The PII Signal Transduction Protein of Arabidopsis thaliana Forms an Arginine-regulated Complex with Plastid N-Acetyl Glutamate Kinase

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The PII proteins are key mediators of the cellular response to carbon and nitrogen status and are found in all domains of life. In eukaryotes, PII has only been identified in red algae and plants, and in these organisms, PII localizes to the plastid. PII proteins perform their role by assessing cellular carbon, nitrogen, and energy status and conferring this information to other proteins through protein-protein interaction. We have used affinity chromatography and mass spectrometry to identify the PII-binding proteins of Arabidopsis thaliana. The major PII-interacting protein is the chloroplast-localized enzyme N-acetyl glutamate kinase, which catalyzes the key regulatory step in the pathway to arginine biosynthesis. The interaction of PII with N-acetyl glutamate kinase was confirmed through pull-down, gel filtration, and isothermal titration calorimetry experiments, and binding was shown to be enhanced in the presence of the downstream product, arginine. Enzyme kinetic analysis showed that PII increases N-acetyl glutamate kinase activity slightly, but the primary function of binding is to relieve inhibition of enzyme activity by the pathway product, arginine. Knowing the identity of PII-binding proteins across a spectrum of photosynthetic and non-photosynthetic organisms provides a framework for a more complete understanding of the function of this highly conserved signaling protein.

In prokaryotic organisms, the PII protein is recognized as the key mediator of energy, carbon, and nitrogen interactions and is referred to as the central processing unit of carbon:nitrogen metabolism (1–4). Escherichia coli PII is a 112-amino acid protein that as a homotrimer senses the cellular status of both ATP and the carbon skeleton 2-oxoglutarate (2KG)3 via allosteric means. Nitrogen status is assessed through glutamine levels by covalent modification (uridylylation) of PII. This metabolic information is signaled to other proteins by protein-protein interaction and produces an appropriate response that alters gene expression and the activity of glutamine synthetase (3, 5). In terms of metabolic sensing, cyanobacterial PII plays a similar role, but in this case, covalent modification is by phosphorylation (6). To date, the processes known to be regulated by PII in cyanobacteria are: ammonium-dependent nitrate/nitrite uptake (7), high affinity bicarbonate transport (8), regulation of the global transcriptional activation by NtcA (9, 10), and arginine biosynthesis (11).

In eukaryotes, PII has only been identified in plants and red algae (12), and its sequence is highly conserved when compared with prokaryotic PII, with Arabidopsis thaliana PII being 50 and 55% identical to E. coli and Synechococcus elongatus PII, respectively. Plant PII proteins have a conserved N-terminal extension that functions as a chloro-protein binding peptide, which is consistent with biochemical data indicating that PII resides in this compartment. We have previously shown that the plant PII protein is not regulated by phosphorylation (13). Like the bacterial protein, plant PII binds 2-oxoglutarate, but only after binding ATP first, and thus likely functions to sense plastid energy and 2KG status (14). PII transcripts appear to be present in all plant organs, and PII protein levels do not change during day/night cycles or N-nutrition (14, 15). Although PII T-DNA knock-out lines show increased sensitivity to nitrite and slight alternations in carbon metabolism and amino acid levels during altered N-nutrition (15), no molecular targets have been firmly established for plant PII. Two preliminary yeast two-hybrid studies have indicated that N-acetyl glutamate kinase (NAGK) is a PII interactor in plants (16, 17). We searched for PII-interacting proteins by performing affinity chromatography with plant PII and have identified the major PII receptor of A. thaliana as the plastid enzyme N-acetyl glutamate kinase. Biochemical studies showed that PII alters the kinetic properties of NAGK and that the downstream end product of this metabolic pathway (arginine) promotes the interaction of PII and NAGK. Interestingly, arginine levels are barely detectable in plants during the light period and are high during the dark (18), suggesting arginine inhibition of NAGK during the light and relief by PII in the dark.

