via the arginine-citrulline cycle leads to the potentially fatal hypotension associated with septic and cytokine-induced circulatory shock. The crystal structures of *Escherichia coli* argininosuccinate synthetase (EAS) in complex with ATP and with ATP and citrulline have been determined at 2.0-Å resolution. These are the first EAS structures to be solved in the presence of a nucleotide substrate and clearly identify the residues that interact with both ATP and citrulline. Two distinct conformations are revealed for ATP, both of which are believed to be catalytically relevant. In addition, comparisons of these EAS structures with those of the apoenzyme and EAS complexed with aspartate and citrulline (Lemke, C. T., and Howell, P. L. (2001) *Structure (Lond.*)* 9, 1153–1164) provide structural evidence of ATP-induced conformational changes in the nucleotide binding domain. Combined, these structures also provide structural explanations of some of the observed kinetic properties of the enzyme and have enabled a detailed enzymatic mechanism of AS catalysis to be proposed.

Argininosuccinate synthetase (AS) catalyzes the reversible conversion of citrulline, aspartate, and ATP to argininosuccinate, AMP, and inorganic pyrophosphate (Fig. 1). There are three important metabolic processes that require the argininosuccinate lyase, together with the flavoprotein nitric-oxide synthase form the arginine-citrulline cycle, an abbreviated urea cycle that provides *de novo* arginine biosynthesis for sustainable overproduction of nitric oxide (NO (2, 3)). NO is a small, membrane-permeable, highly reactive molecule that plays key roles in a wide range of mammalian processes including blood pressure control, neurotransmission, apoptosis, immune system function, and wound healing (for reviews, see Refs. 4–10). AS is the rate-limiting enzyme in both the urea and the arginine-citrulline cycles (11, 12) and is therefore a key participant in all these pathways.

Previously, we reported the structures of uncomplexed EAS and EAS complexed with citrulline and aspartate (EAS-CIT+ASP) (13). Each monomer of this tetrameric protein was found to consist of a nucleotide binding domain and a novel catalytic/multimerization domain. The EAS nucleotide binding domain was found to be structurally similar to the N-type ATP pyrophosphatases, GMP, NAD⁺, and asparagine synthetase (14–16), thus confirming that EAS is a member of this enzyme family. N-type ATP pyrophosphatases all catalyze a substrate adenylation to activate a carbonyl or carboxyl group for the subsequent nucleophilic attack of a nitrogen nucleophile (14). Although our initial structures enabled us to determine the citrulline and aspartate binding sites crystallographically and could be used to model the ATP, the relative positions of the three substrates were such that we predicted that the protein must undergo a large conformational change during its catalytic cycle.

To confirm this hypothesis, we have determined the structures of EAS in complex with intact ATP (EAS-ATP), and in complex with ATP and citrulline (EAS-ATP+CIT). The structures presented here confirm the residues involved in citrulline binding and reveal that ATP can bind to the enzyme in two distinct conformations, both of which are believed to be catalytically relevant. Comparisons of all available EAS structures provide structural evidence that the binding of ATP results in a large rigid body conformational change of the nucleotide binding domain. Furthermore, these comparisons have yielded structural explanations for some of the observed kinetic properties of the enzyme, including the order of substrate binding and the increased formation of the adenylated citrulline inter-
mediate in the presence of aspartate. Together, these structures have provided valuable information about the first steps of AS catalysis and have enabled us to propose a detailed enzymatic mechanism.

**EXPERIMENTAL PROCEDURES**

**Protein Preparation and Crystallization**—The expression and purification of EAS was as described previously (17). The expressed protein consists of full-length EAS and an additional C-terminal 6-histidine affinity tag connected by a three-amin acid linker. Crystallization of the EAS-ATP complex was carried out using the hanging-drop vapor-diffusion technique at room temperature (293 K). Equal volumes (3–5 μl) of protein solution (10 mg ml⁻¹ EAS, 25 mM MES, pH 6.5, 5 mM ATP, 15 mM MgCl₂, 150 mM guanidine hydrochloride) and microseeded solution (2.0 mM Na⁺/K⁺ phosphate, 100 mM MES, pH 6.5, 6.5, 150 mM guanidine hydrochloride, 5 mM ATP, 15 mM MgCl₂) and in cryoprotectant solution (1.6 mM Na⁺/K⁺ phosphate, 100 mM MES, pH 6.5). The EAS-ATP+CIT complex was crystallized in the same manner with the addition of 2 mM citrulline to the protein solution. In each case, crystals appeared within 2–4 days and grew as large chunks, similar to those described in Lemke et al. (17).

