**Background:** Highly conserved P$_{II}$ signaling proteins monitor carbon/nitrogen balance and energy status.

**Results:** Crystals of homotrimeric *Synechococcus elongatus* P$_{II}$ with ADP reveal the structural basis of anti-cooperative ADP binding. Interaction with PipX receptor changes the ATP/ADP preference of P$_{II}$.

**Conclusion:** P$_{II}$ signaling is modulated by conformational states imposed by the receptor protein.

**Significance:** Molecular information is processed through protein structure plasticity.

P$_{II}$ signaling proteins comprise one of the most versatile signaling devices in nature and have a highly conserved structure. In cyanobacteria, PipX and N-acetyl-L-glutamate kinase are receptors of P$_{II}$ signaling, and these interactions are modulated by ADP, ATP, and 2-oxoglutarate. These effector molecules bind interdependently to three anti-cooperative binding sites on the trimeric P$_{II}$ protein and thereby affect its structure. Here we used the P$_{II}$ protein from *Synechococcus elongatus* PCC 7942 to reveal the structural basis of anti-cooperative ADP binding. Furthermore, we clarified the mutual influence of P$_{II}$-receptor interaction and sensing of the ATP/ADP ratio. The crystal structures of two forms of trimeric P$_{II}$ one with one ADP bound and the other with all three ADP-binding sites occupied, revealed significant differences in the ADP binding mode: at one site (S1) ADP is tightly bound through side-chain and main-chain interactions, whereas at the other two sites (S2 and S3) the ADP molecules are only bound by main-chain interactions. In the presence of the P$_{II}$-receptor PipX, the affinity of ADP to the first binding site S1 strongly increases, whereas the affinity for ATP decreases due to PipX favoring the S1 conformation of P$_{II}$-ADP. In consequence, the P$_{II}$-PipX interaction is highly sensitive to subtle fluctuations in the ATP/ADP ratio. By contrast, the P$_{II}$-N-acetyl-L-glutamate kinase interaction, which is negatively affected by ADP, is insensitive to these fluctuations. Modulation of the metabolite-sensing properties of P$_{II}$ by its receptors allows P$_{II}$ to differentially perceive signals in a target-specific manner and to perform multitasking signal transduction.

The P$_{II}$ signal transduction system responds to nitrogen, carbon, and energy of the cell and is highly conserved and widespread in all three domains of life (1–5). P$_{II}$ proteins receive metabolic information by binding ATP, ADP, and 2-oxoglutarate (2-OG)$^4$ and integrate the signal through conformational changes and covalent modifications (1, 3). Depending on the signal, P$_{II}$ proteins control regulatory and metabolic enzymes, transport proteins, and transcription factors involved in central nitrogen metabolism (5).

P$_{II}$ proteins are homotrimers of 12–13-kDa subunits with a cylindrical body and three flexible T-loops that extend into the solvent and take part in binding of effector molecules and protein-protein interactions (3, 5). Each subunit also has two smaller loops, the B- and C-loops, that face each other in the intersubunit clefts and participate in effector binding. The P$_{II}$ trimer thus contains three effector nucleotide-binding sites, one in each intersubunit cleft (6–8), where the adenylly nucleotides ATP and ADP compete for the same sites. In the P$_{II}$ protein from the cyanobacterium *Synechococcus elongatus* PCC 7942, the binding of ATP and ADP to the three sites is anti-cooperative, with ATP having a higher affinity than ADP (9). When Mg$^{2+}$-ATP is ligated to a binding site of bacterial P$_{II}$, the site can also bind 2-OG, whereas ADP binding does not support the binding of 2-OG (10, 11). The 2-OG binding site is located at the base of the T-loop in direct vicinity of the β- and γ-phosphate of ATP, which ligates 2-OG through a chelated Mg$^{2+}$ ion (11, 12). Consequently, binding of ATP and 2-OG is synergistic (13). Because ADP competes with Mg$^{2+}$-ATP in binding to P$_{II}$, it negatively affects the binding of 2-OG and thereby interferes with the ability of P$_{II}$ to sense the signal of nitrogen starvation (10, 14, 15). The three 2-OG binding sites exhibit negative cooperativity relative to each other, which is mediated through intersubunit signaling inside the trimer (9, 11).

In living cells ATP and ADP are simultaneously present, and they compete for the three nucleotide-binding sites of the P$_{II}$

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* This work was supported by the Max Planck Society, the University of Tübingen, Germany, and German Science Foundation Grant FO195/9-1 (to K. F.). The atomic coordinates and structure factors (codes 4C3K, 4C3M, and 4C3L) have been deposited in the Protein Data Bank (http://wwpdb.org/).

1 Present addresses: Unidad de Biofísica (CSIC-UPV/EHU) and Fundación Biofísica Bizkaia, Barrio Sarriena s/n, 48940, Leioa, Vizcaya, Spain and IKERBASQUE, Basque Foundation for Science, E-48011 Bilbao, Spain.

2 Present address: Karlsruhe Institute of Technology, Institute for Applied Biosciences, Dept. of Microbiology, D-76187 Karlsruhe, Germany.

3 To whom correspondence should be addressed: Interfaculty Institute for Microbiology and Infection Medicine, Division Organismic Interactions, University of Tübingen, Auf der Morgenstelle 28, D-72076 Tübingen, Germany. Tel.: 49-7071-2972096; E-mail: karl.forchhammer@uni-tuebingen.de.

$^4$ The abbreviations used are: 2-OG, 2-oxoglutarate; NAGK, N-acetyl-L-glutamate kinase; SPR, surface plasmon resonance; RU, resonance unit; NTA, nitritotiarcitic acid; r.m.s.d., root mean square deviation; FC2 and FC3, flow cell 2 and 3, respectively; PDB, Protein data bank.
trimer. The total concentration of these effectors in bacterial cells is in the millimolar range (16–18), well above the $K_D$ for the $P_i$ binding sites (9); therefore, all three binding sites of $P_i$ are presumably always occupied by either ATP or ADP. Considering that 2-OG is only able to bind to the sites occupied by Mg$^{2+}$-ATP, $P_i$ proteins could theoretically adopt 20 different ligand binding states, but considering that in vivo all sites are filled with either ATP or ADP, only 10 states would be possible. If the ligand binding properties are taken into account, only 5–7 states are probable in vivo. At high ATP concentrations, all $P_i$ sites are filled with Mg-ATP ($P_i$-ATP$_3$) and up to three 2-OG molecules can be bound, thereby measuring this effector with the highest sensitivity. As the ADP concentration increases, the $P_i$-ATP$_2$-ADP$_1$ population forms. Only when the ADP concentration exceeds that of ATP would the $P_i$-ATP$_1$-ADP$_2$ population accumulate, as the binding affinity of ADP is lower than that of ATP. In general, the mixed populations ($P_i$-ATP$_3$-ADP$_1$ and $P_i$-ATP$_1$-ADP$_3$) have fewer 2-OG binding sites than $P_i$-ATP$_3$, and calorimetric titration experiments have shown that at an ATP:ADP ratio of 1:1, only one 2-OG is bound (15). The failure to detect two binding sites for 2-OG under these conditions indicates either that the $P_i$-ATP$_2$-ADP$_1$ species can bind only one 2-OG molecule or, more likely, that in the presence of equimolar ADP and ATP, the $P_i$-ATP$_1$-ADP$_2$ species is preferentially formed.

