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The crystal structure of the complex of P_{II} and acetylglutamate kinase reveals how P_{II} controls the storage of nitrogen as arginine

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2OG, 2-oxoglutarate

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Bank, www.pdb.org (accession code 2V5H for crystal I and 2JJ4 for crystal II).

Abstract

Photosynthetic organisms can store nitrogen by synthesizing arginine, and, therefore, feed-back inhibition of arginine synthesis must be relieved in these organisms when nitrogen is abundant. This is accomplished by the binding of the P_{II} signal transduction protein to acetylglutamate kinase (NAGK), the controlling enzyme of arginine synthesis. Here, we describe the crystal structure of the complex between NAGK and P_{II} of *Synechococcus elongatus*, at 2.75 Å-resolution. We prove the physiological relevance of the observed interactions by site-directed mutagenesis and functional studies. The complex consists of two polar P_{II} trimers sandwiching one ring-like hexameric NAGK (a trimer of dimers) with the threefold axes of these molecules aligned. The binding of P_{II} favors a narrow ring conformation of the NAGK hexamer that is associated with arginine sites having low affinity for this inhibitor. Each P_{II} subunit contacts one NAGK subunit only. The contacts map in the inner circumference of the NAGK ring and involve two surfaces of the P_{II} subunit. One surface is on the P_{II} body, and interacts with the C-domain of the NAGK subunit, helping widen the arginine site found on the other side of this domain. The other surface is at the distal region of a protruding large loop (T-loop) that presents a novel compact shape. This loop is inserted in the interdomain crevice of the NAGK subunit, contacting mainly the N-domain, and playing key roles in anchoring P_{II} on NAGK, in activating NAGK, and in complex formation regulation by MgATP, ADP, 2-oxoglutarate and by phosphorylation of S49.

In photosynthetic organisms nitrogen can be stored by synthesizing arginine (1,2) and, therefore, feed-back inhibition of arginine synthesis must be relieved when nitrogen is abundant. The enzyme of arginine biosynthesis that is the target of arginine inhibition, N-acetyl-L-glutamate (NAG) kinase (NAGK) (1,3-5), was found in cyanobacteria and plants (2,4-8) to be a target of the carbon-nitrogen P_{II} signaling protein (9, 10), forming with it a complex in which arginine inhibition is alleviated (6,7).

P_{II} signaling proteins are homotrimers of a 12-13 kDa subunit that interact with enzymes, transcription factors and ammonia channels, regulating their activity (9,10) and carbon-nitrogen homeostasis. Numerous structures of P_{II} proteins, including those for cyanobacteria and plants (9-12) are known, but it was unclear how P_{II} proteins carry out their functions. The body of the P_{II} trimer is roughly hemispheric. Its subunits have $\beta\alpha\beta\alpha\beta$ topology, with α helices looking outwards and the β sheet inwards and providing the intersubunit interactions. Each subunit has three loops: the B- and C-loops, and the larger flexible T-loop. The T-loop residues Y51 and S49 are, respectively, the sites of the regulatory uridylylation and phosphorylation in enterobacterial and cyanobacterial P_{II} proteins (9,10), with S49 phosphorylation abolishing interaction with NAGK (4,6). ADP, MgATP and 2-oxoglutarate (2OG) bind at the T-loop (13) and modulate the binding of P_{II} to its targets (6,9,10). The recently determined structure of the inhibitory complex of GlnK (a P_{II} protein) with the ammonia channel AmtB of *Escherichia coli* (14,15) showed that the extended T-loop blocks the cytoplasmic opening of the ammonia channel, explaining channel function inhibition. This structure sheds no light on the P_{II}-NAGK complex, since P_{II} activates NAGK (6,7), and since ADP was found in the GlnK-AmtB complex (15) whereas ADP prevents P_{II}-NAGK complex formation (6).