EXPERIMENTAL PROCEDURES

Affinity Chromatography—A. thaliana PII minus the chloroplast transit peptide was expressed and purified as described (19). After dialysis into PBS, 2 mg of PII and 2 mg of Fraction V BSA (Sigma) were separately coupled to 1 ml of CH-Sepharose (Amersham Biosciences) by following the manufacturer’s instructions (coupling was >95%). A. thaliana suspension cells were grown in culture, harvested and lysed as described in Smith et al. (13). Typically, ~300 g of cells were lysed in a French press with 1 volume of 25 mM Tris/Cl, pH 7.5, 1 mM EDTA, 1 mM benzamidine, 0.1 mM PMSF, 0.1 mM (v/v) 2-mercaptoethanol, and 1% (w/v) polyvinylpyrrolidone and clarified by centrifugation at 35,000 rpm for 30 min in a Ti-45 rotor. Following filtration through Miracloth (Calbiochem), the crude extract (~1900 mg of protein) was then split in half and mixed end-over-end at 4 °C with 1 ml of
PII or BSA affinity matrix that was previously equilibrated in buffer A (25 mM Tris/Cl, pH 7.5, 1 mM benzamidine and 0.1 mM PMSF). After 4 h, the mixture was poured into a column and washed with 300 ml of buffer A plus 150 mM NaCl, and protein was eluted with 8 ml of buffer A plus 1.5 M NaCl. Eluted protein was concentrated in a Centriprep 10 (Amicon) and then in a Centricon 10 to 25 μl and boiled in SDS-mixture. In all cases, PII- and BSA-Sepharose chromatographies were performed identically to allow direct comparison.

**Mass Spectrometry and Edman Sequencing—**PII- and BSA-binding proteins were run on SDS-PAGE and Coomassie Blue-stained, and bands were excised, trypsin was digested, and proteins were identified by MALDI-TOF mass spectrometry (20). The only proteins indicated (see Fig. 1A) are those for which a high level of confidence for identification was obtained (see supplementary Table 1). In all cases, the identified protein was the best match to the peptide mass data, the identified protein predicted mass was very near the observed mass on the gel, and the percentage of peptide coverage was high, ranging from 11 to 62% of the total number of residues using a cut-off of less than 35 ppm. For N-terminal sequencing, the PII-Sepharose-eluted proteins were run on SDS-PAGE, blotted to PVM, stained with Amido Black, and washed with water to visualize the 33-kDa protein. This band was excised and submitted to Edman chemistry as described (21).

**Cloning, Expression, and Purification of *A. thaliana* N-Acetyl Glutamate Kinase and Non-tagged PII—**The N-acetyl glutamate kinase was PCR-amplified (to start from the predicted chloroplast cleavage site Ala30) from an *A. thaliana* silique cDNA library template obtained from the *A. thaliana* Biological Resource Centre, Ohio State University, using the following primers: 5’-TATAAGATCTCGACCAGGAATGACCAAGAATCTGGAATTC-3’ and 3’-TATAGAATTCTTATCCAGTAATCATAGTACCAAGAATCTGGAATTC-3’. After digestion with EcoRI and BglII, the PCR product was cloned into EcoRI-BglII-restricted pRSET A plasmid (Invitrogen) to transform *E. coli* was transformed into DH5α. The transformed bacterial cells were grown at 37 °C for 26 h. The cells sedimented, beads were washed two times with 500 μl of PBS, two times with 25 ml Tris/Cl, pH 7.5, 0.05% (v/v) Nonidet P-40, and 20 mM imidazole, and two times again with PBS, and then proteins were released from the matrix by the addition of 0.3 M imidazole, pH 7.5. The following metabolites were included in the incubation mixture to test for their ability to disrupt or enhance the PII-NAGK interaction: Glu, Gln, Asp, ATP, ADP, carbamoyl phosphate, citrulline, and Lys (all at 5 mM), 2KG, AMP, NaNO3 (all at 1 mM), NH4Cl (20 mM), 2KG + ATP (1 and 5 mM, respectively), MgCl2 (10 mM), MgCl2 + ATP (10 and 5 mM, respectively), MgCl2 + ATP (5 and 5 mM, respectively), N-acetyl glutamate (NAG) (20 mM), and Arg (1, 5, 10, and 20 mM). In one series of experiments in which 5 mM arginine was included in the incubation buffer, the same concentration of arginine was included in all wash steps.

