P_{II} signal transduction proteins are ATPases whose activity is regulated by 2-oxoglutarate

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P_{II} proteins are one of the most widespread families of signal transduction proteins in nature, being ubiquitous throughout bacteria, Archaea and plants. In all of these organisms they coordinate many facets of nitrogen metabolism by interacting with, and regulating the activities of, enzymes, transcription factors and membrane transport proteins. The primary mode of signal perception by P_{II} proteins derives from their ability to bind the effector molecules 2-oxoglutarate (2-OG) and ATP or ADP. The role of 2-OG as an indicator of cellular nitrogen status is well understood, but the function of ATP/ADP binding has remained unresolved. We have now shown that the *Escherichia coli* P_{II} protein, GlnK, has an ATPase activity that is inhibited by 2-OG. Hence when a drop in the cellular 2-OG pool signals N-sufficiency, 2-OG depletion of GlnK causes bound ATP to be hydrolysed to ADP, leading to a conformational change in the protein. We propose that the role of ATP/ADP binding in *E. coli* GlnK is to effect a 2-OG-dependent molecular switch that drives a conformational change in the T-loops of the P_{II} protein. We have further shown that two other P_{II} proteins, *Azospirillum brasilense* GlnZ and *Arabidopsis thaliana* P_{II}, have a similar ATPase activity and we therefore suggest that this switch mechanism is likely to be a general property of most members of the P_{II} protein family.

P_{II} proteins are some of the most widely distributed signal transduction proteins in nature. They are ubiquitous in bacteria, archaea and plants where they are involved in the regulation of many aspects of nitrogen metabolism. They function by protein-protein interaction whereby they control the activities of enzymes, transcription factors and membrane transport proteins (1-5).

P_{II} proteins are homotrimers composed of 12-13 kDa subunits with a highly conserved structure. The body of the protein is a compact cylinder, from which three long loops (the T-loops) protrude. These loops have considerable structural flexibility and they constitute the interaction interface for many of the P_{II} target proteins (5). Signal perception by P_{II} proteins can occur at two levels. The primary mode of signal perception appears to be almost universal and involves the binding of the effector molecules 2-oxoglutarate (2-OG) and ATP/ADP within the lateral inter-subunit clefts. A secondary mode of signal perception, which is less conserved, involves covalent modification of a residue within the T-loop.

In the primary mode of signal perception, the binding of 2-OG and ATP is strongly synergistic (6). This synergy is explicable from the structures of the *Azospirillum brasilense* GlnK orthologue, GlnZ, the *Synechococcus elongatus* P_{II} protein, and *Archaeoglobus fulgidus* GlnK3, each with bound 2-OG and MgATP (7-9). In all cases 2-OG binds close to MgATP within the lateral cleft. The Mg^{2+} ion is coordinated by the 2-oxo moiety of 2-OG, together with the three phosphate oxygens of ATP and the side chain of the highly conserved residue Gln39 (*A. brasilense* residue numbering) at the base of the T-loop. The 5-carboxy group of 2-OG forms a salt bridge with another highly conserved residue, Lys58. One of the best characterised P_{II} interactions is its binding to the membrane protein AmtB thereby controlling

the flux of ammonia through AmtB and into the cell. The binding mode of ADP to P_{II} proteins was revealed from the structure of *Escherichia coli* GlnK bound to AmtB in which ADP occupies the same nucleotide-binding site as ATP but Mg^{2+} and 2-OG are absent (10). The absence of 2-OG allows Gln39 to reorientate and form a salt bridge to Lys58 with a concomitant conformational change in the T-loop.

As the intracellular 2-OG pool is directly related to the cellular N-status, 2-OG is a logical effector molecule for P_{II}, but the role of ATP or ADP as P_{II} effector molecules has remained unclear. It was long considered that ATP could not play a regulatory role because its intracellular concentration is typically 1-5 mM whilst the affinity of P_{II} proteins for the nucleotide is in the μM range (K_d ~50 μM) (11, 12). However the subsequent recognition that ADP is also a physiological effector (10) led to a re-evaluation of the role of nucleotides, and a number of studies concluded that P_{II} proteins might also act as sensors of the cellular energy status, as reflected by fluctuations in the ATP/ADP ratio (13-18).