We determine here the crystal structure of the P_{II}-NAGK complex of the cyanobacterium *Synechococcus elongatus* strain PCC 7942. We previously determined the structures of arginine-insensitive (16) and arginine-sensitive NAGKs (17). The latter are hexameric ring-like trimers of dimers with a central hole of 25-30 Å, in which the dimers resemble the homodimeric arginine-insensitive enzyme (16). The NAGK subunit is an open $\alpha_3\beta_8\alpha_4$ sandwich that can be divided into a N-domain and a C-domain. The N- and C-domains host, respectively, the NAG and ATP sites, on the C-edge of the central β -sheet. Arginine-sensitive NAGKs (17) have a N-terminal mobile kinked α -helix (called the N-helix) that, by interlacing with another dimer N-helix, links the dimers into the hexamer. The dimers are tilted relative to the ring plane, and the upper and lower ring surfaces are serrated, with three peaks, one per subunit. Arginine binds on each subunit next to the interdimeric junctions near the N-helices, widening the ring, decreasing the tilt of the dimers and reorienting the N-helices (17). In the present complex, the NAGK closely resembles other arginine-sensitive NAGKs. In contrast, the T-loop of P_{II} adopts a novel compact shape that is specifically adapted to this interaction. We use site-directed mutagenesis, functional assays and binding studies to confirm the *in vivo* relevance of this complex. The structure clarifies regulation of P_{II} binding to NAGK and P_{II} activation of NAGK. Sequence signatures identified here for P_{II} signaling through NAGK account for the restriction of this signaling to photosynthetic organisms.

RESULTS

Crystallization and overall structure of the complex. Crystal forms (Table 1) I and II, diffracted to 2.75 and 3.46 Å resolution, respectively. Phases were obtained for crystal II by molecular replacement using models of *Thermotoga maritima* NAGK (17) and of uncomplexed *S.elongatus* P_{II} (11), yielding a solution consisting of 3 subunits of each protein in the asymmetric unit. In turn, the partially refined structure of crystal II was used for molecular replacement in crystal I, yielding one NAGK hexamer and two P_{II} trimers in the asymmetric unit, forming the complex. All six subunits of each protein in the asymmetric unit have virtually identical structure.

The complex (Fig. 1) has 32 point group symmetry. Two P_{II} trimers sandwich one NAGK trimer of dimers, with their threefold axes aligned. The same complex was generated in crystal II after application of a crystal symmetry, but we will refer to the higher resolution (2.75 Å) crystal I structure. The complex approximates a sphere of radius ~53 Å. The P_{II} trimers are at the poles and contact the NAGK hexamer inner circumference. P_{II} is not packed tightly on NAGK. Each P_{II} subunit interacts with one NAGK subunit, contacting the α_4 region of NAGK towards its connection with the central sheet N-edge (Fig. 2). Two surfaces of each P_{II} subunit mediate the contacts: the B-loop and the β_1 - α_1 connection are exposed in the P_{II} trimer flat side and contact the NAGK subunit C-domain; and the T-loop is inserted in the interdomain crevice making extensive contacts with the NAGK N-domain.

NAGK and its arginine site. The ring-like hexameric NAGK contains one normally bound NAG molecule in each subunit (Fig.2) and resembles closely other arginine-inhibitable NAGKs (17) [Fig. 1 and 2, and supporting information (SI) Fig. 5]. Superimposition of the dimers with those of *T. maritima* NAGK reveals (Fig.3A) a rigid

body displacement of 1.9 Å of the C-domains towards the contacting P_{II} molecules. The hexamer is less wide and its dimers are more tilted than in the arginine-bound NAGK of *T. maritima* (SI Fig. 5). The orientation of the N-helices, identical in all the subunits, represents an approximate average of the closely related orientations of these helices in the arginine-free NAGK of *P. aeruginosa* (17) (Fig. 3B).