$P_i$ proteins from oxygenic phototrophs (cyanobacteria and plants) control arginine biosynthesis through the arginine feedback-inhibited enzyme N-acetyl-l-glutamate kinase (NAGK) (19, 20). $P_i$ binding to NAGK enhances the activity of the enzyme and protects it from feedback inhibition by arginine (21). $P_i$ in the ATP-ligated state avidly binds to NAGK, whereas in the presence of ADP, complex formation is impaired, and as a consequence, NAGK activity is not enhanced (15). Likewise, the effector 2-OG strongly inhibits $P_i$-NAGK complex formation. Thus, the inhibitory effect of 2-OG is not antagonized by ADP and is, therefore, robust against different ATP:ADP ratios (15, 21). In addition to the arginine pathway control, cyanobacterial $P_i$ proteins indirectly control gene expression by sequestering PipX, which is a co-activator of the global nitrogen control transcription factor NtcA (22–24). $P_i$ in the absence of 2-OG, with ADP strongly enhancing the $P_i$-PipX interaction and antagonizing the inhibitory 2-OG effect even at low ADP concentrations (15, 24).

Binding of ADP and ATP is a general property of $P_i$ proteins in living cells (5). In *Escherichia coli*, which has two $P_i$ paralogues, GlnB and GlnK, ADP acts as an antagonist of 2-OG in the GlnB-mediated regulation of the NR1/NRII two-component system and of the adenyltransferase-glutamine synthetase monoycle in vitro. ADP also inhibits uridylation of GlnB by the bifunctional enzyme uridylyltransferase/uridylyl-removing enzyme (UTase) (10, 25). In the presence of ADP, the $P_i$ parologue GlnK forms a complex and thereby blocks the ammonium transporter AmtB, a reaction that is antagonized by Mg$^{2+}$/ATP/2-OG (14). Recently, Radchenko et al. (26) showed that in the absence of 2-OG, GlnK displays intrinsic ATPase activity, slowly converting ATP-GlnK to ADP-GlnK, which then tightly binds AmtB. Sensing of the energy charge levels has also been suggested for the $P_i$ proteins from *Rhodospirillum rubrum* (27, 28). ADP stabilizes the GlnJ-AmtB1 complex (GlnJ is a GlnK parologue) even in the presence of complex-dissociating ATP-2-OG levels (29). The affinities toward ATP and ADP and the anti-cooperativity between the binding sites are key features of $P_i$ proteins that determine their ability to function as energy sensors. In this respect it is important to note that the fine-tuned ligand binding properties of $P_i$ proteins from different sources are variable (30). One of the best-studied $P_i$ proteins is that of *S. elongatus*. Many structure-function details related to 2-OG signaling have been elucidated (3, 11, 24, 31) (an overview of the various *S. elongatus* PII structures is presented in Table 1). However, the structural implications of ADP binding and the functional consequences for the integrated NAGK-$P_i$-PipX interaction network remain elusive.

In the present study we investigated the structural transitions of effector-free and ADP-loaded $P_i$ subunits from *S. elongatus* and propose a mechanism of ADP sensing by $P_i$. We also analyzed the role of energy charge sensing (energy charge measured as the ATP/ADP ratio) for the PipX/NtcA-$P_i$-NAGK interaction network. Based on our data and previously published data, we conclude that the $P_i$ signal transduction system does not work as an on/off switch governed by the intrinsic ATPase activity of $P_i$, but rather as a multitasking signal integration device with flexible signal output dependent on its receptor protein.

### EXPERIMENTAL PROCEDURES

*Overexpression and Purification Of Recombinant $P_i$, NAGK, PipX, and NtcA—*The *glnB* and *pipX* genes from *S. elongatus*, cloned into the Strep-tag fusion vector pASK-IBA3 (IBA, Göttingen, Germany), were overexpressed in *E. coli* BLR9060 (32). The $P_i$ and PipX proteins, each with a C-terminal fused Strep-tag peptide, were purified using affinity chromatography as described previously (20, 22). The genes encoding NAGK and NtcA from *S. elongatus*, cloned in pET15b vector, were overexpressed in *E. coli* strain BL21(DE3) (33), and the His$_6$-tagged recombinant proteins were purified as reported previously (20, 22).

*Surface Plasmon Resonance (SPR) Analysis—*SPR experiments were performed using a Biacore X biosensor system (Biacore AB, Uppsala, Sweden) at 25°C in HBS-Mg buffer containing 10 mM HEPES, 150 mM NaCl, 1 mM MgCl$_2$, and 0.005% Nonidet P-40, pH 7.5, as described previously (21). To immobilize purified His$_6$-NAGK on the Ni$^{2+}$-loaded NTA sensor chip of flow cell 2 (FC2), 50 μL of a 30 nM His$_6$-NAGK solution (of the hexamer) was injected until a binding signal of ~3000 resonance units (RU) was reached, which corresponds to a surface concentration change of 3 ng/mm$^2$. To measure the effect of PipX on the association of the $P_i$-NAGK complex, a solution of 100 mM $P_i$ was injected over the His$_6$-NAGK surface immobilized on the sensor chip in the presence or absence of 100 mM PipX and with various ratios of ATP:ADP (with 1 mM MgCl$_2$). To determine the influence of $P_i$ on the NtcA-PipX interaction, 125 mM His$_6$-NtcA (of the dimer) in a volume of 60 μL was injected onto the Ni$^{2+}$-loaded NTA sensor chip to receive a binding signal of ~1500 RU. PipX (500 nM) was injected in the presence of various effector concentrations, 1 mM MgCl$_2$, and 500 nM $P_i$. To load fresh proteins on the NTA sensor chip,
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TABLE 1
Overview of structural data used in this work