A full understanding of the mode of action of P_{II} effector molecules requires a well-defined model system that can be studied both *in vivo* and *in vitro*, and for which structural information is also available. The interaction of the *E. coli* P_{II} protein, GlnK, with its cognate target, the ammonia channel AmtB, offers just such a model (10, 19, 20). Furthermore, phylogenetic analysis suggests that the regulation by GlnK of ammonia influx into the cell through AmtB is likely to represent the ancestral role of P_{II} proteins (1).

Studies of the *E. coli* GlnK-AmtB system have shown that when cells are nitrogen limited, GlnK is cytoplasmically located and uridylylated within the T-loops; the cellular ATP and 2-OG pools are high and GlnK is expected to contain one molecule of 2-OG, Mg^{2+} and ATP in each of the inter-subunit clefts (7, 20, 21). When nitrogen-limited cells are subject to an extracellular ammonium shock, the 2-OG pool drops rapidly from 1.4 mM to 0.3 mM. GlnK is rapidly deuridylylated and binds in a 1:1 stochiometry to the cytoplasmic face of the AmtB trimer (19, 20). The conformation of the GlnK T-loops changes to adopt an extended form, thereby allowing them to protrude into the cytoplasmic ends of the AmtB conduction channels, and block further ammonium uptake (10). When isolated directly from cells, the GlnK-AmtB complex contains one molecule of ADP, rather than ATP, per GlnK subunit and no 2-OG or Mg^{2+} (10). These *in vivo* changes can be replicated *in vitro*, confirming that complex formation is promoted by ADP and is inhibited by the presence of 2-OG, Mg^{2+} and ATP (20). Whilst these studies confirmed 2-OG as a key effector molecule, they did not fully rationalise the role of ATP and ADP.

We have now carried out a series of studies that lead us to propose that *E. coli* GlnK has an inherent ATP hydrolysis activity that is inhibited by 2-OG. This activity is conserved in other P_{II} proteins, and we therefore suggest that the primary role of nucleotide binding is to facilitate a 2-OG-dependent conformational switch and that this is likely to be a characteristic of most P_{II} proteins.

Results

GlnK is an ATPase that is inhibited by 2-OG.

In our previous *in vitro* studies to investigate the roles of ATP and ADP in regulating complex formation between GlnK and AmtB we used slightly different experimental protocols where

GlnK was only sometimes preincubated with the nucleotide (19, 20). We subsequently recognised a correlation between the observed effects of ATP and the inclusion of a preincubation step. This led us to devise a new series of studies on the effects of both ATP and ADP on association and dissociation of the GlnK-AmtB complex *in vitro*.

In our *in vitro* assay we used wild-type *E. coli* GlnK that had been purified to > 99% purity using chromatography on heparin followed by gel-filtration (see Materials and Methods). When this GlnK was pre-incubated in the presence of the nucleotide, either ATP or ADP promoted complex formation with AmtB (Fig. 1, lanes 2,6), but without pre-incubation only ADP was effective (Fig. 1, lanes 1,5). ATP did not promote complex formation when pre-incubated with the proteins in the presence of Mg^{2+} and 2-OG (Fig. 1, lane 7) (20). We therefore hypothesised that pre-incubation of GlnK with ATP allowed ADP to be formed, but only in the absence of Mg^{2+} and 2-OG. This would explain the observations by Durand and Merrick (19) who used a pre-incubation step and found that ATP promoted complex formation. Furthermore, consistent with this new hypothesis, the non-hydrolysable ATP analogue, AMP-PNP, did not promote complex formation with or without pre-incubation (Fig. 1, lanes 3,4).

The purity and stability of our ATP preparation was assessed by HPLC from which we determined that the ATP solution was >99% pure and that no significant hydrolysis occurred during the pre-incubation period of 15 mins at 30 ºC (Fig. S1). We then considered two hypotheses for the origin of the ATP hydrolysis. Firstly, our GlnK preparation could contain a contaminating ATPase; and such contamination had previously been reported to be present in preparations of the *E. coli* P_{II} protein GlnB (12). Alternatively, GlnK is itself an ATPase, but it only exhibits this activity in the absence of 2-OG.