Each NAGK subunit exhibits one arginine site at its expected location (Fig. 3C and D). This site is widened relative to the high affinity site of *T. maritima* NAGK (Fig. 3C and D), because of movements outwards of the elements forming the site. The N-helix C-terminal portion is positioned as in the empty site of *P. aeruginosa* NAGK, with the phenolic ring of Y23, which should accommodate the C α of arginine (17), dislodged from the site. Strand β 16 is moved towards the P_{II} molecule that binds to its C-end, and the side chain of M287, which would stack the chain of bound arginine, is displaced away ~1 Å. The α H- β 16 connecting loop, which would bind through its main-chain O atoms the guanidinium and α -NH₃⁺ groups of arginine, is overexpanded, possibly because of pulling by the N-helix of the other subunit that runs parallel to it (Fig. 3D). These arginine site changes justify the ~15-fold increase in the half-inhibitory concentration of arginine ($I_{0.5}^{\text{Arg}}$) triggered by P_{II} (Table 2 and SI Fig. 6).

P_{II} exhibits a compact T-loop. *S. elongatus* P_{II} resembles closely the structure of free P_{II} (11) (Fig. 4A-D), except for the T-loop (residues 37-54), which, instead of being extended, is in all the subunits in an identical compact conformation resembling a flexed leg having the proximal segment packed against the P_{II} body and the distal segment (residues 41-52) packed against the proximal segment through a cushion of hydrophobic side chains (Y46, L56 and Y51) (Fig. 4E). Both segments are also linked by a salt bridge (E44:K58). In the distal segment, residues 44-51 form an imperfect β -

hairpin that is centrally involved in the interactions with NAGK and which includes and exposes S49, the residue that when phosphorylated prevents complex formation (4,6). A similarly shaped T-loop was observed very recently in GlnK1 of *Methanococcus jannaschii* bound to MgATP (13). Since the present complex has no nucleotides, each T-loop must be stabilized in the compact conformation by its contacts with NAGK. This compact conformation requires that a salt bridge between R47 and E85 (a B-loop residue) found in free P_{II} (11) be broken, and, indeed, in the complex the partner of E85 in this bond is R233 of NAGK (Fig. 4E). Hydrogen bonds that link the main chain N atoms of R47 and G48 to the εO atom of Q258 from NAGK (Fig. 4E) should stabilize this T-loop compact conformation. Contacts between the T- and B-loops (Q42 and E44 with I86), or even with helix 1 of the adjacent P_{II} subunit also stabilize this compact conformation.

The interaction of P_{II} with NAGK. The contacts with the C-domain of NAGK mediated by the flat side of the P_{II} trimer, bury $\sim 304 \text{ \AA}^2$ per P_{II} subunit (determined with a probe of radius 1.4 Å) and involve the B-loop and the β1-α1 junction (and adjacent residues) of P_{II} and the N-end of helix F and the C-ends of helix G and of β16 (Fig.2) of NAGK. These contacts include hydrophobic interactions (F11 and T83 of P_{II} and I229, I253 and A257 of NAGK), hydrogen bonds, and the already mentioned bond between E85 of P_{II} and R233 of NAGK. These interactions pull the C-domain and β16 towards P_{II}, contributing to the widening of the arginine site (see above) on the other side of the domain.

The other contact surface, provided by the T-loop terminal β-hairpin, buries 393 \AA^2 per P_{II} subunit. This hairpin is inserted at the NAGK interdomain crevice, being surrounded by and making contacts with the last turns of helix G, from the C-domain,

and of helix E from the N-domain, as well as with the $\beta 7$ strand of the $\beta 6$ - $\beta 7$ hairpin at the N-domain surface (Figs. 2 and 4F). This last hairpin and the T-loop terminal hairpin form a hybrid imperfect 4-stranded β -sheet involving five hydrogen bonds, of which two are provided by the OH group of S49. An ion-pair network (Fig.4F) centered in the T-loop residues R45 and E50 (themselves connected by a salt bridge) radiates to the surrounding elements of NAGK, involving R139, D142, E151, E194 and R254, and possibly mediating the decrease in the K_m^{NAG} induced by P_{II} in the absence of arginine (see Discussion).