| Structure PDB entry | Protein(s) and organism | Number of subunits in the AU | Detailed description of AU | Detailed co-factors description | Nomenclature of the PII trimer in this paper | Nomenclature of individual effector sites in this paper | Resolution (Å) | Reference |
|---------------------|-------------------------|-----------------------------|---------------------------|--------------------------------|------------------------------------------|------------------------------------------------------|---------------|----------|
| 4C3L                | PII from S. elongatus   | One monomer                 | None                      | PII NCI                        |                                          |                                                      | 1.6           | This work |
| 4C3M                | PII from S. elongatus   | One trimer                  | None                      | PII NCI                        |                                          |                                                      | 2.2           | This work |
| 4C3K                | PII from S. elongatus   | Two trimers                 | Trimer I                  | One ADP                        | PII ADP1                                 | PII ADP1-S1                                         | 3.1           | This work |
|                     |                         |                             | Trimer II                 | Three ADP                      | PII ADP1                                 | PII ADP1-S1                                         |               |          |
|                     |                         |                             | Trimer III                | Three ADP                      | PII ADP1                                 | PII ADP1-S1                                         |               |          |
| 2XZW                | PII from S. elongatus   | Three trimers               | Trimer I                  | Three ATP                      | PII OG1                                 | PII OG1-S1                                          | 1.95          | (11)     |
|                     |                         |                             | Trimer II                 | Three ATP                      | PII OG1                                 | PII OG1-S1                                          |               |          |
|                     |                         |                             | Trimer III                | Three ATP                      | PII OG1                                 | PII OG1-S1                                          |               |          |
| 2XUL                | PII from S. elongatus   | Two identical trimers       | Trimer I                  | Three ATP                      | PII OGx                                 |                                                      | 2.2           | (11)     |
|                     |                         |                             | Trimer II                 | Three ATP                      | PII OGx                                 |                                                      |               |          |
| 2XBP                | PII from S. elongatus   | One monomer                 | One ATP                   | PII NCI                        |                                          |                                                      | 1.2           | (9)      |
| 4AFF                | PII from S. elongatus   | One monomer                 | One Mg2<sup>2+</sup>       | PII NCI                        |                                          |                                                      | 1.05          | (31)     |
| 1QY7                | PII from S. elongatus   | One trimer                  | One ATP                   | PII NCI                        |                                          |                                                      | 2.0           | (6)      |
| 29D                 | GlnK1 from M. jannashii | Four trimers                | One AMP                   | PII NCI                        |                                          |                                                      | 2.1           | (42)     |
|                     |                         |                             | One AMP                   | PII NCI                        |                                          |                                                      |               |          |
| 1V9Q                | PII from T. thermophiles| One trimer                  | Three ADP                 | PII NCI                        |                                          |                                                      | 1.8           | (8)      |
| 3TA1                | PII from A. fulgidus    | One trimer                  | Three ADP                 | PII NCI                        |                                          |                                                      | 1.9           | (30)     |
| 3NCR                | PII from A. fulgidus    | One trimer                  | Three ADP                 | PII NCI                        |                                          |                                                      | 1.44          | (41)     |
| 2XG8                | PII/PipX from S. elongatus | One trimeric complex    | None                      | PII NCI                        |                                          |                                                      | 3.2           | (24)     |
| 3N5B                | PII/PipX from Nostoc sp.| One monomeric complex      | ADP                       | PII NCI                        |                                          |                                                      | 1.9           | (48)     |
| 2V5H                | PII/NAGK from S. elongatus | One NAGK hexamer      | N-Acetyl-l-glutamate       | PII NCI                        |                                          | N-Acetyl-l-glutamate (bound to NAGK)                | 2.75          | (46)     |

bound proteins were first removed by injection of 25 µl of 0.4 M EDTA, pH 7.5; the chip could then be loaded again with 5 mM NiSO₄ solution and the His<sup>+</sup>-tagged protein.

Isothermal Titration Calorimetry for Determination of Binding Constants—Isothermal titration calorimetry experiments were performed on a VP-ITC microcalorimeter (MicroCal, LCC) in buffer containing 10 mM HEPES, pH 7.4, 50 mM KCl, 50 mM NaCl, and 1 mM MgCl₂ at 20 °C. Isotherms of ADP and ATP binding to P<sub>II</sub> (25 µM trimer concentration) were determined in the presence of 75 µM PipX. For one measurement, 5 µl of 1 mM 2-OG was injected 35 times (2.1–146.8 µM) into the measuring cell containing P<sub>II</sub> protein (cell volume 1.4285 ml) with stirring at 350 rpm. The binding isotherms were calculated from the recorded data and fitted to the three-site binding model using the MicroCal ORIGIN software (Northampton, MA) as indicated.

Crystallization of Recombinant S. elongatus P<sub>II</sub> Protein—P<sub>II</sub> protein was crystallized using the sitting-drop technique by mixing 400 nl of the protein solution with equal amounts of the reservoir solution with the honeybee robot (Genomic Solutions Ltd). Drops were incubated at 20 °C, and images were recorded by the Rocklagger system (Formulatrix, Waltham, MA). For the P<sub>II</sub>-ADP form, the crystallization buffer was composed of 10 mM Tris, pH 7.4, 0.5 mM EDTA, 100 mM NaCl, 1% glycerol, and 2 mM ADP; crystals formed with the precipitant polyethylene glycol (PEG) 4000 at 20% in 100 mM Tris. Crystals in the absence of ADP were achieved under similar conditions with 18% PEG6000 and 100 mM Hepes, pH 6.5. Using glycerol or PEG 400 as the cryoprotectant, crystals were flash-frozen in liquid nitrogen. Diffraction data were collected at the Swiss Light Source (Villigen, Switzerland). Diffraction images were recorded with a MarCCD camera 225 (MAR Research, Norderstedt, Germany), and images were processed using XDS/XSCAL software (34). The crystal structure was solved by molecular replacement using the program Molrep (35) for which the high resolution structure 4AFF was used as a starting model. The structure was rebuilt and refined using the programs Coot and Refmac (36, 37). The quality of the structure was analyzed by the Procheck program (38). Figures were generated using PyMOL. The resolution of the wild type structures can be considered as high (1.6 Å) and medium resolutions (2.2 Å), and hence structural details and changes at this resolution can be clearly monitored. The structure in complex with ADP is at 3.1 Å, and the exact location of residues and co-factors becomes more critical. Details of the structure (such as side chains or termini) were introduced only on the basis of a clear electron density distribution for all atoms. Therefore, we believe that we discuss details that were clearly verified. At this lower resolution the structure is not being discussed on the basis of exact bond lengths and intra-residue distances as these may become
imprecise while we focus on relative changes such as residue presence or absence and residual geometries.

RESULTS

PII Structures Carry Flexible Modules to Adapt to Effector and Protein Interaction—Crystal structures of PII enzymes have shown a variety of conformational states, with transitions typically affecting flexible modules surrounding the structurally invariant β-sheet core. These changes are expressed in particular in the β-loop element and in the C terminus in response to protein-protein interactions or effector interactions, or they are artificially stabilized through crystallographic contacts. More subtle changes, detectable in the vicinity of the ligand-binding sites, may be involved in allosteric regulation of neighboring protein-protein interactions or effector interactions, or they are invariant augmenting core periphery.

In the PII protein from S. elongatus, the conformational states resulting from metabolic signal sensing and signal transduction of the protein have been thoroughly studied. However, snapshots of the protein alone and in complex with ADP have been lacking. Hence, we crystallized the S. elongatus PII protein in the absence and presence of ADP. Two crystal structures of the effector-free protein were resolved, one at 1.6 Å and one at 1.4 Å for PII NC1/PII NC2, resulting in an r.m.s.d. of 1.6 Å for the monomer with 76 of 91 C atoms superimposed, 1.4 Å for PII NC1/1QY7, and 0.9 Å for PII NC2/1QY7. The significant structural deviation between PII NC1 and PII NC2 likely results from the unique crystal packing obtained in the high resolution structure PII NC1, which shows crystal contacts exclusively mediated through anti-parallel strand pairing of the surface-exposed C-terminal β5-strand (Fig. 1A). This structural arrangement known as β-augmentation restricts the back-folding of the C terminus in PII NC1 and thereby prevents the formation of the terminal β6-strand (Fig. 1B). As a consequence, helix α2 is not further stabilized through interactions with the β5/β6-strands and is released to move outward from the β-sheet core (Fig. 1B). Whereas in the mutant structure