We therefore subjected wild-type GlnK to a conventional ATPase assay (see Materials and Methods) with [^{32}P-ATP] as substrate and we analysed the release of [^{32}Pi] by thin layer chromatography. This protocol assumes a relatively high turnover rate and consequently it requires that only a small fraction of the substrate is radiolabelled [^{32}P-ATP]. Using such a protocol we failed to detect any significant hydrolysis by GlnK (Fig. S2). However we reasoned that if GlnK had a low turnover rate of ATP hydrolysis, and if the subsequent rate of dissociation of ADP was also low, such that following hydrolysis ADP effectively remains bound to the protein, then we might not detect hydrolysis of the [^{32}P-ATP]. We therefore repeated the assay using a protocol in which all the substrate was present as [^{32}P-ATP]. Under these conditions the GlnK protein preparation showed a clear ATPase activity that was independent of the presence of Mg^{2+} (Fig 2).

At this point we could still not exclude that the ATPase activity was due to a minor contaminant in our GlnK preparation. However, our studies on *in vitro* formation of the GlnK-AmtB complex had indicated that if the ATPase activity was a property of GlnK, then it should be inhibited by the presence of 2-OG, so that the ADP-bound form of the protein was only formed in 2-OG-limited conditions. This proved to be the case, and furthermore 2-OG was only inhibitory in the presence of Mg^{2+} (Fig 2), thereby supporting the proposal that the ATPase activity is a property of GlnK.

We reasoned that changes in one or more key residues in GlnK might be expected to influence its proposed ATPase activity. P_{II} proteins contain a consensus sequence (T_{83}GKIGDGKIF_{92}) that shares significant homology with the Walker A motif (xGxxGxGKTxx) found in many nucleotide-binding proteins (1, 22), as well as two

conserved arginine residues (R101 and R103) that coordinate the phosphate groups and stabilise the negative charges of the nucleotide triphosphate (22). Furthermore, from observation of the ADP-binding site in the GlnK-AmtB complex, Gruswitz et al had previously suggested that K90, R101 and R103, together with a highly co-ordinated buried water molecule, might facilitate ATP hydrolysis by GlnK (23). We therefore examined the properties of K90A, R101A and R103A variants of GlnK with respect to their ATPase activities and their abilities to form a GlnK-AmtB complex. In addition, we examined variants Q39A, Q39E and K58A, because 2-OG binding to GlnK inhibited ATPase activity and both Q39 and K58 are involved in 2-OG binding (7, 24).

The K90A, R101A and R103A variants of GlnK were all impaired in their ATPase activity when compared with wild-type GlnK protein but, like the wild-type, all of them showed complete inhibition in the presence of 2-OG and Mg^{2+} (Fig. 3A). We hypothesised that the reduced activity of these variants could reflect an effect of the altered residue on the protein's ability to bind ATP rather than its catalytic activity. We therefore measured ATP-binding of each of these variants by isothermal calorimetry and found all three to be significantly impaired in ATP-binding compared with wild-type GlnK. The K_ds for ATP were: wild-type 5µM, K90A - 168 µM, R101A – 260 µM and R103A – 228 µM. All three variants were also assessed for their ability to form a complex with AmtB *in vitro* and their phenotypes in this assay mirrored their ATPase activities. All three variants formed complexes to some degree, with R101A being the least effective and R103A the most effective (Fig. 3B).

GlnK K58A had a markedly reduce ATPase activity when compared to wild-type but this activity was not inhibited by the presence of 2-OG (Fig. 4A). By contrast GlnK Q39A and Q39E showed little or no ATPase activity (Fig. 4A). Both structural data (7) and previous studies of the effector binding properties of a Q39E variant of *E. coli* GlnB (24) indicate that alterations to Q39 will impair 2-OG binding but not ATP binding. Hence these data suggest that Q39 could play a key role in the mechanism of ATP hydrolysis. Consistent with the observed ATPase activities GlnK Q39A and Q39E failed to form complexes with AmtB irrespective of whether 2-OG was present, whereas GlnK K58A still formed complexes albeit less effectively than wild-type GlnK (Fig. 4B).

