Structural Basis for the Regulation of N-Acetylglutamate Kinase by PII in Arabidopsis thaliana

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PII is a highly conserved regulatory protein found in organisms across the three domains of life. In cyanobacteria and plants, PII relieves the feedback inhibition of the rate-limiting step in arginine biosynthesis catalyzed by N-acetylglutamate kinase (NAGK). To understand the molecular structural basis of enzyme regulation by PII, we have determined a 2.5-Å resolution crystal structure of a complex formed between two homotrimers of PII and a single hexamer of NAGK from Arabidopsis thaliana bound to the metabolites N-acetylglutamate, ADP, ATP, and arginine. In PII, the T-loop and Trp22 at the start of the α-helix, which are both adjacent to the ATP-binding site of PII, contact two β-strands as well as the ends of two central helices (αE and αG) in NAGK, the opposing ends of which form major portions of the ATP and N-acetylglutamate substrate-binding sites. The binding of Mg2+-ATP to PII stabilizes a conformation of the T-loop that favors interactions with both open and closed conformations of NAGK. Interactions between PII and NAGK appear to limit the degree of opening and closing of the active-site cleft in opposition to a domain-separating inhibitory effect exerted by arginine, thus explaining the stimulatory effect of PII on the kinetics of arginine-inhibited NAGK.

PII (GlnB) is now recognized as one of the most ancient and conserved signal transduction proteins known, with orthologs spread across the three domains of life (1, 2). Originally discovered as a factor necessary for the inactivation of Escherichia coli glutamine synthetase, PII is now known to play roles in the regulation of gene transcription, enzyme activity, and membrane function, all in response to cellular carbon, nitrogen, and energy status (3–6). PII interprets the metabolic status of the cell by directly binding to ATP and 2-ketoglutarate, and in certain bacteria, nitrogen status is sensed via covalent modification of PII. PII then directly interacts with a variety of proteins to regulate their function in response to the detected metabolic conditions.

A eukaryotic PII protein has been discovered in several algae and higher plants. Arabidopsis thaliana PII is >50% identical to proteobacterial and cyanobacterial PII (7), but unlike these orthologs, it does not appear to be regulated by phosphorylation or uridylylation in response to nitrogen metabolites (8). Plant PII has a conserved chloroplast transit peptide that is cleaved upon entry into the chloroplast, where PII performs its function. PII knock-out plants display altered carbon and nitrogen metabolite levels as well as increased sensitivity to nitrite (9).

To date, the sole interacting protein discovered for plant PII is the chloroplast enzyme N-acetylglutamate kinase (NAGK), which catalyzes the second and rate-limiting step in the pathway of arginine biosynthesis (10). We (11) and others (12) have performed enzyme kinetics to define the role of PII in NAGK activity: although PII activates NAGK slightly, the primary purpose of binding appears to be relief of feedback inhibition by the downstream product arginine. Quite elegantly, the binding of PII changes the kinetics of NAGK from sigmoidal to hyperbolic in the presence of arginine. A similar form of regulation is also found in cyanobacteria (13–15).

Recently, we solved the crystal structure of the first eukaryotic PII protein from A. thaliana (16). As in bacteria, plant PII forms a trimer with three protruding T-loops on one surface as well as a plant-specific N-terminal extension on the opposing surface that may be involved in protein-protein interactions and signaling. We have now determined the structure of A. thaliana PII bound to NAGK. This structure reveals for the first time the molecular mechanism underlying the regulation of an enzyme by PII.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—PII was expressed and purified as described previously (16). The NAGK gene from A.
**Structure of the PII-NAGK Complex**

*thaliana* was amplified by PCR using primers 5′-GGGAATTCCATATGACGTATCACACCCT-3′ and 5′-GAAGATCTTTTATGACGTATCACATGTTCCAGC-3′, Vent polymerase (New England Biolabs), and the His-tagged NAGK expression clone in the pRSET-A plasmid (Invitrogen) as a template (11). The amplified product was digested with NdeI and BamHI and then ligated to pET-3a (Novagen) digested with the same enzymes. The expression plasmid was transformed into Rosetta-gami pLysS cells (Novagen).