| Table 2 | Data collection and refinement statistics |
|---------|-----------------------------------------|
| PII NC1 | PII NC2 | PII ADP |
| **Space group** | P321 | P321 | P6_1 |
| **Cell dimensions** | 65.69, 65.69, 49.20 | 73.42, 73.42, 95.63 | 122.46, 122.46, 80.47 |
| a, b, c (Å) | 90, 90, 120 | 90, 90, 120 | 90, 90, 120 |
| α, β, γ (°) | 90, 82.9, 120 | 93.6-2.15 (2.28-2.15) | 48.7-3.1 (3.29-3.1) |
| Resolution (Å) | 33.1-1.6 (1.7-1.6) | 0.07 (0.34) | 0.13 (0.76) |
| Rmerge or Rmerge | 32.3 (4.9) | 20.2 (2.4) | 8.8 (1.8) |
| Completeness (%) | 99.9 (99.4) | 100.0 (99.8) | 87.8 (90.1) |
| Redundancy | 8.1 (7.8) | 13.7 (13.6) | 3.2 (3.5) |
| **Refinement** | | | |
| Resolution (Å) | 33.1-1.6 (1.7-1.6) | 63.2-1.6 (2.28-2.15) | 45.3-3.1 (3.41-3.10) |
| No. reflections | 16,722 | 16,524 | 0.21/0.23 (0.31/0.37) |
| Rmerge/Reff | 0.21/0.23 | 0.23/0.27 (0.30/0.36) | 0.21/0.27 (0.30/0.36) |
| No. atoms (all) | 48 | 48 | 48 |
| Protein | 258 (97.0) | 258 (100.0) | 546 (96.8) |
| B-Factors | 31.8 | 31.8 | 122.46, 122.46, 80.47 |
| Water | 31.8 | 31.8 | 122.46, 122.46, 80.47 |
| r.m.s.d. | 1.28 | 0.78 | 1.05 |
| Bond lengths (Å) | 0.004 | 0.004 | 0.005 |
| Bond angles (°) | 0.78 | 0.78 | 1.05 |
| Ramachandran statistics | | | |
| Residues in favored region, no. (%) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Residues in allowed region, no. (%) | 253 (95.8) | 245 (97.0) | 546 (96.8) |
| Residues in outlier region, no. (%) | 26 (100.0) | 258 (100.0) | 568 (100.0) |
| PDB entry | 4C3I | 4C3M | 4C3K |
The T-loop is extended in a 90° angle relative to the trimeric molecular axis and is stabilized by crystal contacts, in PII NC1 and PII NC2, the T-loops are entirely disordered (Figs. 1A and 2). Residues at the basis of the T-loop located between β9232 and β9233 strands (Gly-35/Phe-36 and Phe-55/Leu-56) are stabilized through backbone interactions with each other and by backbone interaction with residues Thr-28 and Met-29 of the β9232* strand of the neighboring subunit (Fig. 2A). In the following analysis and comparisons, we selected the PII NC2 structure because its overall geometry is the least affected by crystallographic packing artifacts. The three subunits of this structure are also identical, with an r.m.s.d. of 0.2 Å, and the comparison is not distorted after superposition of individual subunits.

FIGURE 1. Comparison of PII structures from *S. elongatus* in the absence of cofactors. All structures are shown as schematics prepared using PyMOL. A, superposition of the two wild-type structures PII NC1 (green) and PII NC2 (red) with the previously published mutant structure (1QY7; blue). The structures are shown in two perspectives (top and side views), which are related to each other through a rotation around the x axis by 90°. The structures show significant deviations (yellow arrows) in the T-loop conformations and the C-terminal extensions (CT), which strongly depend on the crystallographic environment of the protein trimer. B, crystallographic packing of the PII NC1 structure, which shows that the C-terminal β-strand β6 is not formed due to crystallographic contacts (encircled by dotted lines) of trimers via their β6 strands.

PII S49A the T-loop is extended in a 90° angle relative to the trimeric molecular axis and is stabilized by crystal contacts, in PII NC1 and PII NC2, the T-loops are entirely disordered (Figs. 1A and 2). Residues at the basis of the T-loop located between β2 and β3 strands (Gly-35/Phe-36 and Phe-55/Leu-56) are stabilized through backbone interactions with each other and by backbone interaction with residues Thr-28 and Met-29 of the β2* strand of the neighboring subunit (Fig. 2A). In the following analysis and comparisons, we selected the PII NC2 structure because its overall geometry is the least affected by crystallographic packing artifacts. The three subunits of this structure are also identical, with an r.m.s.d. of 0.2 Å, and the comparison is not distorted after superposition of individual subunits.

Structural Changes upon ADP Binding and Allosteric Modulation—A comparison of the trimeric structure of PII NC2 and of PII ADP1 revealed a small r.m.s.d. of 0.7 Å for 269 Ca atoms, which is in the same range as that determined for the PII ADP1/PII ADP3 trimers, i.e., 0.6 Å for 280 Ca atoms superimposed. After superimposing the trimers, we analyzed changes of mobile elements in these structures (T-loop and C terminus) and in the vicinity of the effector-binding sites S1-S3. The small changes surrounding the binding sites are in particular indica-
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![Diagram](image)

**Figure 3. Transition between structures $P_{II}^{ADP1}$ and $P_{II}^{ADP3}$ and differential binding mode of ADP.** A, superposition of the $P_{II}^{ADP1}$ (orange and yellow) and $P_{II}^{ADP3}$ (dark blue and magenta) structures shown as schematics with side views of the S1 effector binding sites. Secondary structure elements are marked with $\alpha1-\alpha2$ and $\beta1-\beta4$ (with * for the second molecule). The ADP cofactors are represented by stick structures and color-coded according to the protein backbone (orange and blue). B, close-up of the S1 site of $P_{II}^{ADP3}$ highlighting residues (labeled and represented by stick structures) involved in ADP binding. C, comparison of the structures of the S2 binding site of $P_{II}^{ADP1}$ and $P_{II}^{ADP3}$. The color codes are the same as for A, but additional subunits are light orange ($P_{II}^{ADP1}$) and light blue ($P_{II}^{ADP3}$). Significant structural changes in B-loop, T-loop, and C termini induced by ADP binding are enclosed in dotted rectangles. D, close-up of the S2 site of $P_{II}^{ADP3}$. ADP is bound only via main-chain interactions to residues of the B-loop and T-loop.

Aromatic of the anti-cooperative behavior of $P_{II}$ proteins (see data below). Per our definition, the individual sites in the $P_{II}^{ADP}$ state are denoted $P_{II}^{ADP1-S1/S2/S3}$, with site S1 occupied by the single ADP and sites S2 and S3 ligand free. The three occupied sites of the $P_{II}^{ADP3}$ complex are termed accordingly, i.e. $P_{II}^{ADP3-S1/S2/S3}$.

First, to decipher the structural transitions in response to ADP binding, we visualized changes using fully occupied $P_{II}^{ADP3}$ relative to the ligand-free $P_{II}^{NC2}$ structure (Fig. 2B). ADP binding in sites S1 and S2 causes stabilization and translocation of the C terminus toward the ADP-binding sites (Figs. 2B and 3, A and C) through a cascade of small changes in the side and main chains. ADP binding induces the realignment of Arg-101 toward the $\beta$-phosphate. This shift is further transferred to Glu-106 located on the C-loop, which forms a salt bridge with Arg-101. Both side chains contact the backbone of Ala-111, whereby a small helix motif is moved into the subunit interface (Fig. 2B). ADP binding further leads to the stabilization of terminal residues of the T-loop (Gly-37–Arg-38–Gln-39), which form pure backbone interactions with the phosphate moiety, mainly mediated by Arg-38 (Figs. 2B and 3B).