ATP hydrolysis by other P_{II} proteins.

The ability to bind 2-OG and ATP/ADP is an almost universal characteristic of P_{II} proteins and consequently we considered the possibility that other P_{II} proteins would also exhibit an ATPase activity. We tested the ATPase activity of *A. brasilense* GlnZ (a GlnK orthologue), and of the P_{II} protein from the model plant, *Arabidopsis thaliana* using the same assay that we had applied to *E. coli* GlnK. Both these P_{II} proteins also had ATPase activity. The activity of *A. brasilense* GlnZ had an identical profile to that observed with *E. coli* GlnK, whereas *A. thaliana* P_{II} showed inhibition of the ATPase activity by 2-OG both in the presence and absence of Mg^{2+} (Fig. 5).

Discussion

In this study we have shown that the *E. coli* GlnK protein has an ATPase activity that is only revealed in the absence of 2-OG. This observation is a previously unrecognised facet of P_{II} biology and resolves a long-standing question as to the likely role of ATP binding to P_{II} proteins. In N-limited cells P_{II} proteins are expected to have 2-OG, Mg^{2+} and ATP bound in

each of the three ligand-binding pockets and the T-loops of the protein will be relatively unstructured, as seen in the *A. brasilense* GlnZ structure (7, 20). Improvement in the cellular N-status results in a marked drop in the 2-OG pool (20) and as a consequence we expect that 2-OG occupancy of P_{II} will fall and the bound ATP will be concomitantly hydrolysed to ADP. Crystallography of *E. coli* GlnK, *A. brasilense* GlnZ and *S. elongatus* P_{II} has shown that such a switch is predicted to result in a rearrangement of the ligand binding pockets of the protein and a conformational change in the T-loops which adopt an extended more rigid structure (7, 8, 10). The energy released upon ATP hydrolysis could help to drive this conformational change. The ability of ADP to act as a potent inhibitor of ATP binding in low 2-OG has previously been demonstrated *in vitro* (13, 20). Consequently, once the P_{II} protein is in the ADP-bound form, its replacement by Mg-ATP is not favoured until the cellular 2-OG pool rises again. This 2-OG dependence of ATP binding, together with the hydrolysis of ATP to ADP in the absence of 2-OG, provides a robust 2-OG-dependent conformational switch.

The precise mechanism of ATP hydrolysis by GlnK will require further detailed study. However, the key feature in this study is that in all three P_{II} proteins examined here, ATPase activity is inhibited by 2-OG. For both *E. coli* GlnK and *A. brasilense* GlnZ that inhibition also requires Mg^{2+} which is predictable given the known role of Mg^{2+} in coordinating the binding of 2-OG (7, 8). However, under the conditions used, 2-OG inhibition was apparently not dependent on Mg^{2+} in *A. thaliana* P_{II}. Residue Gln39 is very highly conserved in all P_{II} proteins (4) and is required for ATPase activity, suggesting that it plays a key catalytic role.

It is interesting to consider the functional similarities between the role of ATP and ADP, as proposed here for the regulation of P_{II} protein conformation, and that of GTP and GDP in regulating the conformational switch in the signal-transducing G_{α} proteins (25). These small GTPases transition between a MgGTP-bound and a GDP-bound conformation and GTP hydrolysis is controlled by interaction with other effector proteins. The mechanism of GTP hydrolysis by this family of proteins is believed to involve a conserved glutamine residue that contributes to catalysis by orientating a nucleophilic water molecule for attack on the γ-phosphate of GTP (26). Interestingly, the side chain of the catalytic Gln residue only exhibits catalytic geometry in the transition state (27).