**Antibody Production and Localization of NAGK to Chloroplasts—**Recombinant NAGK purified by Ni-NTA-agarose (see Fig. 1B) was used to raise antibodies in a rabbit using standard procedures (19). The antibodies were used in Western blots and immunofluorescence experiments as described previously (13), harvested, and allowed to settle by gravity. Once settled, the supernatant was removed, and the cells were fixed by adding a freshly prepared 4% (w/v) formaldehyde solution made in PBS. Cells were fixed for 15 min before being washed with PBS (3 × 1 ml). The cell wall was partially digested with 500 μl of a 0.1% (w/v) pectolyase Y-23 (Seishen Pharmaceutical Co.) solution made in PBS for 15 min at 30 °C. The cell membrane was permeabilized by incubation with 1% (v/v) Triton X-100 for 5 min at room temperature. The cells were then washed with PBS (3 × 1 ml), blocked in 2% (w/v) BSA in PBS for 10 min, and then incubated overnight at 4 °C in 200-fold diluted crude serum specific for NAGK or PII (14). The following day, the cells were washed with PBS (3 × 1 ml) and incubated with anti-mouse secondary antibody conjugated to the fluorophore Alexa Fluor 488 (Molecular Probes) and in blocking buffer for 1 h at room temperature. The samples were washed again in PBS (3 × 1 ml) before being analyzed with a fluorescence microscope (Leica DMR) using the fluorescein isothiocyanate filter set. For the observation of chloroplasts, chlorophyll fluorescence was achieved using the UV filter set. Images were captured using a cooled CCD camera (Retiga 1350 EX, Qimaging), and image enhancement and deconvolution confocal algorithm manipulations were performed using the Openlab software package (Version 3.0, Improvision). Pseudocoloration and image manipulation was performed in PhotoShop.

**Gel Filtration Chromatography—**Protein samples were diluted into Superdex 200 column buffer (25 mM Tris/Cl, pH 7.5, 150 mM NaCl, 5% (v/v) glycerol) to a final volume of 45 μl centrifuged at 14,000 rpm for 10 min, passed through a Costar 0.22-μm Spin-X filter, and then chromatographed on a Superdex 200 PC 3.2/30 column (Amersham Biosciences) equilibrated at room temperature in column buffer. The fast protein liquid chromatography flow rate was 0.40 μl/min, and 0.10-ml fractions were collected. For experiments that included Mg-ADP, the sample was made 2 mM MgCl2 and 1 mM ADP and chromatographed in
column buffer that included 0.4 mM MgCl₂ and 0.2 mM ADP. The Superdex 200 PC 3.2/30 column was calibrated from a standard plot of \( K_v \) versus molecular mass for ribonuclease A (13.7 kDa), ovalbumin (43 kDa), bovine serum albumin (66 kDa), aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), thyroglobulin (669 kDa), and blue dextran (void). The mass of NAGK (\( n = 5 \)), His₅-PIL (\( n = 7 \)) and the NAGK-His₅-PIL complex (\( n = 3 \)) was determined and is presented as the mean ± S.E.

Isothermal Titration Calorimetry—ITC experiments were performed as described in Smith et al. (14) using His₅-NAGK and His₅-PIL without transit peptides, in the following: 25 mM Tris/Cl, pH 7.5, 5% (v/v) glycerol, and 150 mM NaCl. Each run was corrected for heat of dilution.