**X-ray Analysis**—Before data collection, the EAS-ATP and EAS-ATP+CIT crystals were soaked for 10 min in cryoprotectant solution (350 mg ml⁻¹ trehalose, 1.6 mM Na⁺/K⁺ phosphate, 100 mM MES, pH 6.5, 150 mM guanidine hydrochloride, 5 mM ATP, 15 mM MgCl₂) and in cryoprotectant solution supplemented with 2 mM citrulline, respectively. The crystals were then transferred to rayon CryoLoops (Hampton Research) and flash-frozen in liquid nitrogen. The frozen crystals were subsequently mounted in a continuous cold stream, and data were collected at 100 K on a MAR345 image plate using Cu-Kα radiation. The data were indexed, merged, and scaled using DENZO/SCALEPACK (18). Because the EAS-ATP and EAS-ATP+CIT crystals are isomorphous with the uncomplexed EAS crystals, the uncomplexed structure was used as the starting model for the refinement of both complexed structures. The models were refined using the simulated annealing, individual B-factor refinement, and energy minimization protocols incorporated into the program CNS (19). A maximum likelihood target (20, 21) and a flat bulk solvent correction were used. No or low resolution cutoffs were applied to the data. Each round of refinement was alternated with a round of manual rebuilding using TURBO-FRODO (22). The progress of the refinement was monitored by decreases in R cryst and R free.

The final EAS-ATP model consists of residues 1–182, 187–385, and 394–443, 286 water molecules, 1 ATP molecule, 3 phosphate ions, and 1 guanidine ion. Only water molecules with reasonable hydrogen-bonding geometry and significant density at greater than 3σ on χ2-weighted [F o] − [F c] maps were included. There was insufficient electron density present to model residues 183–186, 386–393, and the 12 C-terminal residues (Lys-444, Gly-445, and Gln-446, along with the appended tripeptide linker and the 6-histidine tag) as well as the full side chains of 7 residues (Lys-4, Asp-22, Tyr-182, Glu-187, Lys-230, Glu-317, and Ser-430). The final EAS-ATP+CIT model consists of residues 1–385, 393–446, 186 water molecules, 1 ATP molecule, 2 citrulline molecules, 1 phosphate ion, and two guanidine ions. There was insufficient electron density to model residues 386–392 and the 9 C-terminal residues (the tripeptide linker and the 6-histidine tag) as well as the full side chains of 6 residues (Lys-5, Lys-74, Lys-183, Glu-187, Lys-230, and Lys-444).

A summary of the refinement statistics for both structures is presented in Table II. The quality of the final structures was assessed using the program PROCHECK (23). For the EAS-ATP structure, 90.0% of the residues were found in the most favored regions of the Ramachandran plot, 8.9% in allowed regions, 1.1% in generously allowed regions, and none in disallowed regions. For the EAS-ATP+CIT structure, 89.7% of the residues were found in the most favored regions of the Ramachandran plot, 8.7% in allowed regions, 1.6% in generously allowed regions, and none in disallowed regions. The overall G factor was 0.3 for both the EAS-ATP and EAS-ATP+CIT structures, indicating excellent geometry and stereochemistry.

**RESULTS AND DISCUSSION**

**Structure Determination**—The structures of uncomplexed EAS and of EAS complexed with aspartate and citrulline were reported earlier (13). Modification of the crystallization conditions used for these structures has yielded isomorphous crystals that are capable of tolerating the addition of ATP, thereby enabling the structure determination of the EAS-ATP and EAS-ATP+CIT complexes (see “Experimental Procedures” and Table I). The key advance that permitted the co-crystallization and soaking of EAS crystals with ATP was the addition of guanidine to the crystallization conditions. In the resulting structures guanidine localizes to a region of intermolecular crystal contacts that we believe would otherwise be compromised by the substrate-induced conformational change. The EAS-ATP and EAS-ATP+CIT structures were refined to an R cryst = 17.7% and R free = 21.6% and an R cryst = 19.2% and R free = 22.9%, respectively (Table II).