Next we compared the single and triple occupied ADP states to identify small changes that may reflect the anti-cooperative behavior of $P_{II}$ from *S. elongatus* (Fig. 3). Analysis of the $P_{II}^{ADP1-S1}$ and $P_{II}^{ADP1-S1}$ binding sites indicates only minor changes in backbone conformations, possibly introduced through the different diphosphate torsion angle geometries (Fig. 3A). The general binding mode of ADP in both S1 sites is based on main-chain H-bond interactions formed with Gly-87 and Asp-88 and a weaker additional contribution with Gly-89 (~3.5 Å apart from ADP) located in the B-loop. The backbone of Arg-38 from the T-loop further stabilizes ADP from the “bottom” side (Fig. 3B). Side chains of Lys-90 and of C-loop residues Arg-101 and Arg-103 contribute to the further alignment and stabilization of the phosphate moiety of ADP from the top side (Figs. 3B and 4B, left). All residues that contribute side chains and glycines that allow backbone flexibility to adapt to the various effector molecules are highly conserved in the canonical $P_{II}$ proteins (31). Interestingly, the coordination of ADP in the $P_{II}^{ADP3-S2}$ and $P_{II}^{ADP3-S3}$ sites is quite different from that in $P_{II}^{ADP3-S1}$, in agreement with this difference, the superposition of the three $P_{II}^{ADP3}$ subunits shows a significantly increased structural heterogeneity at the C-terminal extensions and at the ADP-binding site. Specifically, in the S2 and S3 sites, the side-chain interactions of Lys-90, Arg-101, and Arg-103 with ADP are lacking, and the phosphate moiety of ADP is only stabilized by main-chain interactions with Gly-87, Gly-37, and Arg-38 (Figs. 3D and 4B). Of particular interest is the structural rearrangement of the side chain of Lys-58, which plays a key role in various $P_{II}$ conformations (12). In the S1 site, Lys-58 interacts with the side chain of Gln-39, whereas in the S2 and S3 sites it approaches the ADP-ribose moiety instead (Fig. 3D). Furthermore, in the S1 site, the backbone of B-loop residues Ile-86 and Gly-87 interact with the side chains of Gln-39 and Lys-58, respectively. These interactions result in a trapezoidal arrangement between the B-loop backbone (Ile-86, Gly-
87), Gln-39 from the T-loop basis, and Lys-58 from the core of the PII body (Fig. 5). This arrangement, which shifts the basal part of the T-loop closer to the B-loop in the S1 site, does not exist in the S2 and S3 sites.

With regard to the transition of the S2 site from the PII\textsubscript{ADP1} state to the PII\textsubscript{ADP3} state (see Fig. 3C), the most obvious movement is observed at the C terminus. A short C-terminal \(\alpha\)-helix (\(\alpha_{C-T}\)) of PII\textsubscript{ADP3-S2} is formed and folds back toward the ADP-binding site. This movement is similar to that observed in the transition of the S1 site from the co-factor-free state to the ADP-filled state (see above). By contrast, this transition does not occur in the S3 site (Fig. 4A). The S2 site of the PII\textsubscript{ADP1} state displays several subtle transitions near the ADP-binding site. In the PII\textsubscript{ADP1} state, the N-terminal end of the \(\beta\)-sheet is slightly shifted toward the empty binding pocket (Fig. 3C, \textit{flexed arrow}). Furthermore, subtle changes in the B-loop and C-loop are visible. Interestingly, the side chain of the key residue Lys-58 is displaced in the S2 site of PII\textsubscript{ADP1} as compared with the S1 site of the PII\textsubscript{ADP1} or PII\textsubscript{NC2} state, where it contacts the backbone of Gly-87. These subtle changes in S2 are apparently caused by the binding of ADP to the S1 site in PII and could affect the binding mode of the second ADP molecule described above.

\textbf{Structural Differences between ADP and ATP Binding to PII from S. elongatus—}\ To compare ADP binding of PII\textsubscript{ADP3} with the most closely related PII\textsubscript{ATP}\textsubscript{O1} complex from \textit{S. elongatus}, we decided to use the PII\textsubscript{OG1}\textsubscript{O1} trimer complex with three ATP and one Mg\textsuperscript{2+}/2-OG (note that the PDB entry 2XZW contains three independent trimers: PII\textsubscript{OG1} with one active site occupied by ATP/Mg\textsuperscript{2+}/2-OG and PII\textsubscript{OG2} and PII\textsubscript{OG3} in the asymmetric unit). The superposition of this structure onto PII\textsubscript{ADP3} resulted in an r.m.s.d. of 0.7 Å for 292 Ca atoms. Overall, the changes in the C terminus and in the B-loop are minute (Fig. 4A). However, the binding modes of ADP and ATP are significantly different (Fig. 4B). Binding of ATP is more similar to binding of ADP to the S1 site (involving side-chain interactions of Lys-90 and Arg-101/-103) than to binding of ADP to the weakly coordinated S2 and S3 sites of PII\textsubscript{ADP3} (see above). In contrast to the binding of ADP to the S1 site, ATP interacts with side chains of Arg-38 and Gln-39 of the basal part of the T-loop. As a consequence, the trapezoidal arrangement between the B-loop backbone, Lys-58, and Gln-39 in PII\textsubscript{ADP3-S1} is broken. Overall, the larger number of interactions increases the affinity of ATP toward all three binding sites but leads to only a minor stabilization of the distal part of the T-loop (Fig. 4A).

\textbf{Influence of Interaction Partner PipX on PII Effector Binding—}\ To understand the structural basis for the positive effect of ADP on the interaction of PII with the PII-target protein PipX described previously (15), we compared the PII\textsubscript{ADP3} structure
with the structures of the PII/PipX complex from *S. elongatus* and the PII/PipX-ADP complex from *Anabaena* sp. strain PCC 7120 (Table 1). The PII/PipX complexes from these two cyanobacteria show very similar vertically extended T-loop conformations (Fig. 6, A and B) that clasp the PipX partner protein. In our comparison of these complexes with PII ADP3, the PII conformation in the PII/PipX-ADP complex from *Anabaena* sp. PCC 7120 is almost identical to the structure of the S1 site of PII ADP3 but differs from that of the S2 and S3 sites (Fig. 7). Notably, the hydrogen-bonding network organized by residues Lys-58, Gln-39, Gly-87, and Ile-86 that drags the basal part of the T-loop close to the B loop is conserved, which causes a similar folding of the T-loop basis (see also Fig. 5). We questioned whether this particular T-loop conformation, which appears to be stabilized by PipX binding, influences the interaction between PII and its effectors. Using isothermal titration calorimetry, we determined the binding affinities of ADP and ATP to PII in the presence of equimolar amounts of PipX and PII (monomers). The data obtained were calculated assuming a model with three binding sites and indicated that PipX influences both affinity and anti-cooperativity of the binding reaction (Table 3). In the presence of PipX, binding site S1 has increased affinity for ADP (1.6-fold) but a 6-fold decreased affinity for ATP, which results in an inversion of the adenylate nucleotide preference, with a 3.5-fold higher affinity for ADP than for ATP. For binding site S2, PipX strongly decreases the affinity for both ATP and ADP binding (8- and 13-fold increase in $K_D$, respectively), whereas binding site S3 is almost unaffected by PipX. In conclusion, only the first binding site in the
PipX complex becomes very sensitive to ADP. This behavior fits very well with the asymmetric structure of the PipX-ADP complex; PipX binding stabilizes the S1 site conformation of PipX-ADP, whereas the conformation of the S2 and S3 sites is displaced by PipX. By this mechanism, PipX enhances the sensitivity of PipX toward ADP by favoring the PipX-PipX-ADP, ATP2 population over PipX-PipX-ATP3. Because coordination of ADP prevents the binding of 2-OG, the above results explain our previous observation that ADP can strongly antagonize the inhibitory effect of 2-OG on PipX interaction (15).