Enzyme Kinetics—For enzyme kinetics, all assays were done in duplicate in three or more separate experiments, except in the assays where \( K_m \) and \( V_{\text{max}} \) values were examined for arginine-inhibited NAGK, plus and minus PIL. Here, each assay was done in triplicate. NAGK activity assays with purified proteins were determined as described by Heinrich et al. (11). Unless stated otherwise, the reaction mixture consisted of 300 mM NH₄OH·HCl, pH 7.5, 20 mM Tris/Cl, pH 7.5, 20 mM MgCl₂, 40 mM NAG, and 10 mM ATP and NAGK diluted into 25 mM Tris/Cl, pH 7.5, and 150 mM NaCl such that each assay contained 0.81 μg of enzyme. Reactions were initiated by the addition of reaction mixture, and the incubation was carried out at 37 °C in a volume of 0.10 ml and terminated after 20 min by the addition of 0.1 ml of stop mixture (1:1:1 of 5% (w/v) FeCl₃ · 6 H₂O in 0.2 M HCl, 8% (w/v) trichloroacetic acid, and 0.3 M HCl), and after standing for 5 min at room temperature, the tubes were centrifuged for 1 min at 14,000 rpm. Under these reaction conditions, the assay was linear up to at least 30 min. Blank reactions were performed by omitting N-acetyl glutamate from the assay. One unit of enzyme activity is defined as one mmol of product produced in 1 min calculated with a molar absorption coefficient of 456 M⁻¹ cm⁻¹ at 540 nm for the N-acetylglutamylhydroxamate-Fe³⁺ complex. The non-linear regression program GraphPadPrism 4.0 (GraphPad Software, San Diego, CA) was used for estimating the \( K_m \) and \( V_{\text{max}} \) values for all data except the data obtained from arginine inhibition. The catalytic efficiency and \( K_{cat} \) of NAGK were calculated using \( K_m/V_{\text{max}} \) and \( V_{\text{max}}/[NAGK]_{\text{total}} \), respectively. The \( V_{\text{max}} \) and \( K_m \) values for NAGK were determined by holding the concentration of the second substrate at a saturating level (NAG at 40 mM or ATP 10 mM (MgCl₂ at 20 mM)). The varied substrate was omitted when carrying out blank assays.

To study the effect of PIL on the activity of NAGK, various amounts of A. thaliana PIL (14) were added into the diluted NAGK fraction, and the mixture was placed at 4 °C for 10 min to allow complex formation. The reaction was initiated by the addition of the reaction mixture into the NAGK-PIL complex mixture. For determining the effect of PIL on the \( K_m \) and \( V_{\text{max}} \) for NAG and ATP, PIL was added into the diluted NAGK such that the molar ratio of PIL-polypeptide to NAGK-polypeptide was 2:1 (based on a Bradford assay with BSA as standard) and allowed to interact at 4 °C for 10 min. For studying the NAGK activity in the presence of PIL and its effector 2KG, PIL was incubated with 5 mM ATP and 1 mM 2KG for 3 min at 4 °C followed by the addition of NAGK. For studying the \( K_m \) and \( V_{\text{max}} \) of NAGK for the substrate ATP, PIL was preincubated with 0.05 mM ATP and 1 mM 2KG.

The effect of arginine on NAGK was studied under two different conditions (10 mM ATP and 40 mM NAG). The effect of arginine was also studied at \( K_m \) conditions in which the concentration of NAG was set to 40 mM and the concentration of ATP was set to the \( K_m \) value (1.74 mM). For either condition, various amounts of arginine were added into the NAGK fraction, and the mixture was allowed to stand for 5 min before initiating the reaction by adding substrates. The \( K_m \) and \( V_{\text{max}} \) values for NAGK in which NAG was the varied substrate were studied under three different concentrations of arginine, 0.16, 0.32 and 0.64 mM. Hill plots, with the equation \( \log [V_o/(V_o - V_s)] = N \log [NAG]_0 - \log K' \), were performed for each set of data. The \( V_{\text{max}} \) values were estimated from Eadie-Hofstee plots, and the \( K_m \) values were estimated from plots of velocity versus substrate concentration. To test the effect of arginine on the PIL-NAGK complex, PIL was first added into the diluted NAGK solution, and the two proteins were placed at 4 °C for 10 min to allow complex formation followed by the addition of arginine. The enzyme mixture was allowed to stand for 5 min before the initiation of assays.