In all four EAS structures reported to date, a single monomer of EAS is present in the asymmetric unit. Each monomer consists of two αβ domains, a nucleotide binding domain similar to that of the N-type ATP pyrophosphatase class of enzymes and a novel catalytic/multimerization domain. Two EAS monomers come together to form a dimer, which in turn dimerizes to form the biologically relevant homotetramer (17, 25–28).

**Substrate Binding in the EAS-ATP Complex**—The structure of EAS complexed with ATP has been determined at 2.0 Å resolution. As predicted previously (15), the nucleotide substrate is bound to EAS in a manner similar to that observed in the GMP, NAD⁺, and asparagine synthetase structures (14, 16, 29). The ATP molecule (ATP1) is bound at the topological switch point between the second and fifth β-strands (β2 and β7) of the nucleotide binding domain and interacts directly with residues Ala-16, Ala-42, Gly-128, Thr-130, Asp-135, and Asp-191 of the nucleotide binding domain, 7 water molecules, and an inorganic phosphate molecule scavenged from the crystallization conditions (Fig. 2, a and b). Eight hydrogen bonds secure the adenosine moiety, five to main chain atoms, two to ordered waters, and one to the phosphate bound to the PP loop. The PP loop is the loop between the second β-strand and the first α-helix of the nucleotide binding domain, where free pyrophosphate has been shown to bind in other N-type pyrophosphatases. The absolutely conserved AS residues Arg-106 and Phe-139 are in the vicinity of the adenosine ring but are not sufficiently close to contribute to stacking interactions. The
triphosphate tail of ATP1 is secured by an additional 11 electrostatic interactions, 1 to the amino nitrogen of Thr-130, 1 to a carboxyl oxygen of Asp-135, 2 to a carboxyl oxygen of Asp-193, 6 to ordered waters, and 1 to the phosphate bound to the PP loop. In this conformation, the β- and γ-phosphates of ATP1 extend away from the PP loop to interact with two residues of the aspartate binding site, Thr-130 and Asp-135. Although all three phosphates of ATP are relatively well ordered in this conformation and an excess of Mg2+ was included in the crystallization conditions, no electron density consistent with the ion was observed.

Substrate Binding in the EAS-ATP+CIT Complex—The structure of EAS complexed with both ATP and citrulline has also been determined at 2.0 Å resolution. Two molecules of citrulline and one molecule of ATP were observed in the resulting electron density. The first molecule of citrulline (CIT1) is bound at the citrulline binding site as described in the EAS-ASP structure (13), with few deviations (Fig. 3, a and b). In the current configuration, the hydroxyl oxygen of Ser-191 no longer makes a hydrogen bond to the terminal ureido nitrogen of CIT1, whereas the hydroxyl oxygen of Tyr-291 and a carboxyl oxygen of Glu-202 now make hydrogen bonds to the amino nitrogen and Nε of CIT1, respectively. These conformational changes are small, and it is uncertain that they represent significant differences between the EAS-ASP and EAS-ATP+CIT structures. The second molecule of citrulline (CIT2) is bound adjacent to CIT1 and interacts directly with residues Thr-130, Asn-134, Asp-135, Glu-202, Ser-287 as well as CIT1 and the bound ATP (Fig. 3, c and d). In the presence of equimolar citrulline and aspartate, this site is occupied by aspartate (13). This suggests that the CIT2 binding site is the result of the high concentration of citrulline and the absence of aspartate in the crystallization conditions and, therefore, does not represent a biologically relevant citrulline binding site. In the reciprocal experiment, where EAS is provided with excess aspartate but no citrulline, aspartate is observed in both the aspartate and citrulline (CIT1) binding sites (data not shown).

In the EAS-ATP+CIT structure the ATP molecule (ATP2) is bound in a similar manner as ATP1 in the EAS-ATP structure, with the exception that its β- and γ-phosphates no longer interact with residues of the aspartate binding site but instead pass over the ribose C3 to interact with residues of the PP loop (Fig. 2, c and d). Four residues of the PP-loop (Ser-18, Leu-21, Asp-22, and Thr-23) and a water molecule contribute eight hydrogen bonds to coordinate the γ-phosphate of ATP2, whereas the β-phosphate is held in position by only one hydrogen bond to the carboxyl oxygen of Gly-128. This disparity of binding interactions with the two phosphates results in a much higher mobility for the β-phosphate, which is consequently poorly defined in the experimental electron density (Fig. 2c). As with the EAS-ATP structure, although an excess of Mg2+ was included in the crystallization conditions, no corresponding electron density consistent with the ion was observed.