Sequence comparisons among many P_{II} proteins, together with *in vivo* and *in vitro* studies in a number of model systems suggest that ATP/ADP binding is likely to be a universal feature of the P_{II} family and that 2-OG binding is also highly conserved (7, 13, 20, 22, 28, 29). We have demonstrated that the ATPase activity of *E. coli* GlnK extends to *A. brasilense* GlnZ and *A. thaliana* P_{II}, suggesting that this is indeed likely to be a characteristic of most P_{II} proteins. However, it should be noted that *Bacillus subtilis* GlnK has been reported to bind 2-OG only weakly (30) and *Archaeoglobus fulgidus* GlnK2 apparently shows no 2-OG binding (31) suggesting that these proteins might have a different mode of action.

These studies rationalise a number of previous observations. The crystal structure of the GlnK-AmtB complex described by Gruswitz et al (23) was achieved by co-crystallisation of separately purified GlnK and AmtB proteins at an equimolar concentration of 0.22 mM in the presence of 2mM ATP. However the resultant crystal complex contained ADP bound in all three GlnK nucleotide binding sites. Gruswitz et al (23) speculated that their observation may be explained by an innate ATPase activity of GlnK and our data support that view. Likewise, our previous observation that complex formation *in vitro* is facilitated by ATP (19) can now be explained by GlnK-mediated ADP formation from ATP during the pre-incubation period in those experiments.

Crystal structures for P_{II} proteins with bound ATP but no 2-OG have been reported in a number of cases including *A. fulgidus* GlnK2 and GlnK3 (9, 31), *E. coli* GlnK (22), *Methanococcus jannaschii* GlnK1 (32), *Mycobacterium tuberculosis* P_{II} (33), *S. elongatus* P_{II} (34) and *Thermatoga thermophilus* GlnK (35). Hence in these cases there was either no significant ATPase activity during the crystallisation or the particular P_{II} protein does not exhibit such an activity. It is notable that with one exception (*S. elongatus* P_{II} with bound ATP – PDB: 2XBP) all of the structures listed above were derived in crystallisation conditions with a pH below 7.0 (ranging from pH 3.5 to 6.0) in which case the acidic conditions used might have inhibited the P_{II} ATPase activity. By contrast the GlnK-AmtB structure derived by Gruswitz and colleagues in which the proteins were co-crystallised with ATP but ADP was present in the resultant complex, was obtained with a crystallisation buffer at pH 8.0 (23).

A number of authors have proposed that, in addition to sensing the cellular N-status, P_{II} proteins may also act as sensors of cellular adenylate energy charge (13-18). The majority of those studies report either the differential binding of ATP and ADP to P_{II} proteins *in vitro*, or the *in vitro* effects of ATP and ADP on interactions of P_{II} proteins with a number of their targets. However the cellular adenylate energy charge is quite strongly buffered around a value of 0.9 (36, 37) and in order for the published *in vitro* studies to translate into sensing of cellular energy charge *in vivo* there need to be cellular fluctuations in energy charge that are sufficient to facilitate exchange of ATP for ADP in the nucleotide-binding site. Such fluctuations are challenging to demonstrate experimentally and attempts to manipulate the adenylate pools in *Rhodospirillum rubrum* so as to provoke an effect on the regulatory activity of P_{II} were not successful (16). So whilst the possibility remains that P_{II} proteins might act as sensors of cellular energy charge, this remains to be proven and our studies suggest that the primary source of ADP as a P_{II} ligand is likely to be as a result of P_{II}-mediated ATP hydrolysis.

Materials and Methods

Protein purification. *E. coli* His_6-AmtB was purified from strain GT1000(pAD4) as described previously (19). Wild–type *E. coli* GlnK was purified from BL21(DE3)pLysS (pJT25) using a one–step purification heparin chromatography protocol described previously (20) which gave protein of >95% purity. This was followed by a second gel-filtration chromatography step to give protein of >99% purity. GlnK fractions from the heparin column were pooled and concentrated by centrifugal filtration (Amicon Ultra 10K - Millipore). This sample was then purified on a Superose 12 10/300 GL column (GE Healthcare), in 50mM Tris pH 7.5, 100mM KCl at 0.5ml/min.