Four 1-liter cultures of LB medium supplemented with ampicillin and chloramphenicol were induced with 0.2 mM isopropyl β-D-thiogalactopyranoside and grown for 20 h at 25 °C. Cells were centrifuged, resuspended in 160 ml of Buffer A (20 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.5 mM EDTA, 0.5 mg of DNase, and 0.25 mM phenylmethylsulfonyl fluoride), and lysed by sonication. The extract was clarified by centrifugation, diluted 1:5 with Buffer B (20 mM NaHEPES (pH 7.0), 1 mM NaEDTA, 0.2 mM dithiothreitol, and 10% glycerol), dialyzed containing 20 mM NaCl. NAGK was eluted at 0.15 M NaCl with a 0.02–1 M NaCl linear gradient and loaded onto a 5-ml HiTrap Q HP anion exchange column (Amersham Biosciences) equilibrated with 100 mM NaCl, 10 mM Tris-Cl (pH 8.0), 1 mM NaEDTA, 2 mM dithiothreitol, and 10% glycerol), and loaded at 1 ml/min onto a blue dye column (5 × 2.5 cm; Bio-Rad) equilibrated with Buffer B containing 1.2 m NaCl. Peak fractions were eluted using Buffer B containing 1 ml/min onto a 5-ml HiTrap Q HP anion exchange column (Amersham Biosciences) equilibrated with Buffer C containing 20 mM NaCl. NAGK was eluted at ~0.15 m NaCl using a 0.02–1 m NaCl linear gradient and loaded onto a Superdex 200 10/300 GL gel filtration column (Amersham Biosciences) equilibrated with 100 mM NaCl, 10 mM Tris-Cl (pH 8.0), 1 mM NaEDTA, 2 mM dithiothreitol, and 5% glycerol. The purity of NAGK was assessed by SDS-PAGE, and the protein was purified to homogeneity using Amicon Ultrafree-15 centrifugal concentrators.

**Structure Determination of the PII-NAGK Complex**—NAGK and PII were independently concentrated and mixed at a 2:1 mass ratio to produce a mixture containing 40 mM l-arginine, 20 mM MgCl₂, 10 mM ADP, 10 mM N-acetylglutamate, 4 mg/ml NAGK, and 2 mg/ml PII. Protein concentrations were determined using the Bradford dye binding assay (Bio-Rad) (17). Crystals were grown using the hanging drop vapor diffusion method at room temperature by mixing 2 μl of the protein mixture with an equal volume of reservoir solution containing 8.5% (w/v) polyethylene glycol 8000, 8.5% (w/v) polyethylene glycol 1000, 0.1 mM NaHEPES (pH 7.0), 0.4 mM trimethylamine N-oxide, 50 mM l-arginine, 2 mM dithiothreitol, and 12% (w/v) glycerol. This mixture was seeded with small crystals from a previous crystallization trial under the same conditions and equilibrated against 1 ml of reservoir solution. The crystallizations were dehydrated by adding 50 μl of 80% (w/v) glycerol to the well solution on days 7 and 15 after the initial setup. On day 17, 100 μl of 80% (w/v) glycerol was added. On day 18, a single crystal (cubic shape with edges of ~0.15 mm) was mounted on a polyimide loop (MiTeGen) and flash-cooled at 100 K with a nitrogen gas stream (Oxford Cryostream). The crystal was stored in liquid nitrogen and transferred to Canadian Light Source beamline CMCF-1 08-ID-1 for data collection using a MarCCD detector. Data were measured on 331 images with 1-s exposures and 0.5° rotational steps. Autoindexing, examination of systematic absences, and scaling statistics indicated that the most likely space group was P2₁,3, with unit cell parameters a = b = c = 171.13 Å and α = β = γ = 90°. Data were processed and scaled using HKL2000 (18) and programs from CCP4 (Version 6.0) (19). Crystallographic statistics are summarized in Table 1. The relatively high Rsym values may be due in part to the high level of data redundancy (20).

