Mechanism of Allosteric Inhibition of N-Acetyl-l-glutamate Synthase by L-Arginine*

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N-Acetylglutamate synthase (NAGS) catalyzes the first committed step in l-arginine biosynthesis in plants and micro-organisms and is subject to feedback inhibition by l-arginine. This study compares the crystal structures of NAGS from Neisseria gonorrhoeae (ngNAGS) in the inactive T-state with l-arginine bound and in the active R-state complexed with CoA and l-glutamate. Under all of the conditions examined, the enzyme consists of two stacked trimers. Each monomer has two domains: an amino acid kinase (AAK) domain with an AAK-like fold but lacking kinase activity and an N-acetyltransferase (NAT) domain homologous to other GCN5-related transferases. Binding of l-arginine to the AAK domain induces a global conformational change that increases the diameter of the hexamer by ~10 Å and decreases its height by ~20 Å. AAK dimers move 5 Å outward along their 2-fold axes, and their tilt relative to the plane of the hexamer decreases by ~4°. The NAT domains rotate ~109° relative to AAK domains enabling new interdomain interactions. Interactions between AAK and NAT domains on different subunits also change. Local motions of several loops at the L-arginine-binding site enable the protein to close around the bound ligand, whereas several loops at the NAT active site become disordered, markedly reducing enzymatic specific activity.

l-Arginine biosynthesis in most micro-organisms and plants involves the initial acetylation of l-glutamate by N-acetylglutamate synthase (NAGS, EC 2.3.1.1)² to produce N-acetylglutamate (NAG). NAG is then converted by NAG kinase (NAGK, EC 2.7.2.8) to NAG-phosphate and subsequently to N-acetylornithine (1, 2). Two alternative reactions are used to remove the acetyl group from acetylornithine. The linear pathway uses N-acetylornithine deacetylase (EC 3.5.1.16) to catalyze the metal-dependent hydrolysis of the acetyl group to form l-ornithine and acetate, whereas the acetyl recycling pathway transfers the acetyl group from N-acetylornithine to l-glutamate, producing l-ornithine and NAG. This reaction is catalyzed by ornithine acetyltransferase (EC 2.3.1.35).

In the linear pathway, NAGS is the only target of feedback inhibition by l-arginine. In contrast, in the acetyl cycling pathway l-arginine may inhibit NAGS and NAGK or ornithine acetyltransferase (3). Structure determinations of l-arginine-insensitive (4) and l-arginine-sensitive NAGKs (5) provided insights into the structural basis of l-arginine inhibition of NAGK. l-Arginine-insensitive Escherichia coli (ec) NAGK is a homodimer (4), whereas l-arginine-sensitive NAGKs from Thermotoga maritima (tm) and Pseudomonas aeruginosa (pa) are hexamers formed by pair-wise interlacing of the N-terminal helices of three ecNAGK-like dimers, to create a second type of dimer interface. l-Arginine binding to a site close to the C terminus induces global conformational changes that expands the ring by ~8 Å and decreases the tilt of the ecNAGK-like dimers relative to the plane of the ring by ~6°. The inhibition mechanism was proposed to involve the enlargement of an active site located close to the l-arginine-binding site.

Because of the sequence similarity between NAGK and NAGS, it was speculated that they may have similar l-arginine-binding sites and hexameric ring structures (5). However, our recent structural determination of NAGS from Neisseria gonorrhoeae (ng) revealed the active site to be located in the NAT domain, >25 Å away from the proposed l-arginine-binding site (6). Therefore, the allosteric mechanism of NAGS is likely to be different from that of l-arginine-sensitive NAGKs. Here we compare the structures of ngNAGS in the inactive T-state with l-arginine bound and in the R-state complexed with CoA and l-glutamate and determine the structural basis for the allosteric inhibition of NAGS by l-arginine.

EXPERIMENTAL PROCEDURES

Enzyme Production and Crystallization—Cloning, expression, purification, and crystallization of ngNAGS have been previously described (6). Briefly, the protein was expressed in...
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E. coli BL21(DE3) cells (Invitrogen) and purified with nickel affinity and DEAE columns (GE Healthcare). The best crystals were obtained by the hanging drop vapor diffusion method with crystallization buffer containing 100 mM ammonium citrate, pH 6.4, 8% polyethylene glycol 3350, 100 mM L-glutamate, and 25 mM CoA. L-Arginine-bound crystals were prepared with the same concentrations of CoA and L-glutamate and 100 mM L-arginine in the crystallization buffer. The crystals in the absence of L-arginine were hexagonal prisms, whereas those obtained in the presence of L-arginine were hexagonal plates.