Mutant alleles of *E. coli glnK* were made by site-directed mutagenesis of plasmid pJT25 (20) and the resultant plasmids were used to overexpress GlnK variant proteins Q39A, Q39E, K58A, K90A, R101A and R103A in *E. coli* BL21(DE3)pLysS. However, the variant proteins failed to bind effectively to heparin as observed for the wild-type and consequently an alternative purification method was used. Cells were grown in 20 ml of LB broth supplemented with100 μg/ml carbenicillin for 8 h at 37 °C. This culture was inoculated into 1 liter of autoinduction medium (ForMedium Ltd.); grown overnight at 37 °C; harvested at 6000 × *g*; and resuspended in 50 mM Tris-HCl (pH 7.5), 100 mM KCl, and 20% glycerol. Whole cell extracts of the culture were heated to 80 °C for 4 min, cooled on ice for 10 min, and harvested by centrifugation at 28,000 × *g*. The supernatant was loaded onto a 30ml HiLoad 16/10 Phenyl Sepharose HP column (GE Healthcare) equilibrated with 50mM Tris

(pH 7.6), 100mM NaCl, 1mM EDTA and ammonium sulphate to 25% saturation, and run at 1 ml/min. GlnK was eluted with a gradient to 0% ammonium sulphate over 40ml, at 2ml/min. GlnK fractions were pooled and ammonium sulphate added to 60% saturation with stirring at 4°C to precipitate GlnK. The pellet was harvested by centrifugation at 7,000 x g for 20min at 4°C, then resuspended into a minimal volume of 50mM Tris-HCl (pH 7.5), 100mM KCl and centrifuged at 4°C to remove any remaining solids. A sample volume of 0.5ml was loaded onto a Superose 12 10/300 GL column (GE Healthcare) equilibrated with 50mM Tris pH 7.5, 100mM KCl, and run at 0.5ml/min. Pure fractions of GlnK were identified on 12.5% SDS-polyacrylamide gel and stored at −80 °C. Whereas heparin chromatography resulted in GlnK fractions that contained no contaminating ATPase activity, fractions coming from phenyl sepharose chromatography had to be monitored by TLC for a contaminating ATPase activity that came off the gel-filtration column just ahead of GlnK. Only the later fractions were used in subsequent assays.

A. brasilense GlnZ was purified as described previously (7) and was the kind gift of Dr. Xiao-Dan Li. *A. thaliana* P_{II} was purified from strain BL21(DE3)pLysS pET29-PII-HT. Cells were grown in 1 liter of autoinduction medium (ForMedium Ltd) supplemented with 15 μg/ml kanamycin overnight at 28 °C, harvested at 6000 × *g* and resuspended in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10mM imidazole and 5% glycerol. Whole cell extracts of the culture were applied to a 5ml His Trap (GE Healthcare) column equilibrated with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 10mM imidazole. The column was washed with 50 mM Tris-HCl (pH 7.5), 1000 mM NaCl, 35mM imidazole and 0.1% Tween 20 to remove contaminating proteins and the *A. thaliana* P_{II} was eluted with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 500mM imidazole. 1ml fractions were collected and the flow rate used was 1ml/min throughout. Fractions from the elution peak were pooled and dialysed for 1 hour against 50mM Tris (pH 7.6), 100mM NaCl, 1mM EDTA then ammonium sulphate was added to 25% saturation. Protein was applied to a 30ml HiLoad 16/10 Phenyl Sepharose HP column (GE Healthcare) equilibrated with 50mM Tris (pH 7.6), 100mM NaCl, 1mM EDTA and ammonium sulphate to 25% saturation, and run at 1 ml/min. P_{II} was eluted with a gradient to 0% ammonium sulphate over 40ml, at 2ml/min. Pure fractions were analysed on a 15% SDS-polyacrylamide gel, pooled, buffer exchanged by filtration into 50mM Tris (pH 7.5), 100mM KCl, and then concentrated to 38 μM.