The structure of the PII-NAGK complex was determined with the molecular replacement method using the structure of PII from *A. thaliana* (100% sequence identity; Protein Data Bank code 2O66) (16) and the structure of NAGK from *Pseudomonas aeruginosa* (~45% sequence identity; Protein Data Bank code 2BUF) (21) as search models. The solvent content of the crystal was calculated to be 73% (Vₘᵢₙ = 1.9 Å³/Da) if two PII and two NAGK protomers are present in the asymmetric unit (22). Molecular replacement calculations were carried out using PHASER (23), yielding only a weak solution for the placement of two copies of the NAGK search model (z = 6.0). After refining and fixing the solutions of these two NAGK protomers, a very strong solution for the placement of two copies of the PII protomer (z = 33) was obtained, allowing for the calculation of good electron density maps for model building (R = 0.37 and dᵣₘᵢₙ = 2.6). REFMAC (Version 5.2.0019) (24) was used for

**Table 1Crystallographic statistics**

| Table 1 Crystallographic statistics |
|-------------------------------------|
| Data collection                     |
| Space group                         |
| Unit cell lengths (Å)               |
| Unit cell angles (Å)                |
| Wavelength (Å)                      |
| Resolution (Å)                      |
| High resolution (Å)                 |
| Total reflections                   |
| Unique reflections                  |
| Completeness (%)                    |
| Rmerge (%)                          |
| Rfree (%)                           |
| Refinement                          |
| No. of atoms                        |
| Protein                             |
| Ligand                              |
| Solvent and ions                    |
| r.m.s. deviations from ideal geometry |
| Bond lengths (Å)                    |
| Bond angles (°)                     |
| Average temperature factors (Å²)    |
| Wilson plot                         |
| Protein                             |
| Ligand                              |
| Water                               |
| Ramachandran plot (% residues in regions defined by PROCHECK (26)) |

Values from the outermost resolution shell are given in parentheses.

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refinement calculations, and Xfit (25) was used to build models into electron density maps. PROCHECK (26) was used to evaluate the stereochemical parameters in the models. Some residual electron density was noticed near the thiol group of Cys65 in both protomers of NAGK. This electron density may signify the presence of a covalent adduct for which we were unable to devise a sensible model.

RESULTS

Structure of the PII-NAGK Complex—To understand the molecular mechanisms of enzyme regulation by PII, we determined the three-dimensional structure of a complex formed between two homotrimers of PII and a single hexamer of NAGK. The structure of the PII-NAGK complex was solved by molecular replacement using the structures of PII from A. thaliana (Protein Data Bank code 2O66) (16) and NAGK from P. aeruginosa (Protein Data Bank code 2BUF) (21) as search models. Although many different crystal forms were obtained under a wide range of conditions, well diffracting crystals were obtained only in the presence of Mg2+, ADP, N-acetylglutamate, and arginine. All four of these ligands are bound to NAGK and are well defined in electron density maps. Surprisingly, ATP and not ADP is clearly bound to PII. Because no ATP was added during purification or crystallization, the ATP was likely bound to PII when the protein was produced from E. coli.

The asymmetric unit of the PII-NAGK complex crystal (space group P213) contains two protomers of NAGK and two protomers of PII. A crystallographic 3-fold rotational symmetry axis coincides with the molecular 3-fold axis of the complex to generate the dodecameric complex that forms in solution (Fig. 1). The overall dimensions of the complex are ~115 × 105 × 105 Å, with the longest dimension spanning along the 3-fold rotational axis reaching from the N-terminal segments of one PII trimer to the N-terminal segments of the second PII trimer.

Each PII trimer forms intimate interactions with opposing faces of the NAGK hexamer, with each PII protomer contacting a single separate NAGK protomer (Fig. 2 A). Only two short stretches of PII contact NAGK: Trp22 at the end of the α1-helix and residues in the T-loop of PII contact residues on helices E and G as well as strands β6 and β7 on the side of NAGK opposite the active-site cleft. A relatively small amount of solvent-accessible surface area (SASA) (532–616 Å2) for each PII protomer is buried upon complex formation, and each PII trimer appears to bind independently on opposite faces of the NAGK hexamer. This constitutes 7% of the total SASA for each PII protomer and 5% of the total SASA for each NAGK protomer. Given the extensive interactions between separate protomers in each PII trimer and NAGK hexamer, however, the total amount of SASA (1600–1850 Å2) buried upon binding of a single PII trimer to the NAGK hexamer represents 11% of the total SASA for the PII trimer and 3% for the NAGK hexamer. Each PII trimer binds independently on opposite faces of the NAGK hexamer.