**RESULTS**

Identification of A. thaliana PIL-binding Proteins—To identify PIL-binding proteins, an extract from A. thaliana was incubated with PIL coupled to Sepharose beads. After extensive washing, bound proteins were eluted with 1.5 M NaCl and run on SDS-PAGE (Fig. 1A). No proteins were eluted from the control matrix (BSA-Sepharose), whereas several proteins were consistently enriched on the PIL matrix. The eluted proteins were identified by MALDI-TOF mass spectrometry and are indicated on the gel (Fig. 1A, and see also Supplemental Table 1). The major PIL-binding protein of 33 kDa was identified as a putative plastid N-acetyl glutamate kinase (gi 15230338). Other minor bound proteins were the cytosolic α, β, β’, and γ subunits of the coatomer protein complex I vesicle coat protein complex and several of the smaller cytosolic 60 S ribosomal proteins. ChlP40 analysis (ChlP 1.1 Server) of the NAGK sequence predicts that this gene product is chloroplast-localized, and cleavage of the chloroplast transit peptide is likely between Lys³⁸ and Ala⁵⁰, yielding a protein of 31.1 kDa. Edman sequencing of the PIL-purified NAGK yielded the sequence TSTPPS¹⁸. This confirmed that mature NAGK has its putative chloroplast transit peptide cleaved, but between Ala⁵⁰ and Thr, and thus likely localizes to the chloroplast. This is consistent with the expected localization of a PIL-interacting protein. Being the most abundant PIL-binding protein and likely residing in the same compartment, we further characterized the interaction of PIL and NAGK. A. thaliana NAGK was cloned minus the chloroplast transit peptide, expressed in E. coli as a 6-histidine fusion protein, and purified (Fig. 1B).

NAGK and PIL Are Chloroplast-localized Proteins—The chloroplast localization of NAGK and PIL was confirmed by immunolocalization.
experiments using antibodies raised against each protein. The NAGK antibody was first tested by probing Western blots of pure enzyme and an *A. thaliana* crude fraction. The diluted crude serum could easily detect less than 1 ng of recombinant NAGK and one major band of 33 kDa in a crude extract (data not shown). Both PII and NAGK stained in small structures that resemble chloroplasts and were subsequently colocalized with chlorophyll fluorescence (Fig. 2). The immunolocalization of PII and NAGK to the chloroplast is consistent with previous biochemical studies of *A. thaliana* PII (22) and the predicted chloroplast transit peptides of both PII and NAGK.

**Reverse Affinity and Gel Filtration Chromatography Confirms an Interaction between PII and NAGK**—Knowing that PII and NAGK localize to the same compartment supports the idea that they are true interacting partners. To further confirm an interaction between PII and NAGK, we performed the reverse experiment in which His-tagged NAGK was incubated with non-tagged PII and the complex was pulled out with Ni-NTA-beads. Fig. 3A shows that NAGK binds PII and that PII in the presence of an unrelated His-tagged protein (PR65) is not retained on the beads, nor is non-tagged PII retained on the beads alone (data not shown).

The mass of the *A. thaliana* purified NAGK was determined by gel filtration chromatography after elution from the PII matrix. Peak fractions demonstrated a mass of ~215 kDa (Fig. 3B). This 33-kDa band was confirmed to be NAGK by Western blot analysis (data not shown) and suggests that the native protein exists as a multimer of six or seven 33-kDa subunits. Fig. 3, C–F, show the elution profiles for recombinant PII alone, NAGK alone, and a mixture of PII plus excess NAGK. Tagged PII displayed a mass of ~45 ± 0.5 kDa (14), which is consistent with a mass of a trimer of 16-kDa subunits, whereas recombinant NAGK eluted at ~276 ± 2 kDa, which is consistent with a multimer of six or seven 33-kDa subunits (the recombinant protein contains a tag of ~5 kDa in addition to the native protein). If the recombinant protein exists as a hexamer, subtracting six tags gives a mass similar to the native protein, giving us confidence that the bacterial produced protein is behaving like the purified plastid enzyme. When the two proteins are mixed (Fig. 3, E and F), the PII elution profile shifts dramatically, suggesting that PII and NAGK form a complex that displays a mass of ~300 ± 1.5 kDa. PII and NAGK chromatographed in the presence of 0.4 mM MgCl₂ and 0.2 mM ADP displayed the same mass (data not shown).