Control of the PipX-NtcA Interaction Network by the ATP/ADP Ratio—PipX is a co-activator of transcription factor NtcA (23). When loaded with 2-OG, the dimeric NtcA protein binds two PipX monomers (24). The interaction of PipX with PipX serves to sequester PipX and to prevent NtcA activation. From the biochemical properties it is evident that 2-OG levels control the distribution of PipX between NtcA and PipX (the PipX-NtcA interaction network), with higher 2-OG levels shifting the equilibrium toward NtcA. However, the modulation of 2-OG-controlled PipX-NtcA interaction by ADP raises the question of whether PipX-NtcA interaction in the presence of PipX may respond to the cellular energy state (measured as ATP/ADP ratio). To resolve this issue, we investigated the PipX-NtcA interaction network using an SPR spectroscopy-based assay in which the NtcA protein was fixed on the surface of a sensor chip and challenged with a mixture of PipX and PipX in the presence of ATP and ADP at various ratios and with a fixed concentration of 2-OG. In the presence of 2-OG, PipX binds to NtcA unless prevented by PipX. Therefore, an increase in RU, which measures the binding of PipX to NtcA, indicates the distribution of PipX between PipX and PipX in control experiments in the absence of PipX, the adenylyl nucleotide regime did not influence the PipX-NtcA interaction (Fig. 8A, gray lines). In the presence of PipX and 2 mM Mg2+-ATP plus 4 mM 2-OG, the NtcA-PipX interaction was not affected because PipX in its ATP-2-OG conformation is not suitable for PipX binding (24). In the presence of 2 mM ADP and 4 mM 2-OG, however, PipX has a higher affinity for PipX and suppresses the NtcA-PipX complex formation as a competitive factor. In the presence of ATP and ADP at a 1:1 ratio and 4 mM 2-OG, the association of PipX to immobilized NtcA was reduced to one-third that in the absence of PipX.

We then investigated the effect of subtle changes in the energy state (ATP/ADP ratio) on the PipX-NtcA interaction network at high (4 mM) and intermediate (1 mM) 2-OG levels using the SPR assay described above. The response signal at the end of the PipX injection phase (t = 197 s) was taken as the value of protein binding (Fig. 8). In the presence of 1 mM 2-OG, the NtcA-PipX-PipX network was extremely sensitive to the ATP/ADP ratio (Fig. 8A). Even small amounts of ADP (at an ADP:ATP ratio of 0.05:1) allowed PipX to reduce the binding of PipX to NtcA by 40% (setting the binding of PipX to NtcA in the presence of 2-OG and ATP to 100%). When the 2-OG concentration was increased to 4 mM, the effect of ADP (in presence of 1 mM ATP) was greatly diminished. For example, 0.5 mM ADP decreased NtcA-PipX interaction only by 35% (Fig. 8B) compared with >80% reduction in the presence of 1 mM 2-OG.

Competition between NAGK and PipX for PipX—In addition to interacting with PipX, PipX is further involved in the activation of NAGK in S. elongatus. In vitro the interaction of NAGK with PipX is antagonized by both ATP/Mg2+ /2-OG and ADP. High ADP/ATP ratios, which are probably non-physiological, are required to noticeably impair NAGK activation by PipX (39). Because PipX and NAGK compete for the 2-OG-free PipX populations, we investigated the possibility that PipX may successfully compete with NAGK for PipX in an energy state (ATP/ADP ratio)—depen-

FIGURE 8. SPR analysis of PipX binding to NtcA in the presence of competitor PipX. PipX was bound to FC2 of a Ni2+-loaded NTA sensor chip (see “Experimental Procedures”), and FC1 was used as a control. The response difference (ΔRU) between FC2 and FC1 is shown. A, PipX was preincubated with PipX in the presence of 4 mM 2-OG and with various concentrations of ATP and ADP (solid line, 2 mM ATP; dashed line, 2 mM ADP; dot-dashed line, 2 mM ATP plus 2 mM ADP) and then injected onto immobilized NtcA. The PipX binding experiments in the presence of PipX are shown in black, the control binding experiment without PipX is in gray. B, PipX was preincubated with PipX in the presence of 1 mM ATP together with various amounts of ADP in the presence of either 1 mM (B) or 4 mM (C) 2-OG and then injected onto immobilized NtcA. The response signal at t = 197 s after the start of the injection phase was taken as a measure of protein binding. Error bars indicate standard deviations from three independent measurements.
dotted recruited PII from its complex with PipX, which in turn slows both conditions, the binding of PII to NAGK was decreased reproducibly slower than in the absence of PipX (Fig. 9).

NAGK in the presence of 1 mM ATP regardless of the presence down complex formation. In the presence of 1 mM ADP, that used for the analysis of PII

PipX, the association of the PII

GlnK2 (first/second sites: 10-fold; second/third sites: +4-fold) (41). Although these differences are obvious, they require explanation on a structural basis. Yet structural asymmetry is often not analyzed in detail even though previously published structures from different organisms indicate such an asymmetric behavior of PII proteins. As found for PII from S. elongatus, some binding sites in the same trimeric molecule of some structures were occupied, and others were unoccupied. For example, in the structure of GlnK1 from the archaeon Methanocaldococcus jannaschii crystallized in the presence of ADP with four trimers in the asymmetric unit, one or two ADP molecules occupied the binding sites (see Table 1) (42). Interestingly, all these partially occupied trimers occurred in the same asymmetric unit. Similarly, the ATP-Mg2-OG complex of S. elongatus PII could be crystallized with three PII trimers in the asymmetric unit containing one, two, or three bound 2-OG molecules. The increase in $K_D$ value from site S1 ($5.1 \pm 4.0 \mu M$) to site S2 ($11.1 \pm 1.8 \mu M$) to site S3 ($106.7 \pm 14.8 \mu M$) were in line with changes in the structures. The absence of 2-OG in the S2 and S3 binding sites was accompanied by the absence of Mg2+. Moreover, binding of the first 2-OG/Mg2+ molecules introduced a structural transition in the geometry of the neigh-