Activity and Inhibition Assays—NAGS activity measurements were performed using a modification of the assay described by us previously (7, 8). The reaction was carried out at 30°C for 1 or 5 min in 100 μL of 50 mM Tris–HCl buffer, pH 8.5, containing 100 mM NaCl and 0.2 μg of enzyme (unless otherwise specified) and quenched with 100 μL of 30% trichloroacetic acid. The product, NAG, was quantified using liquid chromatography–mass spectroscopy. In the absence of L-arginine, AcCoA or L-glutamate concentration was varied in the range 0.5–140.0 mM, and 1.6 mM NAGS activity was measured over a range (0.01–10.0 mM) of L-arginine concentrations fixed at 15 or 4 mM, respectively (see Fig. 1, A and B). These titration data were fit to Michaelis–Menten kinetics using the program GUNPLOT. Inhibition of NAGS activity was measured over a range (0.01–10.0 mM) of L-arginine concentrations, and the concentrations of L-glutamate and AcCoA were fixed at 15 and 4 mM, respectively (see Fig. 1C). The data were fitted to the expression: 

\[ v = A + B \times \left(1 - \frac{[Arg]}{[Arg]_0} + \frac{[Arg]}{K_{[Arg]} + [Arg]}\right), \]

where \( v \) and \( A \) are activities at a given [Arg] and at [Arg] = 0; \( B \) is the reduction in activity caused by [Arg] = 0; \( I_{[Arg]} \) is the half-maximal activity [Arg]; and \( n \) is the Hill coefficient. The L-glutamate titration data measured in the presence of 0.1 and 1.0 mM of L-arginine were fit to sigmoidal kinetics, using the equation: 

\[ v = V_{max} \times \frac{[Glu]^{n}}{K_{[Glu]}^{+} + [Glu]^{n}}, \]

where \( v \) and \( V_{max} \) are activities at a given [Glu] and at [Glu] = 0; \( K_{[Glu]} \) is half-maximal activity [Glu]; and \( n \) is the Hill coefficient. In the presence of 0.1 mM of L-arginine, L-glutamate concentrations were varied between 0.5 and 24.0 mM. In the presence of 1.0 mM of L-arginine, L-glutamate concentrations were varied in the range 0.5–140.0 mM, and 1.6 μg of enzyme was used. At both L-arginine concentrations, AcCoA was fixed at 4 mM (see Fig. 1D). Protein concentration was determined using a commercial reagent (Bio-Rad) by the Bradford assay (9) with bovine serum albumin as standard.

Analytical Gel Filtration Chromatography—The molecular weight of ngNAGS with or without L-arginine (1.0 mM) was determined with a Superdex 200 HR 10/30 column (Amersham Biosciences) as previously described (6).

Data Collection and Processing—Diffraction data were collected from a single crystal at 100 K on beamline Southeast Regional Collaborative Access Team 22-ID equipped with MAR300 CCD at the Advanced Photon Source Argonne National Laboratory. The crystals were cryo-protected by supplementing the well solution with 30% glycerol. The data were indexed, integrated, and scaled with the software package HKL2000 (10) and reduced using the program TRUNCATE in the CCP4 suite (11). The CoA- and L-glutamate-bound crystals belonged to the trigonal space group P312 with unit cell parameters: \( a = b = 98.7, c = 89.8 \) Å. The L-arginine bound crystals were obtained in the presence of 1.0 mM of L-arginine, L-glutamate concentrations were hexagonal prisms, whereas those obtained in the absence of L-arginine were hexagonal plates.