The structures of the two PII trimers in the complex appear to be very similar to the structure of free PII, with the exception of the T-loop (residues 49–66) and the C-terminal segment.
the side chain of the plant-specific Trp\textsuperscript{22} at the N terminus of the first α-helix of PII forms a hydrogen bond with the main chain carbonyl group of Ala\textsuperscript{264} of NAGK as well as van der Waals contacts with Gln\textsuperscript{265} and Gly\textsuperscript{266} (Fig. 2C).

The PII-NAGK complex also reveals that the NAGK hexamer consists of dimers arranged about a 3-fold rotational axis, as seen previously in the bacterial and archaeal NAGK hexamers from \textit{P. aeruginosa} and \textit{Thermotoga maritima} (21). Each NAGK protomer adopts an αβ-fold characteristic of the amino acid kinase family, consisting of an N-terminal domain that binds \(N\)-acetylglutamate and a C-terminal domain that binds the substrate ATP (21, 27). Each protomer is related to adjacent protomers through 2-fold rotational axes lying at two distinct interfaces, A and B. Interface A is formed primarily by packing interactions between the edge of the central 8-stranded β-sheet, an adjacent loop, and two flanking α-helices (Fig. 1C). Interface B is formed between the N-terminal helices of adjacent protomers (Fig. 1D). Interface A appears to be found in all bacterial and archaeal NAGK enzymes, but interface B and the N-terminal helix seem to be found only in organisms in which NAGK exists as a hexamer and arginine acts as an allosteric inhibitor (21), as in plants (11). As seen previously in the NAGK hexamers from \textit{P. aeruginosa} and \textit{T. maritima}, arginine binds near the C-terminal end of the N-terminal helix and may mediate inhibition by stabilizing an open conformation of NAGK (Fig. 1D), as described further below.

\textbf{Structural Basis for the ATP-dependent Binding of NAGK by PII}—The structure of the PII-NAGK complex reveals for the first time the molecular basis for the regulation of an enzyme by PII. In bacteria, Archaea, and plants, PII regulates key metabolic enzymes by integrating signals dependent on the cell’s energy, carbon, and nitrogen status. Energy status appears to be reflected in PII proteins primarily through the binding of ATP. The PII-NAGK complex strikingly reveals the molecular mechanism through which ATP binding modulates the regulation of an effector enzyme.

The binding of ATP to PII clearly favors a conformation of the T-loop that interacts with NAGK. First, the adenosine base
of ATP exerts important changes on the structure of PII and its interactions with NAGK. The base lies in a hydrophobic pocket that is occupied by Met126 in the absence of ATP (Fig. 3). Displacement of Met126 from the ATP-binding pocket allows the movement of the C-terminal segment away from an area occupied by the T-loop in the PII-NAGK complex. As noted previously, Met126 is perfectly conserved in plants and lies in a C-terminal segment found only in plant PII (16). The coupled changes in the conformations of the C-terminal segment and the T-loop may be essential components of an ATP-dependent switch found only in plants. In prokaryotic PII, the binding of the adenosine base may displace a shorter C-terminal segment in a similar manner that is also likely involved with conformational signaling.

The triphosphate group of ATP also plays a key role in promoting a conformation of the T-loop that favors interactions with NAGK. The triphosphate group coordinates Mg$^{2+}$ in conjunction with the main chain carbonyl oxygen of Gly48 at the base of the T-loop to promote the conformation of the T-loop required for interactions with NAGK (Fig. 3). Notably, Gly48 is conserved in PII from bacteria, Archaea, and all eukaryotes (supplemental Fig. S2). A previous mutagenesis study in E. coli has also revealed that the replacement of the equivalent residue, Gly37, with Ala can disrupt PII interactions with bacterial receptors as well as the binding of 2-ketoglutarate and ATP (28).