**Isothermal Titration Calorimetry**—ITC allowed us to further address the stoichiometry of binding for PII and NAGK. Titration of PII into a solution of NAGK (Fig. 4) gives an *n* value of 0.43, suggesting that each PII trimer binds ~7 NAGK subunits. In the reverse experiment, in which NAGK was titrated into a solution of PII, *n* = 1.77, suggesting that about five NAGK molecules interact with each PII trimer. In combination with the gel filtration data, this supports the idea that one PII trimer interacts with one NAGK hexamer and that native NAGK is indeed a hexamer.

*Altered Kinetic Behavior of NAGK in the Presence of PII and Its Effectors*—NAGK catalyzes the second step in the arginine biosynthetic pathway (see Supplemental Fig. 1). The reaction catalyzed by NAGK involves two substrates, ATP and NAG, in which NAGK transfers the γ-phosphate group from ATP to NAG to form N-acetyl-γ-glutamyl phosphate. For free NAGK (not complexed with PII), the apparent *V*ₘₐₓ and *Kᵥₘ for NAGK obtained when NAG was the variable substrate were 10.6 (±0.3 S.E.) units/mg and 7.08 (±0.49 S.E.) mM, respectively, yielding a catalytic efficiency (*kₘₐₓ*Kᵥₘ) of 872 s⁻¹ M⁻¹. For ATP, the apparent *Kᵥₘ for NAGK* was 1.74 (±0.21 S.E.) mM and *V*ₘₐₓ for NAGK was 9.99 (±0.29 S.E.) units/mg, yielding a catalytic efficiency of 3350 s⁻¹ M⁻¹. The plot of velocity versus substrate concentration for NAG (Fig. 5A) displayed a classic Michaelis-Menten kinetic profile (a Michaelis-Menten profile was obtained for ATP as well).

As shown in Fig. 5B, the *V*ₘₐₓ of NAGK increased by a maximum of 30% when it was in complex with PII. When NAGK forms a complex with PII, the *V*ₘₐₓ for the NAG substrate increased to 13.2 (±0.3 S.E.) units/mg, and its *Kᵥₘ increased slightly to 7.55 (±0.56 S.E.) mM. The resulting catalytic efficiency of 1020 s⁻¹ M⁻¹ corresponds to a modest increase in catalytic efficiency for PII-complexed NAGK, in comparison with free NAGK. When NAGK forms a complex with PII, the *V*ₘₐₓ and *Kᵥₘ for ATP both increased to 11.6 (±0.25 S.E.) units/mg and 2.03 (±0.15 S.E.) mM, respectively, giving an overall catalytic efficiency of 3320 s⁻¹ M⁻¹ that was different from the one obtained for free NAGK.

2-Ketoglutarate is an effector molecule that binds to PII and signals carbon status in the chloroplast (2, 14). It binds PII only when the ATP binding site in PII has ATP bound (14). In the presence of PII-ATP-2KG complex, the *V*ₘₐₓ of NAGK increased ~2-fold (20.2 ± 0.3 S.E. units/mg) when NAG was the variable substrate and 1.3-fold (12.7 ± 0.3 S.E. units/mg) when ATP was the variable substrate. The *Kᵥₘ for NAG and ATP also increased slightly to 9.85 (±0.41 S.E.) mM and 2.47 (±0.16 S.E.) mM, respectively. The catalytic efficiency obtained for NAGK when NAG was the variable substrate was 1200 s⁻¹ M⁻¹ and corresponded to an 18% increase in catalytic efficiency in NAGK activity in

![Figure 2. PII and NAGK are chloroplast-localized proteins.](image)
A. thaliana PII-binding Proteins