### Table 1

| Data collection and refinement statistics | Data 1 | Data 2 |
|-----------------------------------------|--------|--------|
| **Data collection**                     |        |        |
| Bound ligands                           | CoA and L-glutamate | CoA and L-arginine |
| Space group                             | P312   | P312   |
| Wavelength (Å)                          | 1.0    | 1.0    |
| Resolution (Å)                          | 30.2–2.2 (2.29–2.21) | 30.2–2.56 (2.63–2.56) |
| Unit cell parameters (Å)                | \( a = b = 98.7, c = 90.1 \) | \( a = b = 107.1, c = 185.5 \) |
| Measurements                            | 248,975 | 125,545 |
| Unique reflections                      | 24,544 (1,836) | 35,747 (3,330) |
| Redundancy                              | 10.1 (4.6) | 3.5 (2.6) |
| Completeness (%)                        | 96.5 (72.9) | 91.2 (86.4) |
| \( R_{merge} (%) \)                     | 37.5 (1.3) | 13.2 (1.5) |
| \( R_{merge} \text{free} (%) \)        | 7.0 (73.4) | 11.5 (70.1) |

Structure Determination and Refinement—Coordinates of the CoA- + NAGS-bound ngNAGS structure (Protein Data Bank code 2BBG) were used for the phase determination of the CoA- + L-glutamate bound structure because they are isomorphous. The L-arginine-bound structure was solved by molecular replacement using the AcCoA-bound ngNAGS structure (Protein Data Bank code 2R8V) as the search model with the program Phaser (13). The search model was divided into the AAK domain (residue 5–284) and the NAT domain (residue 285–426). The two AAK domains and two NAT domains placed in an asymmetric unit with a log likelihood gain of 1640.2 automatically formed an ecNAGK-like dimer, confirming the correctness of the solution. The ring-like hexamer formed from three dimers by the crystallographic 3-fold axis supports the molecular replacement solution. Rigid body refinement with the program CNS (14) brought the \( R_{work} \) and \( R_{free} \) values to 42.1 and 42.2%, respectively, further confirming the structural solution.

The program COOT was used for viewing initial electron density maps (15). Bound CoA and L-glutamate were clearly visible in the crystals grown without L-arginine after rigid body refinement, and the ligands were built into the model. Similarly, L-arginine was clearly visible in a site close to the junction...
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between the ecNAGK-like dimers in the L-arginine-bound structure after rigid body refinement. The models were iteratively built with the programs COOT or O (16) and refined using the program CNS (14). Lastly, the structures were further refined by a combination of TLS and restrained refinement using program REFMAC5 (17). Translation/libration/screw groups were selected using the TLSMD server (18). The figures were drawn using the programs Situs 2.3 (19), VMD (20), and PyMOL (21).

RESULTS AND DISCUSSION

Biochemical Characterization of ngNAGS and Inhibition by L-Arginine—In the absence of L-arginine, recombinant ngNAGS displays Michaelis-Menten kinetics for both L-glutamate and AcCoA. L-Glutamate or AcCoA was varied in the range 0.5–6.0 and 0.05–0.5 mm, respectively, with AcCoA and L-glutamate fixed at 4 or 15 mm, respectively. C, dependence of enzyme activity at saturating substrate concentrations on the concentration of L-arginine between 0.01 and 10.0 mm. D, dependence of enzyme activity on the concentration of L-glutamate in the presence of 0.1 and 1.0 mm of L-arginine. L-Glutamate was varied between 0.5 and 24.0 mm in the presence of 0.1 mm L-arginine and between 0.5 and 140.0 mm in the presence of 1.0 mm L-arginine.

The data displayed in Fig. 1C clearly indicate that ngNAGS activity is inhibited by L-arginine. The inhibited enzyme has a residual specific activity of 4.4 ± 0.3 µmol/min/mg, a decrease of 86% compared with uninhibited activity, with a half-inhibition L-arginine concentration of 0.19 ± 0.01 mm. The inhibition appears to be cooperative with a Hill constant of 1.7 ± 0.1.

A titration curve for L-glutamate in the presence of non-saturating L-arginine (0.1 mm) and saturating L-arginine (1.0 mm) is shown in Fig. 1D. The apparent $K_m$ of L-glutamate increases to 6.6 ± 0.8 mm in the presence of 0.1 mm of L-arginine and 6-fold, to 27.5 ± 2.3 mm, in the presence of 1.0 mm of L-arginine. The apparent $V_{max}$ decreases to 48.6 ± 3.3 µmol/min/mg in the presence of 0.1 mm of L-arginine and 4-fold, to 15.8 ± 0.6 µmol/min/mg, in the presence of 1.0 mm of L-arginine. In addition, at both L-arginine concentrations, binding becomes cooperative, with a Hill coefficient of 1.5 ± 0.2, providing evidence for an allosteric transition from an R- to T-state. These data agree well with earlier results for $\rho_{NAG}$ and NAGS from other organisms (22–25). They clearly indicate that L-arginine alters both the affinity of the enzyme for L-glutamate and the catalytic efficiency of the enzyme, and thus are consistent with the conclusions drawn from the crystal structures.