Overall, the NAGK-P_{II} interface is remarkably open, being composed of six small contact surfaces for each P_{II} trimer, totalizing 2,094 Å² buried area, or, for the NAGK hexamer, 1,826 Å² buried area per P_{II} trimer. These buried surfaces only represent 14.3 % of the exposed surface of P_{II}, and, for both P_{II} trimers, only 5.8% of the exposed surface of the NAGK hexamer, accounting for the transient nature of the interactions between NAGK and P_{II}.

Signature sequences for P_{II}-NAGK signaling. The restriction to photosynthetic organisms of P_{II}-NAGK signaling reflects sequence and structural specializations. The T-loop residues R45 and S49 and the B-loop residue E85 have paramount roles in complex formation (Fig. 4E and F) and are conserved in photosynthetic organisms but not in other organisms. The same applies to the NAGK residues E194, R233, R254 and Q258 of NAGK, all involved in the interactions, (Figs. 4E and F), and to A257, which centers the hydrophobic patch linking both proteins. The simultaneous presence of these residues in either P_{II} or NAGK is a signature for the involvement of these proteins in P_{II}-NAGK signaling: these residues did not concur in any of 214 or 183 available P_{II} or NAGK sequences from non-photosynthetic organisms, but they were present in 46 out

of 48 and in 41 of 43 available P_{II} and NAGK sequences of photosynthetic organisms (Swissprot Database, <http://www.expasy.org>). The two red algae without the NAGK signature, *Gracilaria tenuistipitata* and *Cyanidioschyzon merolae* (Swissprot files Q6B8Z0 and Q85FW5) have no P_{II} gene in their chloroplast genomes (NCBI Genomes database files NC_006137 and NC_004779; <http://www.ncbi.nlm.nih.gov/sites/entrez>) and may not use P_{II} signaling.

Functional relevance of the complex revealed by site-directed mutagenesis and binding studies. Using the yeast two-hybrid system that revealed the P_{II}-NAGK interaction (5), no interaction was detected between wild type NAGK and the P_{II} mutants F11A, R45A, Y46A, R47A, S49A, S49D, S49E and E85A, or between wild type P_{II} and the NAGK mutants R139A, I229A, R233A, R254A, L256A and Q258A, (SI Fig.7), whereas interaction between P_{II} subunits or between NAGK subunits were not hampered by these mutations. All these residues (except Y46, and L256, which are indirectly involved) are directly involved in P_{II}-NAGK contacts. In contrast, mutations to alanine of seven P_{II} residues and fifteen NAGK residues mapping at points of the molecular surfaces not directly involved in P_{II}-NAGK interactions did not prevent complex formation (SI Fig.7, *in blue*). Thus, residues involved in the contacts in the crystalline complex are important residues for complex formation *in vivo*.

NAGK activity assays (Table 2) revealed that the P_{II} mutations F11A, F11Q, R45A, S49D or E85A, or the NAGK mutations R139A, I229A, I229N, R233A or R254A, abolished or greatly reduced the increase in the $I_{0.5}^{Arg}$ triggered by P_{II} when using the wild type proteins (Table 2). For most of these mutants, including the inactive (although soluble and hexameric) Q258A NAGK mutant, surface plasmon resonance assays showed abolished or decreased P_{II}-NAGK binding (Table 2). Since these

mutations affect residues in which the side-chains are directly involved in the P_{II}-NAGK contacts, the complex reflects the genuine interactions between NAGK and P_{II}. R47 of P_{II} only contacts NAGK through its main-chain atoms, and Y46 of P_{II} and L256 of NAGK are not directly involved in the contacts, and thus, the mutations at these residues had less important (R47 and Y46) or no (L256A) effect. As expected, the NAGK mutant D250A, used as an internal negative control, behaved just as wild type NAGK. Overall, the three assays agree, and leave little doubt that the present complex reflects the physiological interactions between P_{II} and NAGK. Further, the 2:1 P_{II} trimer:NAGK hexamer stoichiometry of the complex is the same in the crystal and in solution, as shown in binding studies in which we used ultrafiltration to separate free P_{II} from NAGK-complexed P_{II}, carried out with accurately quantitated protein solutions (see SI text). The linear Scatchard plots (SI Fig. 8) revealed a single type of site occurring in a number of ~2 per NAGK hexamer.