ATPase assay by thin-layer chromatography. Initial ATPase assays were ATPase assays were performed in 50 mM Tris-HCI pH8, 100 mM NaCl, 10% v/v glycerol, 0.05% w/v LDAO (lauryl dimethylamine oxide). Assays were performed at 30 °C for 1 hr in a final reaction volume of 10 μl. Reactions were initiated by addition of ATP (0.8 μl of 100mM ATP plus 1 μ1 [γ^{32}P]-ATP (3000 Ci mmol, Amersham Biosciences). The final ATP concentration in the assay was 4 mM and the protein concentration was 7 μM. The reactions were terminated by adding one tenth vol of 5% SDS, 20 mM EDTA followed by freezing in liquid nitrogen.

Subsequent ATPase assays contained only [γ^{32}P]-ATP and these reactions were carried out in 50 mM Tris HCl pH 8.0, 100 mM KCl, 6 mM $MgCl_2$ in a total volume of 10 μl. P_{II} protein, and effector molecules where required (2-OG 1.5 mM , $MgCl_2$ 6 mM), were mixed and the reaction was initiated by addition of [γ^{32}P] ATP (Perkin-Elmer 3000 Ci/mmole) to a final concentration of 3 μM. The final protein concentration varied between 100 and 150 μM according to the expt. Reactions were incubated for 30 min at 30°C.

In all cases the chromatography membrane (Polygram CEL300 PEI/UV_{254} 20 x 20cm, Macherey-Nagel) was pre-run in water and dried prior to loading. 1-2 μl of the reaction was loaded onto the membrane and rapidly dried (~ 5 sec) with a hair-dryer. The membrane was then run 0.75 M KH_2PO_4 pH 3.5, dried and the resultant signals were detected by a 2min exposure to a BAS-MP 2040S image plate (Fujifilm) and analysed using a Phosphor-Imager (FLA-7000 Image reader - Fujifilm).

***In vitro* GlnK-AmtB complex formation.** This was carried out as described previously (20). The final concentrations of effectors used in these experiments were 5mM ATP, 1.5 mM 2-OG, 6mM Mg^{2+}.

ATP analysis by HPLC. Aqueous solutions of adenosine phosphate standards or samples were injected onto a Poros HQ50 strong anion exchange column for analysis by HPLC (Dionex Ultimate 3000). The column was first equilibrated with 5 column volumes of 5 mM ammonium bicarbonate buffer, then elution profiles for each standard or sample were developed over a linear gradient of ammonium bicarbonate from 5mM - 250mM at a flow rate of 8 ml/min. An on-line detector was used to monitor A_{265}.

Isothermal calorimetry. This was performed as described previously (20).

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Figures

Fig. 1. Nucleotide-dependence of GlnK-AmtB complex formation.

Complex formation between His_6-AmtB and GlnK in the presence of various effectors was assessed by elution from a His-Select Spin column. In lanes 1, 3 and 5 there was no preincubation of GlnK with the effector; in lanes 2, 4, 6 and 7 GlnK was preincubated with the effector for 15 min at 30 °C prior to loading onto the column. The nucleotide used was: ATP - lanes 1,2; AMP-PNP – lanes 3,4; ADP – lanes 5,6; ATP+Mg^{2+}+2-OG – lane 7.

Fig. 2. ATPase activity of *E. coli* GlnK.

Analysis of ATP hydrolysis by thin-layer chromatography (TLC) as described in Materials and Methods. All ATP was present as [γ^{32}P] ATP. ATPase activity is indicated by release of free Pi.

Fig. 3. ATPase activity of *E. coli* GlnK variants K90A, R101A, R103A.

A. Analysis of ATP hydrolysis by thin-layer chromatography (TLC). B. Complex formation between His_6-AmtB and GlnK *in vitro* assessed by elution from a His-Select Spin column. Lanes 1-5, in absence of 2-OG; Lanes 6-9, in presence of 2-OG (1.5 mM). Mg^{2+} (6 mM) was present in all cases. Lanes 1,6 – wild type GlnK; lanes 2,7 – K90A; lanes 3,8 – R101A; lanes 4,9 – R103A, lane 5 – no GlnK.

Fig. 4. ATPase activity of *E. coli* GlnK variants Q39A, Q39E, K58A.