Analytical gel chromatography analysis demonstrated that ngNAGS elutes as a symmetric peak, regardless of enzyme concentration, with or without L-arginine. The calculated molecular mass is ~330 kDa, indicating that the ngNAGS protein in solution is a hexamer as in the crystal.

Structure of D-Arginine-bound NAGS—The T-state structure complexed with CoA and L-arginine has two subunits in the asymmetric unit related by 2-fold noncrystallographic symmetry. Their structures are identical with rmsd of 0.59 Å for all 424 equivalent Ca atoms. The subunits interact via their AAK domains to form an ecNAGK-like dimer (4). A 3-fold crystallographic symmetry axis corresponds to a molecular 3-fold axis and generates a hexamer.

The structure refined at 2.6 Å resolution has 89.5% of the residues within the most favorable region in the Ramachandran plot, assessed with PROCHECK (26). Interestingly, residues 112–122, which were poorly defined in the R-state structure and therefore not included in the previous model, are now clearly visible. In contrast, the electron density for the loops that are involved in binding L-glutamate becomes poorly resolved. No electron density was visible for residue 308–311 and 422–425, and they were not modeled. No density for L-glutamate in its potential binding site could be seen, and the electron density for CoA is weak, reflecting partial binding to the active site. In contrast, clear electron density was observed for L-arginine, as shown in Fig. 2A.

Structure of NAGS with CoA and L-Glutamate Bound—Clear electron density can be seen for both CoA and L-glutamate (Fig. 2B). The N-terminal His6 tag, the first four amino residues of the protein, and residues 112–122 still have weak electron density, even though the resolution of the structure has improved to 2.2 Å, and were not modeled. 89.9% of the residues fall within the most favorable region in the Ramachandran plot, assessed with PROCHECK (26). This structure is basically identical to the previously determined structures of ngNAGS with AcCoA or CoA + NAG bound, with rmsd values of 0.50 and 0.43 Å, respectively. However, because this structure was refined at higher resolution than those previously determined, it will be used to compare the R-state and T-state structures.

Active Site and Catalytic Mechanism—L-Glutamate was found to bind to the same site as NAG in our previously reported structure (6). The CoA- and L-glutamate-bound ternary structure further confirms that L-glutamate binds in a site within the NAT domain. As seen with NAG, L-glutamate uses the side chains of Arg256, Arg416, Arg425, and Ser27 and the main chain atoms of Leu314, Cys456, and Leu399 for binding (Fig. 2B and Table 2). However, unique to the present structure, the attacking $\alpha$-amino group of L-glutamate forms hydrogen bonding interactions with the S atom of CoA and a water molecule (H2O43), which links it to the main chain O of Ile354, forming a perfect proton shuttle for the catalytic reaction. Compared with
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FIGURE 2. The α-arginine-binding site in the T-state structure (A), CoA and L-glutamate binding sites in the R-state structure (B), and amino acid sequences around α-arginine-binding site for NAGS sequences from X. campestris (C). Sequence alignment around α-arginine-binding site for NAGS sequences from N. gonorrhoeae, X. campestris, P. aeruginosa, zebrafish, and human; NAGK sequences from Pseudomonas aeruginosa and Arabidopsis thaliana; and bifunctional NAGS/K sequences from X. campestris and Maricaulis maris. Fully conserved residues are highlighted in pink, and partially conserved residues are in blue.