The presence of PII and 2KG in comparison with PII alone. PII with 2KG bound increased the catalytic efficiency of NAGK by 38% overall. Feedback inhibition of NAGK—NAGK catalyzes the committed step in the biosynthesis of arginine, and in cyanobacteria, it has been found to be feedback-regulated by the end product of this biosynthetic pathway (arginine) (23). Under V_max conditions, free A. thaliana NAGK exhibited feedback inhibition by arginine with a half-maximum inhibition concentration (IC_{50}) of 0.73 mM, and in the presence of PII, less inhibition of NAGK was observed, with the IC_{50} value increasing ~3.4-fold to 2.5 mM (Fig. 6A).

To investigate arginine inhibition of NAGK under K_m conditions, the concentration of ATP was set at its K_m value (1.74 mM), whereas the concentration of NAG was set at a saturating level. The IC_{50} for arginine obtained for free NAGK was 0.33 mM, and similar to arginine inhibition at V_max conditions, PII relieved the inhibition of NAGK activity by arginine, yielding an IC_{50} of 0.83 mM (Fig. 6B). The effect of 2KG on the feedback inhibition of NAGK by arginine was studied only at V_max conditions, and the resulting inhibition curve gave a half-maximal inhibition value of 2.4 mM, which was indifferent from the IC_{50} obtained for PII-complexed NAGK in the absence of 2KG (data not shown).

Next, we chose to study how the K_m and V_max values change for NAGK for three different concentrations of arginine (0.16, 0.32, and 0.64 mM). This was accomplished by examining the V_max and K_m of NAGK where NAG was the variable substrate and the concentration of ATP was held fixed at a saturating level. For both free NAGK and PII-complexed NAGK, V_max decreased and K_m increased as the concentration of arginine increased (Fig. 7A). Overall, the catalytic efficiency of PII-complexed NAGK was higher than free NAGK, and the difference between the two increased as arginine concentration increased, reaching to a 2.3-fold activation of NAGK by PII at 0.64 mM arginine (435 versus 186 s^{-1} M^{-1}). Another interesting feature observed in this experiment was the change in the shape of the plot for free and PII-complexed NAGK when arginine was present. The velocity versus substrate concentration plot for free NAGK inhibited by 0.32 mM arginine showed a sigmoidal curve with a Hill coefficient of 2.3, and a similar plot for NAGK in complex with PII showed a Michaelis-Menten curve (Fig. 7B). The corresponding Hill plot of the data for PII-complexed NAGK gave a Hill coefficient of 1. In general, the Hill coefficient increased as the amount of arginine increased in the sample. For free NAGK, the Hill slope coefficient increased to ~3 when the concentration of arginine was 0.64 mM.

The following compounds were tested for their ability to inhibit or activate NAGK and the NAGK-PII complex and shown to have little or no effect on activity. The concentration tested in the assay was 5 mM for Glu, Gln, Asp, Asn, Ser, Thr, His, Lys, Gly, Pro, and 5'-AMP or the...
indicated range of values for NaNO₂ (0.025–2.5 mM), carbamoyl phosphate (0.5–10 mM), NH₄⁺ + Cl⁻ (5–100 mM), and citrulline (1–100 mM).