**DISCUSSION**

Structures of PII signaling proteins, with their archetypical motifs including a conserved quaternary structure, T-, B-, and C-loops, and effector binding, have been studied in detail. However, most of the structural studies interpret the complex as being symmetric, with ligand-binding sites treated equivalently with respect to the stereochemistry. Moreover, the T-loop as prototype of a highly sophisticated signaling device has been observed in many different, mostly elongated conformations, which are always artificially stabilized by crystal contacts. By contrast, most of the PII structures from the cyanobacterium S. elongatus are not influenced by any crystallographic artifacts, and hence this system may provide the appropriate structural information to understand the function of PII. Our work presented here and previous studies have led to a repertoire of structures of PII from S. elongatus: PII without cofactors, PII+ADP1, PII+ADP2, PII+OG1, PII+OG2, PII+OG3, PII in complex with NAGK or PipX, and the mutant structures PII(S49A) and PII(I86N) as well as PII(I86N) in complex with ATP/Mg2+ and citrate in place of 2-OG. Hence, only PII in complex with ATP and mixed ADP/ATP structures are lacking for describing the structural basis of PII physiology. A summary of the various cyanobacterial structures together with other PII structures discussed in the following is shown in Table 1. A comprehensive table summarizing all PII structures resolved up to 2013 is given in Huergo et al. (40).

**Structural Basis of Anti-cooperativity**—A number of functional studies have shown that binding of effector molecules may occur cooperatively and/or anti-cooperatively. For PII from S. elongatus, anti-cooperativity for all three sites has been reported (9, 11). By contrast, the first and second 2-OG sites of the archaeal Archaeoglobus fulgidus GlnK3 protein show strong anti-cooperativity (−40-fold), but the second and third binding sites show cooperativity (+10-fold) (30). A similar anti-cooperative/cooperative coupling occurs with ATP binding to A. fulgidus GlnK2 (first/second sites: −10-fold; second/third sites: +4-fold) (41). Although these differences are obvious, they require explanation on a structural basis. Yet structural asymmetry is often not analyzed in detail even though previously published structures from different organisms indicate such an asymmetric behavior of PII proteins. As found for PII from S. elongatus, some binding sites in the same trimeric molecule of some structures were occupied, and others were unoccupied. For example, in the structure of GlnK1 from the archaeon Methanocaldococcus jannaschii crystallized in the presence of ADP with four trimers in the asymmetric unit, one or two ADP molecules occupied the binding sites (see Table 1) (42). Interestingly, all these partially occupied trimers occurred in the same asymmetric unit. Similarly, the ATP-Mg2-OG complex of S. elongatus PII could be crystallized with three PII trimers in the asymmetric unit containing one, two, or three bound 2-OG molecules. The increase in $K_D$ value from site S1 ($5.1 \pm 4.0 \mu M$) to site S2 ($11.1 \pm 1.8 \mu M$) to site S3 ($106.7 \pm 14.8 \mu M$) were in line with changes in the structures. The absence of 2-OG in the S2 and S3 binding sites was accompanied by the absence of Mg2+. Moreover, binding of the first 2-OG/Mg2+ molecules introduced a structural transition in the geometry of the neigh-

**Target-dependent ADP Sensing by a PII Signal Processor**

![Figure 9. PipX effect on the PII-NAGK interaction.](image-url)

For SPR measurements NAGK was bound to FC2 of a Ni2+-loaded NTA sensor chip (see “Experimental Procedures”), and FC1 was used as a control. The response difference ($\Delta RU$) between FC2 and FC1 is shown. A, association of PII-NAGK complex influenced by PipX and effectors; 100 nM PII was preincubated with 1 mM ATP (solid line), 1 mM ATP + 100 nM PipX (dotted line), 1 mM ADP (dashed line), or 1 mM ADP + 100 nM PipX (dot-dot-dashed line) and afterward injected onto immobilized NAGK. B, association of PII-NAGK complex influenced by PipX and ATP and ADP at different ratios: 1 mM ATP + 1 mM ADP without PipX (solid line), 1 mM ATP + 1 mM ADP + 100 nM PipX (dotted line), 1 mM ATP + 2 mM ADP (dashed line), and 1 mM ATP + 2 mM ADP + 100 nM PipX (dot-dot-dashed line).
boring ATP molecule of the S2 site. In site S3, the ATP was clamped differently than in sites S1 and S2, and structural changes in the vicinity of S3 were visible (e.g. C terminus does not fold back to the ligand). In our presented studies, we wanted to analyze structural changes in PII after binding to ADP to trace the changes induced by the effector molecule. We obtained a crystal with two PII molecules in the asymmetric unit, one of which contained only one ADP molecule whereas the second was fully occupied. In the latter structure the second and third ADP molecules are less tightly coordinated, and the binding pocket is not shielded by a structured T-loop basis. These observations are in line with the binding constants of ADP, showing only one high affinity site. A similar effect is seen in the GlkK1 structure of M. jannaschii occupied by ADP (42), where one of four trimers is occupied by two ADP molecules. The first ADP molecule is tightly coordinated (involving the C-loop contacts similar to the S1 state of S. elongatus PII<sub>ADPS</sub>), and the binding site is in a closed conformation with a distance between T- and B-loop (measured between Gln-39 and Pro-86) of 5 Å. By contrast, the second site shows fewer coordinations and is in an open conformation (distance Gln-39 – Pro-86 of 7.8 Å). Asymmetry in ADP binding occurs also in the TT1020 protein structure, a PII homolog from Thermus thermophilus. Superposition of PII<sub>ADPS</sub> with TT1020 reveals that the corresponding S1 sites yield the lowest r.m.s.d. values for the overall structure geometry. All these structures are in support of an asymmetric arrangement of PII proteins, which is obviously not unique to the PII protein from S. elongatus. A recent dynamic correlation analysis of 2-OG binding to S. elongatus PII (43) calculated the binding pocket sizes and revealed that ligation of 2-OG to site S1 alters the binding pocket size in sites S2 and S3. In accord with this result, the binding of ADP to site S1 of S. elongatus PII leads to subtle structural changes in the other two sites, which results in their reduced affinity toward ligands. Although it seems plausible that the transmission of intersubunit signals occurs along the central β2/β3 core of PII, which directly links the effector binding sites with each other (see Figs. 1A and 2A), a detailed atomic description of structural transitions is limited by the resolution of the structure of 3.1 Å.

T-loop Conformations—The only conformation of a fully resolved T-loop unaltered by crystal contact has been trapped in the structure of the S. elongatus PII<sub>I86N</sub>-186N variant in the presence of ATP/Mg<sup>2+</sup>; the structure shows the T-loop stabilized through the formation of an engineered additional intramolecular H-bond (9, 31). None of the structures of wild-type S. elongatus PII<sub>I</sub> occupied by effector molecules (ADP, ATP, ATP/Mg<sup>2+</sup>/2-OG) shows the T-loop in an ordered conformation. In a number of PII<sub>I</sub> proteins crystallized so far, an artificial T-loop structure is strongly stabilized by crystal contacts, and individual changes due to effector binding are, therefore, difficult to detect (e.g. PDB entries 3TA1, 3NCR, 2J9D; see Table 1). Yildiz et al. (42) argue that upon binding of ADP, the entire T-loop becomes stabilized. In the four trimers of the asymmetric unit, all five T-loops associated with AMP or ADP were strongly stabilized by crystallographic contacts and showed significantly different orientations. Moreover, in one binding site not occupied by any effector molecule, the T-loop was stabilized only through crystallographic contacts. This finding coincides with a structure of the S49A variant of PII<sub>I</sub> from S. elongatus, where T-loops are also stabilized in the absence of effector molecules by simple crystallographic contacts (6). A plausible explanation for this variability assumes the T-loop as a very flexible “device” in solution that can adapt very easily to protein complexes of different size and follow different structural constraints given by proteins such as PipX, NAGK, or AmtB.