TABLE 2

| Ligand atoms | Protein or CoA atoms | Distance | Å |
|--------------|----------------------|----------|---|
| CoA          | SBV                  | Ser392 OG| 2.83 |
|              | NBS                  | Leu307 O | 2.39 |
|              | OBR                  | Thr305 OG1 | 2.60 |
|              | OBM                  | Val303 N | 3.13 |
|              | OBK                  | Glu304 OE1 | 2.82 |
|              | OBD                  | Asp305 N | 3.07 |
|              | OBC                  | Gly304 N | 3.14 |
|              | OAZ                  | Glu307 N | 3.15 |
|              | OAY                  | Gly307 N | 2.75 |
|              | O4*                  | Thr306 NE1 | 2.96 |
|              | O3*                  | Arg314 NH2 | 3.15 |
|              | OAS*                 | Arg314 NH2 | 2.96 |
|              |                     | Lys323 NZ | 2.69 |
|              | N1                   | H2O61 OH2 | 2.76 |
|              | N6                   | Asn394 O | 3.75 |
| Glutamate    | O                    | Arg216 NH2 | 3.35 |
|              |                     | Cys216 N | 2.69 |
|              | OXT                  | Arg156 NH1 | 2.63 |
|              |                     | Leu154 N | 3.02 |
|              | OE1                  | Arg156 NH2 | 3.07 |
|              | OE2                  | Ser307 OG | 2.08 |
|              | N                    | Arg375 NH2 | 3.09 |
|              |                     | H2O13 OH2 | 2.77 |
|              |                     | CoA1 SBV | 3.32 |

* Lys181 and Arg216 are residues from the K domain of an adjacent monomer (symmetry operation: −y+1, x, −y, z).

The α-arginine binding we defined previously, small differences can be observed because of the higher resolution of the present data. For example, the N-1 atom of CoA interacts with the main chain N of Glu397 via a water molecule (H2O61) in the present model, rather than with the side chain of Glu397, as indicated in the previous model.

Superimposition of the AcCoA-bound structure with the present structure indicates that the attacking α-amino nitrogen atom of L-glutamate is only 3.1 Å distant from the carbon atom of the acetyl group of AcCoA. The present structure further confirms that the enzyme uses a one-step direct transfer in the catalytic reaction.

Allosteric L-Arginine-binding Site—L-Arginine was found to bind to the AAK domain, and L-arginine and the surrounding protein structure are well defined (Fig. 2A). L-Arginine is surrounded by the central β-sheet (strands B13, B17, and B18), the C-terminal segment of the N-terminal helix, and the loop connecting helix H9 and B18 (residues 270–280). The conformation of this loop is maintained by hydrogen bond interactions of the side chain of Thr273 with the main chain O of Glu269, and the side chain of Glu270 with the main chain O of Ile277. These interactions orient several main chain O atoms in this loop toward the site, enabling them to hydrogen bond with L-arginine (Table 3). Residues Thr277 and Glu270 are highly conserved across phyla (Fig. 2C).

On the other hand, there are only three residues, Lys201, Gln257, and Glu270, whose side chains are involved in binding L-arginine. However, Met21 and Tyr17, located near the amino group, and Ser280 and Glu334, located around the side chain of L-arginine, may also be important for L-arginine binding. The location and orientation of the L-arginine binding in ngNAGS is the same as that identified in tmNAGK (5), and conservation of the L-arginine site between NAGS and NAGK is supported by site-directed mutagenesis studies of recombinant polNAGS (25), mouse NAGS and bifunctional Xanthomonas campestris (xc) NAGS/K (27). Mutation of key residues binding L-arginine significantly decreased inhibition but had little effect on the kinetic parameters of the protein.

L-Arginine Binding Tightens Its Binding Site, Propagating a Signal to the N-terminal Helices—Superimposition of AAK domains (residues 5–284) from the R-state and T-state structure results in a 0.76 Å rmsd for 267 equivalent Cα atoms. The major differences are close to the L-arginine-binding site (N-terminal helices, H9–B18 L-arginine-binding loop, B14–B15 loop, and H4–B5 loop). When L-arginine binds, these elements move to tighten the protein structure around the bound ligand (Fig. 3A). The H9–B18 L-arginine-binding loop and the C-terminal portion of the N-terminal helix move ~1.7 and ~1.0 Å toward the site, respectively. As a result, the side chain conformations of numerous residues change. The phenolic ring of Try17, which points out from the site in the R-state, now swings
into the site to accommodate the Cα atom of l-arginine. The side chain of Arg274 moves to form a close contact with the side chain of Phe117, which is disordered in the R-state. The signal seems to propagate to the N-terminal helix, which moves 2–4 Å (Fig. 3A). Surprisingly, the l-arginine-induced B14-B15 loop movement in ngNAGS is in a direction opposite to that in NAGK (5).