Conformational Changes Observed upon Substrate Binding—Several interrelated conformational changes have been identified between the main-chain atoms of the native and substrate bound models of EAS (Fig. 4). The principal conformational change is brought about by ATP binding. In both ATP complexed structures, nucleotide binding causes the amino nitrogen of Gly-128 to move 2 Å to hydrogen bond with the 2’ and 3’ hydroxyl oxygens of ATP, whereas the carboxyl oxygen of Ala-16 moves 1.5 Å to hydrogen bond with the 3’ hydroxyl oxygen. To allow these residues to interact with ATP, significant changes occur in the C-terminal portions of strands β2, β7, and β8. Strands β2 and β7 move closer together, resulting in a new β-sheet hydrogen bond between the nitrogen of Ala-16 (β2) and the oxygen of Gly-126 (β7) and the loss of a β-sheet hydrogen bond between the nitrogen Asp-127 (β7) and the carboxyl oxygen of Tyr-153 (β8) (Fig. 4c).

This conformational change is propagated throughout the domain in two ways. First, the reorganization of the central β-sheet causes a major shift of the residues following β8 through to the end of the domain (residues 154–188; Figs. 4b and 5). Secondly, this shift of residues 154–188 is augmented by a concerted rigid body movement of the majority of the nucleotide binding domain with respect to the body of the synthetase domain (Fig. 4a). This movement approximates a rotation of the entire nucleotide binding domain (residues 1–72, 101–188), with the exception of helices α4 and α5 (residues 101–120 and 135–146, respectively), which are closely associated with the synthetase domain and remain relatively unmoved. The direction of these conformational changes is consistent with a movement of the nucleotide binding domain toward the synthetase domain, which forms the core of the tetrameric enzyme. The active site, located at the interface of the two domains, becomes significantly narrowed as a result of this movement.

Although these conformational changes are evident in both structures presented in this paper, differences between the two suggest that the conformational changes in the EAS-ATP+CIT complex have progressed further than in the EAS-ATP complex (Fig. 5). The most striking difference is the degree to which the nucleotide binding domain has rotated with respect to the synthetase domain. In the EAS-ATP structure the rotation measures ~3°, whereas in EAS-ATP+CIT structure the rotation is almost 5°. This difference is corroborated by structural alignments of both nucleotide-bound models with the uncomplexed structure. Although the residues that form the body of the synthetase domain (residues 73–100, 189–375) have a relatively low main-chain root mean square deviation (r.m.s.d.) of 0.3 Å for both structures, the main-chain r.m.s.d. of the nucleotide binding domains (residues 1–72, 149–189) of EAS-ATP and EAS-ATP+CIT are 1.2 Å and 1.8 Å, respectively.

There are two other significant differences between the two nucleotide-bound structures. The first is a deviation at the C-terminal end of helix α4. In the EAS-ATP+CIT structure, a rearrangement of the hydrophobic interface between α4 and β7 distorts the final two turns of α4, resulting in a greater deviation of this region than observed in other EAS structures. The second is a unique variation in the Type I β-turn between β13 and β14 (residues Arg-281–Glu-284; Fig. 4d). In the EAS-ATP+CIT structure, a near 180° rotation in ψ of the i+2
residue (Ile-283) results in the loss of the $i$ to $i+3$ $\beta$-turn hydrogen bond and the addition of a new hydrogen bond between the carbonyl oxygen of Ile-283 and the amide nitrogen of Ala-285. This distortion is compensated for by main chain conformational changes in Glu-284 through Lys-286 that allow regular $\beta$-sheet hydrogen bonding to resume at Ala-285. This conformational change may be caused by the binding of CIT2. However, although CIT2 interacts with the side chains of Arg-

| TABLE II |
| --- |
| Refinement statistics |
|  |
| **EAS-ATP** | **EAS-ATP + CIT** |
| Resolution limits | 45–2.0 | 41–2.0 |
| Number of reflections used in refinement | 34031 | 32696 |
| Number of reflections used to compute $R_{\text{free}}$ | 3360 | 3272 |
| $R_{\text{free}}$ ($R_{\text{free}}$ b) (%) | 17.67 (21.64) | 19.16 (22.94) |
| Number of non-hydrogen atoms |  |
| Protein | 3359 | 3419 |
| Solvent | 305 | 199 |
| Substrate | 31 | 55 |
| Root mean square deviation from ideal values |  |
| Bond length (Å) | 0.009 | 0.010 |
| Bond angles (°) | 1.54 | 1.47 |
| Dihedral angles (°) | 22.74 | 22.91 |
| Improper angles (°) | 0.87 | 0.91 |
| Mean B value (Å²) |  |
| Protein | 31.53 | 35.05 |
| Solvent | 37.69 | 39.33 |
| ATP | 44.16 | 47.03 |
| Citrulline | – | 44.07 |