PII Protein-Effector Distribution in Cells—As a consequence of the extensive (anti-)cooperative properties of PII<sub>I</sub> proteins, distinct PII<sub>I</sub> populations will form in cells depending on the effector concentrations, with each of them having its own signaling properties. Moreover, modulation of signal perception by receptor interaction allows the PII-receptor system to respond with different sensitivity toward the different metabolites. For example, regulation of NAGK by PipX is highly sensitive to 2-OG but only weakly responds to fine-tuned changes in ADP levels, whereas the PII<sub>I</sub>PipX-NtcA interaction network responds strongly to the ADP/ATP ratio. Of the 10 theoretical states of PII<sub>I</sub> with completely occupied ATP/ADP sites, only 5 states are physiologically meaningful: the 3 PII<sub>I</sub>-ATP<sub>3</sub> states with 1–3 bound 2-OG molecules and the 2 PII<sub>I</sub>-ATP<sub>2</sub>-ADP<sub>1</sub> states with 0 or 1 2-OG molecule bound. The 2-OG-free PII<sub>I</sub>-ATP<sub>3</sub> state is unlikely to be formed as the K<sub>a</sub> of the first site (5 μM) is much lower than physiological 2-OG concentrations. The theoretically expected species PII<sub>I</sub>-ATP<sub>2</sub>-ADP<sub>2</sub> with two molecules of 2-OG is probably not formed, as concluded from previous isotherm titration calorimetry analysis (15). This implies that ADP, bound to one site of the PII<sub>I</sub> trimer, not only prevents 2-OG from binding to its own site but also has a negative impact on 2-OG binding to one neighboring subunit. The PII<sub>I</sub>-ATP<sub>2</sub>-ADP<sub>2</sub> species is probably irrelevant in S. elongatus cells, as its formation requires an excess of ADP over ATP, which is unlikely in living cells. The distribution of the various PII<sub>I</sub> states is further affected by the interaction with receptor proteins, as shown by the effect of PipX on ATP and ADP affinities for PII<sub>I</sub>. The fully ATP-ligated form of PII<sub>I</sub> will probably prevail in the absence of PipX, and this form clearly shows the highest sensitivity toward 2-OG and regulates NAGK. However, in complex with PipX, one binding site of PII<sub>I</sub> has a preference for ADP. Binding of ADP tunes down the antagonistic effect of 2-OG on NtcA-PipX interaction, and therefore, the PII<sub>I</sub>PipX-NtcA interaction network responds to the energy status: at very low 2-OG levels, which represents nitrogen excess (44), PipX cannot bind to NtcA and, hence, will always be bound to PII<sub>I</sub> irrespective of the ADP/ATP ratio. In the presence of intermediate concentrations of 2-OG (in vitro at 1 mM), PipX could bind to the NtcA-2-OG complex. As long as the ADP levels are low enough to enforce the PII<sub>I</sub>-ATP<sub>2</sub>-2-OG<sub>1</sub> state, PII<sub>I</sub> is unable to compete for PipX binding, and PipX is in fact bound to NtcA. However, a slight increase in ADP levels (0.05 mM) already affects the network; at a 0.05:1 ADP:ATP ratio, NtcA-PipX interaction is reduced by 40%. PipX increases the ADP sensitivity of PII<sub>I</sub> and thereby displaces 2-OG, allowing formation of the PII<sub>I</sub>·PipX complex. At even higher ADP levels (0.5 mM), most PipX (>80%) is bound to PII<sub>I</sub> and removed from NtcA. In essence, these data demonstrate that the PipX distribution between PII<sub>I</sub> and NtcA at intermediate (physiological) 2-OG levels is highly sensitive to the ADP/ATP ratio. At very high 2-OG levels (4
mm), the interaction between PipX and NtcA is so stable that ADP has only a marginal influence on the distribution of PipX. In physiological context, these results suggest that a main function of PipH in the NtcA-PipX-PipH network is to switch off PipX-activated NtcA gene expression when the energy supply of the cell ceases transiently under conditions of intermediate cellular 2-OG levels. 

Recent Advances in PipH Biology or Is PipH a Slow ATPase?—In the GlnK-AmtB complex, the T-loop blocks the pore for ammonium transport (5). As in the PipX interaction in cyanobacteria, the GlnK-AmtB complex in E. coli is formed in the presence of ADP and dissociates in the presence of ATP-Mg/2-OG. Although the regulated processes are completely different, the PipH response to metabolites and target regulation appeared to undergo the same conserved mechanism. A recent paper challenged this view; Radchenko et al. (26) showed that in the absence of 2-OG, GlnK displays intrinsic ATPase activity, slowly converting ATP-GlnK to ADP-GlnK, which then binds AmtB. The authors suggested that it is the 2-OG-regulated slow conversion of ATP-GlnK to ADP-GlnK, which then binds AmtB. The authors suggested that it is the 2-OG-regulated slow conversion of ATP-GlnK to ADP-GlnK, which then binds AmtB. Although this mechanism makes sense for PipH complexes stabilized by the ADP state, it contradicts the formation of PipH-receptor complexes stabilized by the ATP state of PipH, such as the PipH-NAGK association. PipH binds in its ATP state in the absence of 2-OG to its target NAGK. The 2-OG-regulated ATPase activity of PipH would prevent PipH-NAGK interaction due to formation of the ADP state of PipH. However, the experimental evidence of PipH-NAGK interaction contradicts this assumption (15, 39, 45, 46). Therefore, even if the intrinsic ATPase activity of GlnK is relevant for AmtB interaction, this mechanism cannot explain the diversity of PipH signal transduction in the whole PipH family. Numerous examples in other organisms have demonstrated that the PipH regulatory network is as complex as that in cyanobacteria. The combined effect of the metabolites ADP, ATP, and 2-OG on PipH signaling has been studied in E. coli and R. rubrum, and the sensing mechanism undergoes the same principle as in cyanobacteria (10, 25, 27, 29, 47). However, the aspect of competition between different PipH targets in these organisms has not yet been addressed and further complicates the system. 

In summary, the array of PipH signal-input states is not only organized by (anti-)cooperative intersubunit and effector molecule interactions but is further modulated by the receptor protein to which PipH is signaling. This enables the PipH proteins to simultaneously regulate multiple targets with different sensitivity in response to its metabolic effectors ATP, ADP, and 2-oxoglutarate. The asymmetry behavior of PipH proteins and its modulation by target proteins appears to be a key requisite for its function as a multitasking information processor. 

Acknowledgments—We thank Jan Lüddecke for critically reading the manuscript, Karen A. Brune for linguistic revision of the manuscript, Reinhard Albrecht and Vasuki Ranjani-Chellamuthu for help with crystallization and crystal handling, and the entire beamline staff of PXII at the Swiss Light Source for beamline maintenance and continuous support.

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