The binding of the triphosphate moiety of ATP in A. thaliana PII differs significantly from the metal-independent modes of ATP-binding seen in PII-ATP complexes from E. coli (29, 30) and Thermus thermophilus (31) as well as GlnK-ADP-AmtB complexes from E. coli (32, 33). However, the Mg$^{2+}$-dependent mode of ATP binding is similar to the metal-dependent binding mode seen in PII from Methanococcus jannaschii (34). The positions of the triphosphate moiety as well as Mg$^{2+}$ in both A. thaliana and M. jannaschii superimpose closely. However, the orientation of the carbonyl oxygen of Gly48 coordinating Mg$^{2+}$ in A. thaliana differs dramatically. In M. jannaschii, the peptide bond following Gly48 is flipped in the opposite direction, and the carbonyl oxygen does not interact with Mg$^{2+}$. These structural observations indicate that the binding of Mg$^{2+}$ to the phosphate groups of ATP may contribute to the effects of ATP binding on the structure of the receptor-binding surface of PII. As observed previously, the binding of ATP to PII also allows 2-ketoglutarate to interact with PII, thereby integrating the carbon signal after assessment of energy status (5, 35).

As noted above, it is surprising that ATP and not ADP is clearly bound to PII. A refined omit electron density map reveals that the entire ATP molecule is well ordered when bound to PII (Fig. 3D). Three regions of extremely strong refined omit electron density (>14σ) correspond perfectly with the refined positions of the phosphorus atoms in the triphosphate moiety of ATP. Because ATP was not added during purifi-
Structural characterization or crystallization, the ATP was likely bound to PII when the protein was produced from *E. coli*.

Open and Closed Conformations of NAGK Protomers—An unusual feature of the PII-NAGK complex is that the two protomers of NAGK found in the asymmetric unit adopt dramatically different conformations. Protomer A, which is found in the "lower" (green) trimeric ring when the complex is viewed as in Fig. 1, adopts an "open" conformation compared with protomer B. When the two conformations are compared, the N-terminal domain (residues 15–211) of protomer A is rotated by 11° away from the C-terminal domain (residues 212–295). Fig. 4 is viewed down this rotational axis, as identified by the program DynDom (36), which passes between the two key hinge residues Leu<sup>211</sup> and Ile<sup>212</sup>. The closed conformation of NAGK brings the two substrates, *N*-acetylglutamate and ATP, closer together. During the process of crystallization, ADP was added as a product/inhibitor to avoid the formation of a mixture of substrates and products if ATP were present. The closed conformation brings the β-phosphate of ADP within 5.5 Å of the carboxylate oxygen atoms of *N*-acetylglutamate. In the open conformation, however, the distance between these two groups increases by 1.5 Å. In addition, hydrogen bonds donated by the side chain amide group of Asn<sup>194</sup> and the main chain amide group of Gly<sup>77</sup> to the carboxylate oxygen phosphate acceptor appear to be formed only in the closed conformation of the substrate-binding site for NAGK.

Previously determined structures of NAGK from bacteria and Archaea also indicate that a mixture of conformational states with variable degrees of domain closure can be found in different protomers within the same crystal as well as in protomers from different crystals (21). This suggests that variable degrees of domain closure may be an intrinsic property of NAGK structure that is critical to function. It seems likely that the closed conformation promotes the formation of the transition state, as has been seen in the related NAGK enzymes from bacteria and Archaea (21, 37). In contrast, the more open conformations likely promote the entry of substrates and the release of products.

**FIGURE 4.** Open and closed conformations of NAGK drawn as α-carbon traces. Shown are the open (A) and closed (B) conformations of *A. thaliana* NAGK and the open conformation of *T. maritima* NAGK bound to arginine (C) (21). ADP and *N*-acetylglutamate (NAG) are drawn in stick representation. The N-terminal domain (N-term; residues 15–211) is colored blue, and the C-terminal domain (C-term; residues 212–297) is colored red. The black dot marks the position of the rotational axis that is normal to the plane of the page. Rotating the N-terminal domain of the open form of NAGK by 11° about this axis in the direction indicated by the arrow in A would result in the closed conformation shown in B. The right panels show a "top" view similar to that used in Fig. 2A. This view is perpendicular to the rotational axis, which is drawn as a light gray arrow.
DISCUSSION