$^a R_{\text{free}} = \Sigma |F_o| - |F_c| / \Sigma |F_o|$, where $F_o$ and $F_c$ are the observed and calculated structure amplitudes of a reflection, respectively.

$^b R_{\text{free}}$ was calculated by randomly omitting 10% of the observed reflections from the refinement.

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**FIG. 2.** The EAS ATP binding site. Shown is the $\alpha$, weighted $|F_o| - |F_c|$ omit map contoured at 3σ showing the ATP1 (a) and ATP2 (c) binding sites in the EAS-ATP and EAS-ATP + CIT structures, respectively. Schematic representations of the interactions between ATP1 (b), ATP2 (d), and the protein is shown. The dashes represent electrostatic interactions between atoms with distances given in Å.
281 and Ser-287, the main chain deformation of these residues is slight. The only residue of this β-hairpin whose main chain and side-chain positions are significantly altered in the conformational change is that of Glu-284. In the EAS-ATP structure, the carboxyl group of Glu-284 makes two electrostatic interactions with Arg-407 and Arg-334, whereas in the EAS-ATP+CIT...
The enzymatic mechanism of EAS—Previous work has determined that the enzymatic mechanism of AS begins with the ordered binding of ATP, citrulline and aspartate (30). Once the first two substrates have bound to the protein, the nucleophilic attack by the ureido oxygen of citrulline on the \(\alpha\)-phosphate of ATP can occur, resulting in an activated citrulline-adenylate intermediate (Fig. 1) (31). Although formation of this intermediate does not require aspartate, the rate of intermediate formation has been shown to be enhanced in its presence (32). Together, the structures presented previously (13) and in this paper explain why both ATP and citrulline bind before aspartate and why the reaction between the two is enhanced by the presence of aspartate.

The citrulline binding site is a deep pocket at the interface of the nucleotide binding and synthetase domains into which citrulline binds in an extended conformation with its ureido group exposed at the opening (Fig. 6). The aspartate binding site, thus necessitating an ordered binding of the two substrates. Similarly, the binding site of aspartate also influences ATP binding. As demonstrated by the EAS-ATP model, in the absence of competition, the \(\gamma\)-phosphate of ATP binds to residues of the aspartate binding site. Assuming that these interactions are required for the efficient recruitment of ATP and that aspartate binding, like the CIT2 molecule, would disrupt these interactions, it is clear that the enzyme must bind ATP before aspartate.

The conformation of the triphosphate group of ATP also affects the ability of the enzyme to form the adenylated citrulline intermediate. It is most likely that substrate adenylation occurs by an \(S_2\) displacement mechanism, in which the nucleophilic attack of the citrulline ureido oxygen on one side of the \(\alpha\)-phosphate of ATP results in the concomitant release of pyrophosphate from the other. With the \(\gamma\)-phosphate of ATP bound at the aspartate binding site, the \(\beta\)-phosphate is situated between the \(\alpha\)-phosphate of ATP and the ureido oxygen of citrulline, preventing formation of the intermediate (Fig. 7). Upon aspartate binding, however, the \(\gamma\)-phosphate is displaced to interact with residues of the PP-loop. In this second conformation the \(\alpha\)-phosphate is exposed for nucleophilic attack by the ureido oxygen of citrulline, making it possible for catalysis to proceed. Thus the observed increase in the rate of adenylated citrulline formation in the presence of aspartate is explained.