l-Arginine Binding Changes the Conformation of l-Glutamate-binding Loops—The rmsd between NAT domains in two different orientations in R-state (green trace) and T-state (red trace) structures. Bound l-arginine is shown as pink sticks. The conformations of the l-arginine binding loop (H9-B18 loop), N-terminal helix, and B14-B15 loop are significantly different. B, stereo view of the superimposition of NAT domains of the R-state (green trace) and T-state (red trace) structure. The conformation of l-glutamate binding loops (H10-H11 and H14-B25) and the B20-B21 loop are significantly different. Bound CoA and l-glutamate in the R-state structure are shown as pink and yellow sticks, respectively.

FIGURE 3. Comparison of R- and T-state AAK and NAT domains. A, superimposition of AAK domains in two different orientations in R-state (green trace) and T-state (red trace) structures. Bound l-arginine is shown as pink sticks. The conformations of the l-arginine binding loop (H9-B18 loop), N-terminal helix, and B14-B15 loop are significantly different. B, stereo view of the superimposition of NAT domains of the R-state (green trace) and T-state (red trace) structure. The conformation of l-glutamate binding loops (H10-H11 and H14-B25) and the B20-B21 loop are significantly different. Bound CoA and l-glutamate in the R-state structure are shown as pink and yellow sticks, respectively.

FIGURE 4. Comparison of R- and T-state subunit structures. A, ribbon diagram of T-state (left panel) and R-state (right panel) monomer. B, detailed view of the interactions between NAT and AAK domains within the same subunit in the T-state structure. The ribbons are shown in rainbow colors from blue (N-terminal) to red (C-terminal). C, ribbon diagram of subunit 1:subunit 5 dimer interface in T-state structure (left panel) and R-state structure (right panel). Bound l-arginine and l-glutamate are represented as space-filling models. Bound CoA is shown as light blue sticks.

| Table 3: Interactions between l-arginine and protein atoms |
|----------------------------------------------------------|
| Arginine atoms | Protein atoms | Distance (Å) |
|----------------|---------------|--------------|
| N              | Glu270 O      | 2.76         |
| Lecu273 O      | 2.71          |
| Thr274 O       | 3.19          |
| O              | Lys291 NZ     | 2.91         |
| Glu293 O       | 2.83          |
| OXT            | Lys291 NZ     | 2.67         |
| Glu293 NE2     | 3.10          |
| NE             | Glu272 O      | 2.95         |
| Lys201 NZ      | 3.11          |
| Asn279 O       | 2.85          |
| Glu293 O       | 3.01          |
| Asn275 O       | 2.95          |
| Glu293 O       | 2.97          |
| NH1            | Gly278 O      | 3.00         |
| Asn275 O       | 2.79          |
| NH2            | Gly278 O      | 2.66         |
| Ser280 N       | 3.19          |
|                | Ser280 N      | 3.15          |
actions that are absent in the R-state. L-Arginine binding appears to "glue" the two domains together. Residues that interact directly in the T-state structure include Arg255–Asp334, Tyr17–Asn336, and Arg274–Gln362 (Fig. 4B). His333 and L-arginine interact via a water molecule, and several other water-mediated interactions between two domains have been identified. Domain-domain interactions are further enhanced by the H4-B5 loop (residue 112–122) of the adjacent subunit on the opposite trimer (Fig. 4C). This loop, which is disordered in the R-state structure, becomes ordered and binds in the cleft between the AAK and NAT domains.

**L-Arginine Binding Enhances K1:K5-type Dimer Interactions in the Hexamer, but Does Not Alter K1:K4-type Interactions**

As in the R-state structure, the stacked trimer rings in the T-state hexamer are linked by two types of AAK dimer interfaces: K1:K5-type (formed by interlacing their N-terminal helices) and K1:K4-type (analogous to those found in the ecNAGK dimer) (Fig. 5A). Binding of L-arginine alters K1:K5-type interfaces, but not K1:K4-type interfaces.