DISCUSSION

This work reveals how P_{II} and NAGK interact, while also describing the structure of *S. elongatus* NAGK and a novel conformation of the P_{II} protein from this organism. The NAGK closely resembles other arginine-sensitive NAGKs (17), and appears representative of plant NAGKs, which are hexameric and similar in subunit mass (7,18) and sequence (~60% identity). Since plants and cyanobacteria have highly similar P_{II} proteins (11,12) and conserve key interacting residues, the present structure should also represent the complex in plants.

The importance of the compact T-loop conformation for complex formation is highlighted by the involvement of three of the eight residues of the P_{II}-NAGK signaling signature (E85 of P_{II} and R233 and Q258 of NAGK) in triggering or in stabilizing the compact T-loop shape. By similarity with *M. jannaschii* GlnK1, MgATP and ADP binding to P_{II} should trigger, respectively, the compact and highly extended T-loop conformations (13,15), explaining the dissociation of the P_{II}-NAGK complex by ADP but not by MgATP (6). These MgATP and ADP effects on the T-loop fit the proposed P_{II} role in energy signaling in cyanobacteria (10).

Nitrogen abundance is signaled by P_{II} through the inversely related 2OG levels (10), but there is conflict on the 2OG effects on NAGK-mediated signaling: 2OG hampered P_{II}-NAGK complex formation in plasmon resonance assays (6), but not in pulldown or gel filtration assays (4), and it slightly increased instead of decreasing NAGK activity in the presence of P_{II} (4). With *Arabidopsis thaliana* proteins (7), 2OG did not prevent P_{II}-triggered increase in I_{0.5}^{Arg}. Since the structure of *M. jannaschii* GlnK1 (13) suggested that 2OG binding may stabilize the compact T-loop conformation triggered by MgATP (13), 2OG may not abolish P_{II}-NAGK interaction. However, the increased negative potential due to 2OG binding (13) may hamper complex formation

by electrostatic repulsion, given the negative potential of the NAGK ring faces (not shown). This electrostatic effect should be strongly influenced by the ionic strength, possibly accounting for the variable 2OG effects in different assays (4,6).

In *S. elongatus*, the phosphorylation of S49 of P_{II} is stimulated by 2OG and prevents P_{II}-binding to NAGK (10). The present structure clarifies this effect of S49 phosphorylation. One donor hydrogen bond formed with NAGK by the S49 OH group is lost upon phosphorylation. More importantly, steric and electrostatic clash with NAGK binding should be triggered by the bulkiness and negative charge of the phosphate, given the involvement of S49 in the contacts with NAGK and the negative potential of the NAGK surface (not shown).

Protein-protein complex formation involves an initial encounter complex in which one or few side chains of one protein act as anchors (19). The only residue in the complex that meets all the characteristics of an anchor is the T-loop residue R45 (19): it belongs to the smaller protein and it is exposed in the unbound form; it binds a preformed groove in the receptor, burying a positively charged (and thus polar) group and $>100 \text{ \AA}^2$ (115 \AA^2 in the case of R45) of solvent-accessible surface. R45 is a key element of the ion-pair network linking the T-loop and NAGK. This network may mediate the decrease in the K_m^{NAG} triggered by P_{II} on *S. elongatus* NAGK in the absence of arginine. The network involves D142, which stems from a loop that is glued to the mobile lid of the NAG site (Fig. 4F). The pull on D142 towards P_{II} upon network completion may drag the lid towards its lowered position (16,17) found in the high affinity form of the NAG site. This explains why the lid is lowered in an empty NAG site of crystal II (not shown), and the lack of effect of P_{II} on the K_m^{NAG} in the absence of arginine in the NAGK of *A. thaliana* (7), which has D142 replaced by N.