In current structures of EAS, the intermediate formation is not possible due to the large distance between the citrulline and ATP. Originally, when ATP was modeled into the uncomplexed EAS structure, the \(\alpha\)-phosphate of ATP and the ureido oxygen of citrulline were estimated to be \(\sim 7.9\) \(\text{Å}\) apart (13). The conformational changes observed in the structures presented in this paper have reduced this separation to 5.8 \(\text{Å}\), which although considerably closer, is still too large a distance for catalysis to proceed. Although some of this distance could be spanned by a conformational change in citrulline, in the struc-
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The relative positions of the EAS substrates. Stereo view of the relative positions of the EAS substrates from the EAS-ATP (ATP1; faded), EAS-ATP+CIT (ATP2 and CIT1), and EAS-ASP+CIT (aspartate) structures. The binding of aspartate would result in a steric clash with ATP1 and thereby effect the conformational change to ATP2.

structures determined to date maximum extension of the molecule cannot bring the ureido oxygen of citrulline into van der Waals contact with the α-phosphate of ATP. Therefore we believe that the conformational changes observed within the nucleotide binding domain represent only the beginning of the movement that this domain undergoes to effect catalysis. We estimate that an additional 3–5° rotation of the nucleotide binding domain (without further conformational changes occurring) would bring the ATP sufficiently close to the ureido oxygen of citrulline for the adenylation reaction to proceed. Unfortunately, this additional conformational change is prevented from occurring in the I222 crystal form by intermolecular crystal contacts.

Although properly aligned for substrate adenylation, the conformation of the ATP2 in the EAS-ATP+CIT structure is different from the conformation of the ATP observed in the NAD+ synthetase ATP complex, the only other available N-type ATP pyrophosphatase structure available with ATP. ATP2 adopts a U-shaped conformation such that the α, β, and γ phosphates are linearly arranged (Fig. 8b), whereas the ATP complexed with NAD+ synthetase is S-shaped (Fig. 8c), with the triphosphate tail bent such that the pyrophosphate moiety can interact with the PP-loop. Interestingly, six of the eight interactions of the γ-phosphate of ATP2 with the PP-loop are analogous to those made by the β-phosphate of ATP in the NAD+ synthetase complex. Although the conformation of ATP2 is at odds with the ATP observed in complex with NAD+ synthetase, it is remarkably similar to the ATP molecules observed in the structures of several type II tRNA synthetases (33–37) (Fig. 8a). Type II tRNA synthetases catalyze similar substrate adenylation reactions but do not have a PP-loop homologous to that of the N-type ATP pyrophosphatases. Catalysis could therefore proceed with ATP in either of these conformations.

In similar adenylation reactions it has been suggested that a general mechanism for substrate adenylation may involve conserved arginine or lysine residues and two or three divalent cations that stabilize the pyrophosphate leaving group and the pentavalent transition-state intermediate formed during adenylation (38, 39). Although we have been unable to locate magnesium ions in the current EAS structures, such stabilizing ions have been identified in the other N-type ATP pyrophosphatases and are reported to be required for maximal AS activity (30). On the other hand, although the participation of a positively charged side chain has not yet been reported for other members of the N-type ATP pyrophosphatase family, in the EAS-ATP structure the 100% conserved residue, Arg-106, is in the vicinity of the α-phosphate of ATP and may be positioned for such a stabilization role.

The second nucleophilic attack, that of aspartate on the adenylated citrulline intermediate, appears to be relatively straightforward. Because of the proximity of the aspartate to the synthetase domain, the proposed conformational change of the nucleotide binding domain is not expected to significantly affect its position. Once the adenylated citrulline is formed the aspartate amino group appears to be well positioned for imme-

diate attack on the activated ureido carbon of the intermediate, thereby leading to the production of AS, AMP, and PPi. As suggested previously (13), the salt bridge between the carboxyl group of Asp-135 and the amino nitrogen of aspartate may serve to deprotonate the amino nitrogen before its nucleophilic attack on the ureido carbon of adenylated citrulline or to stabilize the positive charge accumulated on the nitrogen during the formation of argininosuccinate. In addition to this, modeling studies have shown that the extension of citrulline toward the α-phosphate of ATP can position both ureido nitrogens of citrulline within hydrogen-bonding distance of the carboxyl group of Asp-135. In this conformation, Asp-135 would serve to enhance the negative charge of the amino nitrogen nucleophile as well as the positive charge on its activated carbon target.

After catalysis is completed, the reported release of products from the enzyme is AS, PPi, then AMP (30). Given that the aspartate and citrulline binding sites are significantly less accessible than the ATP binding site and that AMP is only bound to the enzyme by a small number of interactions, it is peculiar that AS should be released from the enzyme first. Perhaps this discrepancy will be clarified with the structure of the fully closed EAS in complex with the adenylated citrulline intermediate or the reaction products.

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