Comparisons with E. coli GlnK-AmtB Complexes—The structure of the PII-NAGK complex reveals a number of surprising similarities to and differences from with the recently determined structures of GlnK bound to the AmtB ammonium transporter (32, 33). At present, the GlnK-AmtB complex is the only other three-dimensional structure of a complex containing a PII-like protein. In both complexes, the T-loop of PII provides the focal point of interaction, and the trimeric structure of PII matches the trimeric structure of the binding partner perfectly. Despite the overall similarities, however, the details of interactions between the T-loop and NAGK differ dramatically from the interactions seen with AmtB.

The T-loop of E. coli GlnK in the AmtB complex is nearly fully extended along the 3-fold rotational axis of the GlnK trimer. In contrast, the T-loop of A. thaliana PII forms a right-angled kink as it extends from the surface of the PII trimer, pointing roughly perpendicular to the 3-fold rotational axis of the trimer (Fig. 1D). The dramatic differences in T-loop conformation seen in the two different complexes indicate that the flexibility of the T-loop may be exploited in different ways to interact with a wide range of target effector enzymes and transporters.

Relief of Arginine Inhibition by the Binding of PII—Arginine is the final product of the pathway in which NAGK resides as the second but rate-limiting step. Arginine is a feedback inhibitor and binds to NAGK between the N-terminal α-helix and the C-terminal domain at a site near interface B of adjacent protomers (Fig. 1D). This binding site is formed primarily by a pocket in the C-terminal domain of NAGK that is similar to the binding site seen previously in the arginine-sensitive archaeal NAGK enzyme from T. maritima (21). Comparisons of the structures of arginine-free NAGK from P. aeruginosa and arginine-bound NAGK from T. maritima indicate that the binding of arginine may stabilize a more open conformation of NAGK by forming bridging interactions between the C-terminal domain and the N-terminal helix. The high degree of similarity in sequence and structure between the plant and archaeal NAGK enzymes suggests that the binding of arginine may also stabilize a more open conformation in plant NAGK. If arginine binding promotes or stabilizes the more open conformation of NAGK, then arginine inhibition may result from structurally destabilizing the closed conformation that is necessary for transition state formation.

The binding of PII to NAGK in plants (11, 12) and cyanobacteria (13–15) has been observed to relieve the inhibition caused by arginine. The structure of the PII-NAGK complex suggests a mechanism by which PII may relieve this inhibition. PII can interact similarly with both open and closed conformations of NAGK. The location of the PII-binding site is found on the side of NAGK opposite the opening of the active-site cleft and nearly perfectly centered on the axis of rotation for separating the two domains (Fig. 4). This location allows PII to interact with a range of NAGK conformations in a similar manner because relatively small changes arise in this region of the enzyme. Thus, PII binding may not strongly favor either the open or the closed conformation of NAGK, both of which are necessary for normal enzyme function.

Even though PII can interact similarly with both open and closed conformations of NAGK, the PII-NAGK structure suggests that PII may restrict NAGK from accessing more extreme conformations. The arginine-inhibited structure of NAGK from T. maritima reveals a larger degree of separation between the N- and C-terminal domains compared with the open conformation of NAGK seen in the plant PII-NAGK complex as well as an additional opening of a flap-like lid region (residues 85–102 in A. thaliana and residues 71–87 in T. maritima) (Fig. 4C). Both of these changes appear to lead to a further displacement of the adjacent strands β6 and β7. Because these strands contain the side chains of Arg145 and the main chain groups of Val159 and Ala160, which form key interactions with PII (Fig. 2), these interactions may limit larger degrees of domain separation and movements of the lid region of NAGK. If these more extreme conformational changes are nonproductive catalytic states that may be promoted by the binding of arginine, then the constraints imposed by PII binding may counter this effect and relieve inhibition. Examining the kinetics of NAGK and PII mutants targeting interactions seen in the NAGK complex and determining the structures of A. thaliana NAGK in the presence of arginine would help test the validity of this hypothesis.

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