In the T-state structure, K1:K5 interfaces are enlarged, and the changes in their position, orientation, and contact area appear to be the primary trigger for the large quaternary structure alteration that results from L-arginine binding. In addition to the N-terminal helices, the H4-B5 loops (residues 112–122), H10 helices (residue 299–305), and residues 336–337 from the NAT domains are involved in interactions at this interface in the T-state (Fig. 4C). The buried surface area of this interface, calculated with a probe radius of 1.4 Å using program AREAIMOL in the CCP4 suite (11), increases from 1340 Å² in the R-state to 2205 Å² in the T-state.

In contrast, binding of L-arginine has no effect on interactions at K1:K4-type interfaces. However, as discussed in detail below, these dimers change their position relative to both the 2- and 3-fold molecular axes of the molecule.

**L-Arginine Binding Induces Major Quaternary Changes in the Hexamer**

When L-arginine binds, the hexamer contracts ~20 Å along its 3-fold axis, and its internal cavity width increases by ~10 Å (Fig. 5, A and B). As mentioned above, this global conformational change is driven by changes in the orientation of the AAK domain K1:K4 dimers, which rotate around their 2-fold axis, increasing their tilt relative to the 3-fold molecular axis, and moving outwards along their 2-fold axes. To quantify this rotation, the tilt angle of the dimer relative to the hexameric plane was calculated as the angle of the centroid vector between the center of mass for the common β-sheets core in K1 and K4 domains relative to the hexamer plane. When L-arginine binds, the tilt angle decreases ~4°, from 39.7° to 36.0°, causing the molecular envelope of the dimer to move outwards along its 2-fold axis by ~5 Å.

The magnitudes of these changes are comparable with those found in L-arginine-sensitive NAGK structures and appear to be driven by the changes in orientation of the N-terminal heli-
ces (5) and K1:K5-type interface. Tilt angles of all known NAGK and NAGS-like structures are compared in Table 4.

**L-Arginine Binding Changes the Interactions between AAK and NAT Domains in Adjacent Subunits within a Trimer**—The interdomain rotation in the monomer induced by L-arginine binding also alters the K2:S1-type interactions between the AAK and NAT domains of adjacent subunits within the trimeric rings of the hexamer. Plausible hydrogen bonding interactions in the R- and T-states are listed in Table 5 and shown in Fig. 5C.

The superficial stem loop (residues 134–145) of the AAK domain appears to play a key role in maintaining the NAT domain of the adjacent subunit in a catalytically active form. In all three R-state structures, this loop interacts with two well-defined loops in the NAT domain of the adjacent subunit (residues 392–401 and 425–427) that contain residues that form part of the active site. Arg151, Ser407, and Leu230 are involved in binding of L-glutamate, whereas Asp249 and Trp498 are involved in the binding of CoA.

In the T-state structure, the superficial stem loop interacts with residues 322–323 in the NAT domain, and the interactions with loops 392–401 and 425–427 are eliminated. As a result, two regions that bind L-glutamate in the R-state (residues 308–311 and 422–425) become disordered, reducing the affinity of the protein for L-glutamate. L-Glutamate titration data in the presence of saturating L-arginine concentrations (1.0 mM) confirmed reduced affinity of L-glutamate ($K_m$ 17.5 ± 2.3 mM) in comparison with the absence of L-arginine ($K_m$ 3.5 ± 0.3 mM).

**The Mechanism of the Allosteric Transition and L-Arginine Inhibition**—As outlined above, binding of L-arginine to the AAK domain induces a series of structural changes that ultimately transmit a signal to the active site in the NAT domain. Key local changes include the tightening of the L-arginine-binding site that alters the position of the H9-B18 L-arginine binding loop and, in turn, the orientation of the adjacent N-terminal helix. These changes propagate to the hexamer, causing the K1:K4-type dimers to rotate and enlarge the hexameric ring. The local changes and the rotation of the dimer are similar to those observed for L-arginine-sensitive NAGK (5). However, whereas in NAGK, the signal is transmitted to an active site within the same domain, in NAGS, the catalytic site is within the NAT domain, and structural changes specific to the NAT domain are required.