The present structure clarifies how P_{II} binding renders NAGK less sensitive to arginine. Given the low activity and high apparent affinity for arginine of *S. elongatus* NAGK in the absence of P_{II}, and its increased activity and decreased affinity for arginine in the complex with P_{II} (4,6), the binding of P_{II} must stabilize an active NAGK form having low affinity for arginine. Previous work (17) with other NAGKs equated this form with a narrow ring conformation of the NAGK hexamer presenting highly tilted dimers and an orientation of the interlaced N-helices that is restricted within narrow spatial margins. The NAGK in the present complex conforms with these requirements for narrowness, high dimer tilt (SI Fig. 5) and N-helices orientation (Fig. 3B). Through their linkage to P_{II}, the NAGK subunits should be pulled towards both poles of the complex, favoring the high tilt of the NAGK dimers and the concomitant narrowing of the NAGK ring, and indirectly forcing the N-helices that interconnect the dimers into the observed orientation, associated with a low affinity form of the arginine site. In addition, the interactions of the P_{II} body with the C-domains of NAGK pull from β 16, contributing to arginine site widening. In any case, the effects of P_{II} on NAGK functionality are long-distance effects, since they do not involve P_{II} participation in substrate sites or P_{II} occlusion of the arginine site. This fits the observation that the increase in $I_{0.5}^{\text{Arg}}$ is saturated with increasing P_{II} concentrations (SI Fig. 6): there should not be such saturation for physical competition between P_{II} and arginine for the arginine site.

The binding of two P_{II} trimers per NAGK hexamer revealed by the present structure and binding studies fits prior determinations with *S. elongatus* NAGK and P_{II} (6), provided that these earlier data are corrected for apparent NAGK overestimation by a factor of 1.73 (due to the use in (6) of a too small ϵ^{280} for NAGK). A stoichiometry of 1:1 P_{II} trimer:NAGK hexamer reported (7) for the complex of *A. thaliana* may need

revision since molar concentrations of the proteins may not have been accurate and since the assays involved potentially damaging long incubations at 25°C. Clearly, our crystallographic evidence and binding assays strongly indicate that each NAGK molecule has two apparently identical sites for P_{II}. Considering the abundance of P_{II} in *S. elongatus* (K. Forchhammer, unpublished), NAGK should mainly exist in complex with two P_{II} trimers when nitrogen is abundant.

The indirect effects of P_{II} on NAGK functionality contrast with the direct role of GlnK in blocking the ammonia channel AmtB (14,15). In the latter case the tip of the ADP-binding and more extended T-loop, including non-uridylylated Y51, blocks the cytoplasmic opening of the channel. As in the complex with NAGK, the flat side of the GlnK trimer looks towards the target, but nearly all the contacts are mediated by the T-loop, the anchor residue appears to be R47, and the T-loop conformation resembles one conformation of the ADP-bound loop of *M. jannaschii* GlnK1 (13). More examples of structures of P_{II} complexed to other targets will have to be characterized to ascertain whether the present structure and that of the GlnK-AmtB complex are paradigms for the interaction of P_{II} with its targets, or whether P_{II} is a highly plastic protein that is able to adapt its T-loop in diverse ways for interaction with different targets.

MATERIALS AND METHODS

Full protocols are available in *SI Text*.

Preparation of P_{II} and NAGK. The *argB* and *glnB* genes of *S. elongatus* strain PCC7942, encoding NAGK and P_{II}, were PCR-cloned from plasmids pUAGC62 and pUAGC12 (5), respectively, in the *Nde*I and *Xho*I or *Nde*I and *Hind*III sites of pET-15b or pET-22b plasmids, being separately expressed in *E. coli* BL21 cells. Purification of N-terminally His-tagged NAGK (N-terminal sequence MGSSH₆SSGLVPRGSHM, ending in M1 of wild type NAGK) and of P_{II} were described (6,11).