**DISCUSSION**

The chloroplast of plants functions not only as the site of photosynthesis and carbon fixation but also as the location of many metabolic biosynthetic pathways, including the assimilation of NO₂⁻ into an organic form and the synthesis of nucleotides and amino acids. As such, the chloroplast is the primary site for the interface of carbon and nitrogen metabolism and is also the compartment where plant PII resides. We have previously shown that like prokaryotic PII proteins, plant PII senses the carbon skeleton 2Kg in a manner dependent upon first binding (or sensing) ATP. After sensing metabolic status, PII provides this information to other proteins to allow them to adjust their function or output accordingly. Although PII has been suggested to be critical to carbon/nitrogen sensing and signaling in plants (12, 22), our understanding of how it performs this role is still very limited. This work was initiated to define the binding partners of *A. thaliana* PII as to date, plant PII partners have only been suggested through a yeast two-hybrid screen (16, 17). We used an affinity chromatography-proteomics approach to look for PII-interacting proteins and identified NAGK as the primary PII-binding protein of *A. thaliana* cells, consistent with the yeast two-hybrid result. NAGK has been proposed to reside in the plastid where PII is localized. Sequence analysis tools suggest that NAGK has a chloroplast transit peptide, and we have confirmed by Edman sequencing that the mature PII-associated NAGK is cleaved between Ala⁵⁰ and Thr⁵¹, consistent with cleavage by the transit peptide protease. To further support this observation, we employed immunolocalization to place both PII and NAGK in the chloroplast of *A. thaliana* cells. Previously, Sugiyama et al. (17) showed that GFP-NAGK was chloroplast-localized. Knowing that the putative interactors reside in the same compartment, we further explored the interaction using pull-down, gel filtration, and ITC experiments. All of these approaches confirmed that PII and NAGK form a complex. In addition, the gel filtration and ITC data support the idea that native NAGK, as well as the recombinant enzyme, functions as a hexamer like the enzyme from *Thermotoga maritima* and *Pseudomonas aeruginosa* (24). Recently, *S. elongatus* PII was shown to interact with the hexameric NAGK of that species (23).

One of our goals was to define the function of PII interaction with NAGK. Our enzyme kinetic analysis demonstrated that PII modestly activated the plant enzyme. This is in contrast to the *S. elongatus* NAGK, where the *Vₘₐₓ* increases 4-fold by binding PII and the *Kₘₐₓ* for NAG displays a 10-fold decrease (23). It is notable that the *A. thaliana* enzyme alone has a *Vₘₐₓ* near that displayed for PII-activated *S. elongatus* NAGK, and therefore, we are not surprised that binding PII only increased activity slightly. We found that in the plant system, the primary function of PII binding to NAGK is to relieve feedback inhibition by the downstream pathway product, arginine, shifting the substrate saturation curve to hyperbolic from sigmoidal in the presence of arginine. It is interesting to note that unlike the dimeric *E. coli* NAGK, which is not regulated by arginine, all hexameric NAGKs appear to be
that it is not regulated by phosphorylation (13), and this appears to be true for some cyanobacterial PII proteins as well (25, 26).

Why would such an ancient molecular interaction exist? It is interesting to note that in plants, the level of arginine is nearly undetectable during the light period (18). This implies that arginine is primarily synthesized during the dark, under the control of PII, and thus may function as a signaling molecule in plants. This type of event is not unprecedented as it has been shown that the amino acid leucine can control the signaling events downstream of the target rnapy protein kinase (27), and this signaling cascade is key to the response to energy and amino acid status (28, 29). At this point, we must note that the plant PII protein levels are constant during light/dark cycles and various N-regimes (15) and thus could function in a rapid response to changing cellular conditions as necessary. As discussed above, the phosphorylation state of PII does not appear to control the binding of PII to NAGK in plants (unlike _S. elongatus_), and the only metabolite found to control the interaction was arginine itself, which enhanced binding and therefore should increase its own synthesis. In a system in which arginine appears to promote its own synthesis, it is as yet unclear what the molecular switch is that turns the pathway on and off. Perhaps NAGK is regulated by covalent modification and the interaction of NAGK with PII may be regulated by this event. Nevertheless, plant PII plays a regulatory role in a pathway that produces the amino acid arginine, and importantly, flux through this pathway results in the consumption of the key amino acids glutamate, aspartate, and the carbon/nitrogen donor molecule carbamoyl phosphate, thus further establishing the link to nitrogen metabolism. Carbamoyl phosphate is also used in the biosynthesis of the pyrimidines, and in plants, this pathway is localized to the plastid compartment (30).

In conclusion, we have characterized the first eukaryotic PII-binding protein interaction after identification using proteomics. Identification of NAGK as the primary PII target in plants makes this the missing and critical link of plant PII to nitrogen metabolism. With plant PII having the same primary target as photosynthetic bacteria, this further establishes the importance of this ancient signaling cascade.

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