Therefore, in addition to the structural changes described above, L-arginine binding increases the interactions between AAK and NAT domains within the same subunit. These results indicate that binding of L-arginine causes many direct and water-mediated interactions between the AAK and NAT domains of the same subunit. The thin neck that links the two domains in the R-state becomes thicker, and the ordering of the H4-B5 loop from an adjacent subunit creates additional interactions between the two domains and between adjacent dimers. As a result, the orientation of the NAT domain relative to the AAK domain of the same subunit changes substantially. In addition, the interactions of the AAK domain with the NAT domain of the adjacent subunit in the trimer change. These changes, together with the alteration in the intrasubunit NAT-AAK interactions, cause the L-glutamate-binding loops (H10-H11 and H14-B25) to become disordered and less able to bind L-glutamate to carry on the catalytic reaction.

These structural studies indicate that the AAK domain plays two roles. Its first role is to form an R-state hexamer that promotes the catalytic reaction via inter-subunit AAK-NAT domain interactions that maintain the L-glutamate-binding loops in the correct conformation. A short version of NAGS from *Mycobacterium tuberculosis* has an extremely low affinity for L-glutamate, probably because it lacks the AAK domain (28). The second role is to reduce catalytic activity in the presence of L-arginine. As shown here, binding of L-arginine switches the AAK domain into a T-state conformation. This signal is transmitted to the NAT domain by a series of structural changes that ultimately result in enzyme inactivation by destabilizing the L-glutamate-binding loops.

**Structural Basis for L-Arginine-resistant Mutants**—Several L-arginine-resistant mutants of ecNAGS (H15Y, Y19C, S54N, R58H, G287S, and Q432R) have been previously identified by our group (29). These residues correspond to the *ng*NAGS sequence of Glu13, Tyr17, Gln52, Arg56, Gly278, and Asn423, respectively.

Because Glu13 and Tyr17 belong to the N-terminal helix and Gly278 belongs to the H9-B18 L-arginine-binding loop, mutating these residues would be expected to eliminate L-arginine inhibition, because they are part of the L-arginine-binding site. Gln52 is located at the C-terminal end of H2 and close to the C-terminal end of the N-terminal helix from the adjacent subunit. It interacts with the H4-B5 loop (residues 112–122) in the L-arginine-bound T-state structure. This residue appears critical in mediating the L-arginine-induced signal via the H4-B5 loop to the NAT domain. Arg56 forms a salt bridge interaction with Asp423 near the K1:K4 AAK dimer interface. This interaction may be important for R- and T-state structural conversion.

| R-state structure | T-state structure |
|-------------------|-------------------|
| **AAK domain** | **NAT domain** | **Distance** | **AAK domain** | **NAT domain** | **Distance** |
| Ile216 O | Asn298 OD1 | 2.60 | Arg124 NE | Asn213 OD1 | 3.23,2.94 |
| Val218 N | Thr358 O | 2.66 | Pro355 O | Asn233 ND2 | 3.17,3.04 |
| Val218 O | Thr358 OG | 2.75 | Arg122 NH2 | Glu222 OE2 | 2.67,2.42 |
| Asp213 OD2 | His429 NE2 | 2.56 | Arg122 NH2 | Glu222 O | 3.25,3.25 |
| Arg211 NH1 | Glu401 OE2 | 3.30 | Ser419 OG | Glu222 OE1 | 3.51,3.45 |
| Arg211 NH2 | Glu401 OE1 | 3.54 | Gly478 N | Glu222 OE1 | 2.83,2.93 |

The values are for two subunits (subunit A and B) in the asymmetrical unit, respectively.

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The basis of the effect of the Q342R mutation is less clear. One possibility is that it offsets the destabilization of the L-glutamate binding loops produced by L-arginine binding.

Conservation of NAGS l-Arginine-binding Site across Phyla—
The sequence around the L-arginine-binding site (residues 270–280) is highly conserved across classic NAGS, vertebrate-like bacterial bifunctional NAGS/K and vertebrate NAGS (Fig. 2C). The L-arginine-binding sequence signature, E(I/L)(F/M)(T/S)XGTXGTX, can be used not only to identify L-arginine-sensitive NAGK (5) but also to identify L-arginine-binding sites in NAGS proteins across phyla. Mutagenesis studies of this region in mouse NAGS and bifunctional xcNAGS/K (27) and paNAGS (25) support the universality of the L-arginine-binding site in NAGS and NAGK. The conservation of this site in mammalian NAGS is noteworthy, because L-arginine activates mammalian NAGS.

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