Crystallization of the complex. A mixture of 0.5 mg/ml NAGK and 0.1 mg/ml P_{II}, in a solution containing 20 mM Hepes (pH 7.5), 1 mM dithiothreitol, 40 mM NaCl, 10 mM MgCl₂ and 20 mM NAG, incubated 10 min at 23°C, was concentrated to 5 mg/ml by ultrafiltration. The complex was crystallized in two forms (Table 1) at 21°C in hanging drops made of 1µl each of protein and precipitant solution [crystal I, 0.1 M Tris-HCl (pH 8.5), 0.15 M Na acetate and 20% (wt/vol) polyethylene glycol 4K; crystal II, 0.1 M Na cacodylate (pH 6.5), 0.25 M Mg acetate, 10% (wt/vol) polyethylene glycol 8 K].

Data collection and structure Determination. See Table 1 and *SI Methods* for details.

Other methods. Site-directed mutagenesis of yeast two-hybrid plasmids (5) was carried out using the Quickchange II system from Stratagene (La Jolla, CA) following the manufacturer's instructions. Yeast two-hybrid analysis of NAGK and P_{II} mutants was carried out as described (5). For activity and plasmon resonance assays (4,6), the *argB* and *glnB* mutants were PCR-subcloned into, respectively, pET-22b and the *Strep*-tag fusion vector pASK-IBA3 (IBA GmbH, Göttingen, Germany), and the mutant proteins were overexpressed and purified (4,6). When comparing with these mutants, wild-type P_{II} was also fused to the *Strep*-tag II peptide (4). All the purified mutants of P_{II} and NAGK were soluble and formed the expected oligomer (checked by polyacrylamide gel

electrophoresis under non-denaturing conditions). The binding of P_{II} to NAGK was quantitated by centrifugal ultrafiltration through a membrane of cutoff 100 kDa, monitoring the decrease in the amount of P_{II} crossing the membrane when NAGK was added.

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FIGURE LEGENDS

Fig. 1. P_{II}-NAGK complex. NAGK, P_{II} and NAG in surface, ribbon and spheres representations, respectively. NAGK dimers and P_{II} subunits are colored independently. Views are along the threefold axis (*A*) or the twofold axis (*B*).

Fig. 2. P_{II} subunit-NAGK subunit contacts. P_{II}, NAGK and NAG are in string, ribbon and spheres representations, respectively. The contacting parts of the T-loop, B-loop and β 1- α 1 connection, including some interacting side chains (in sticks), are blue, red and green, respectively. The surfaces provided by these elements form meshworks of the same colors. The NAGK central β sheet is green, and other β strands and the α helices are brownish and grayish for N- and C-domains, respectively. Some NAGK elements and P_{II} residues are labeled. The inset details the structure of bound NAG, encased within its electron density omit map contoured at 2.5σ .

Fig. 3. Changes in the NAGK dimer, the N-helix and the arginine site. (*A*) Stereo view of backbone superimposition of NAGK dimers of *S. elongatus* (green) and *T. maritima* (red). Two P_{II} subunits (blue; labeled **P_{II}**) are bound to *S. elongatus* NAGK. The twofold axis (black ellipse) is perpendicular to the paper. For clarity, not all elements are shown in NAGK. Note the displacement towards P_{II} of the C-domain, and the opposite displacement of the N-helix, of *S. elongatus* NAGK. (*B*) N-helix orientation in the NAGKs of *S. elongatus* (green), *T. maritima* (red) and in two NAGK subunits of *P. aeruginosa* (cyan and yellow). (*C*) Arginine site surface in *T. maritima* NAGK (upper panel) with bound arginine (in sticks), and in *S. elongatus* NAGK (lower panel). (*D*) Stereo view of the superimposed arginine sites of *T. maritima* (black; bound arginine in yellow) and *S. elongatus* (green) NAGKs. The next subunit N-helix portion (in fainter color) runs parallel to the α H- β 16 loop. Red broken lines are polar contacts with

arginine and the black broken line is one hydrogen bond between the α H- β 16 loop and the adjacent subunit N-helix.

Fig. 4. The P_{II} trimer and the T-loop. (A-D) Ribbon representations of the trimer, with independent coloring of each subunit, except the T-loop, B-loop and β 1- α 1 junction, colored blue, red and green, respectively. The P_{II} conformation in the present complex (A, B) and in free *S. elongatus* P_{II} (C, D), are compared. (A,C) Views along the threefold axis from the side that contacts NAGK, or (B,D) perpendicularly to the vertical threefold axis, with the face that interacts with NAGK looking down. (E) T-loop (in blue) contacts with NAGK subunit (yellow semi-transparent surface) showing interacting residues. Parts of the B-loop (in red) and of another P_{II} subunit (green) that interact with the T-loop are also represented. (F) Ion-pair network (red broken lines) centered in the T-loop (blue), involving NAGK subunit N-domain (yellow) and C-domain (grayish) elements. The β 6- β 7 hairpin and the neighboring β 3- β 4 hairpin forming the NAG site lid (with bound NAG as spheres), are in string representation, and are compared with the corresponding hairpins of *T. maritima* NAGK (in green). Residue side-chains are as sticks, and hydrogen bonds as black broken lines.

Table 1. Data collection and refinement statistics

	Crystal I	Crystal II
Space group	P2 ₁	C222 ₁
Unit cell (a, b, c), Å	90.4, 161.0, 91.6	106.9, 149.5, 162.2
	$\beta = 106.5^\circ$	
Resolution, Å*	87.71-2.75	54.07-3.46
	(2.90-2.75)	(3.65-3.46)
Completeness, %*	100 (100)	100 (100)
Multiplicity*	5.1 (5.2)	4.1 (4.1)
I/σ^*	6.9 (2.0)	6.3 (1.9)
R_{sym} , %*	8.5 (36.7)	9.9 (39.1)
Reflections		
total/unique	334,220/65,178	70,396/17,338
$R_{\text{cryst}}/R_{\text{free}}$, %	20.0/23.6	23.3/29.4
rmsd bond length, Å	0.011	0.010
rmsd bond angles, °	1.37	1.13
No. of atoms/average B-factors, Å ²		
Protein atoms	17,902/38.3	8,531/73.4
N-Acetyl-L-glutamate	6/45.7	2/73.2
Water	187/31.8	
Ramachandran plot, %		
favored	92.0	89.2
allowed	7.8	10.2
generous	0.2	0.4
disallowed	0	0.2

*Values in parenthesis are data for the highest resolution shell.

Table 2. Influence of mutations of P_{II} or of NAGK on the arginine concentration causing 50% inhibition of NAGK ($I_{0.5}^{Arg}$) in the presence of P_{II}, and on surface plasmon resonance assay of P_{II}-NAGK complex formation.

P _{II} form	NAGK form	$I_{0.5}^{Arg}$ (μ M)*	Plasmon resonance signal (%)
None	wild type	39	0
wild type	wild type	572	100
F11A	wild type	46	0
F11Q	wild type	52	not assayed
R45A	wild type	41	0
Y46A	wild type	376	43
R47A	wild type	380	16
S49D	wild type	52	0
E85A	wild type	91	4
wild type	R139A	33 \pm 12	10
wild type	I229A	10 \pm 9	not assayed
wild type	I229N	100	not assayed
wild type	R233A	65 \pm 4	0
wild type	D250A	466	100
wild type	R254A	128	12
wild type	L256A	517	100
wild type	Q258A	No activity	0

The $I_{0.5}^{Arg}$ value was determined from plots of NAGK activity versus arginine concentration, in assays containing 6 μ g/ml NAGK (29 nM hexamer) and 2.4 μ g/ml P_{II} (65 nM trimer). The NAGK mutations did not alter substantially the $I_{0.5}^{Arg}$ in the

absence of P_{II}, except for mutation L256A (I_{0.5}^{Arg} in the absence of P_{II}, 115±13 μM). For Biacore surface plasmon resonance, His₆-tagged NAGK proteins were immobilized on Ni²⁺-NTA sensor chips, and P_{II} proteins (87.5 μg/ml, 2.37 μM trimer) were the analytes.

*Unless indicated, standard errors did not exceed 5 % of the mean values given.