A. Analysis of ATP hydrolysis by thin-layer chromatography (TLC). B. Complex formation between His_6-AmtB and GlnK *in vitro* assessed by elution from a His-Select Spin column. Lanes 1-5, in absence of 2-OG; Lanes 6-9, in presence of 2-OG (1.5 mM). Mg^{2+} (6 mM) was present in all cases. Lane 1 – no GlnK; lanes 2,6 – wild type GlnK; lanes 3,7 – Q39A; lanes 4,8 – Q39E; lanes 5,9 – K58A.

Fig. 5. The ATPase activities of *A. brasilense* GlnZ and *A. thaliana* P_{II}.

Analysis of ATP hydrolysis by thin-layer chromatography (TLC) as described in Materials and Methods. All ATP was present as [γ^{32}P] ATP. ATPase activity is indicated by release of free Pi.

Fig. S1 ATP stability at 30 °C

The stability of ATP after incubation at 30 °C for 15 min was assessed by HPLC as described in Materials and Methods. A - ATP sample after incubation, B – ATP control (no incubation), C- ADP control (no incubation), contains significant amounts of AMP.

Fig. S2 ATPase activity of *E. coli* GlnK using standard protocol.

Analysis of ATP hydrolysis by thin-layer chromatography (TLC) as described in Materials and Methods. Reactions were initiated by addition of ATP (0.8 □l of 100mM ATP plus 1 □1 [□^{32}P]-ATP (3000 Ci mmol, Amersham Biosciences). The final ATP concentration in the assay was 4 mM.

Figure 1

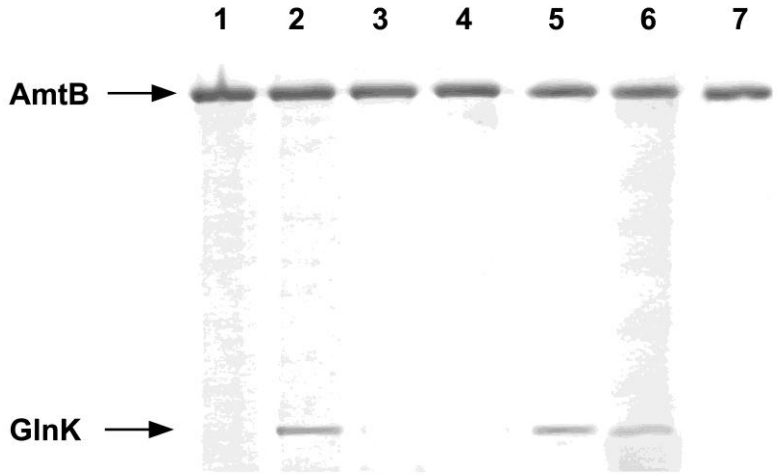

Figure 2

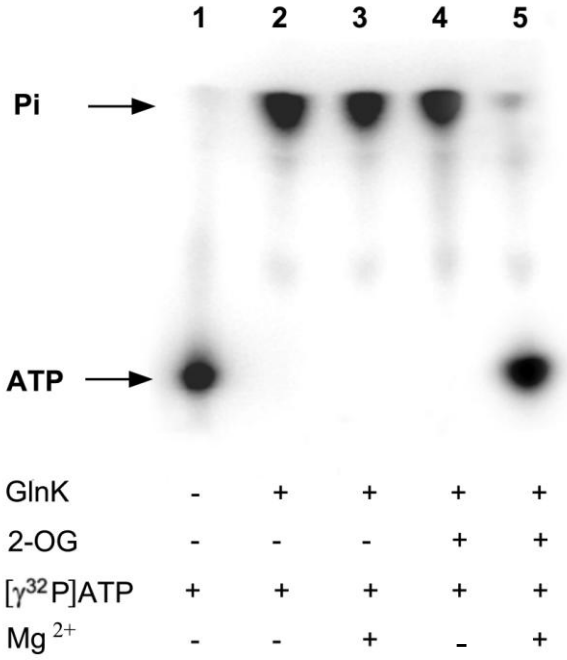

Figure 3

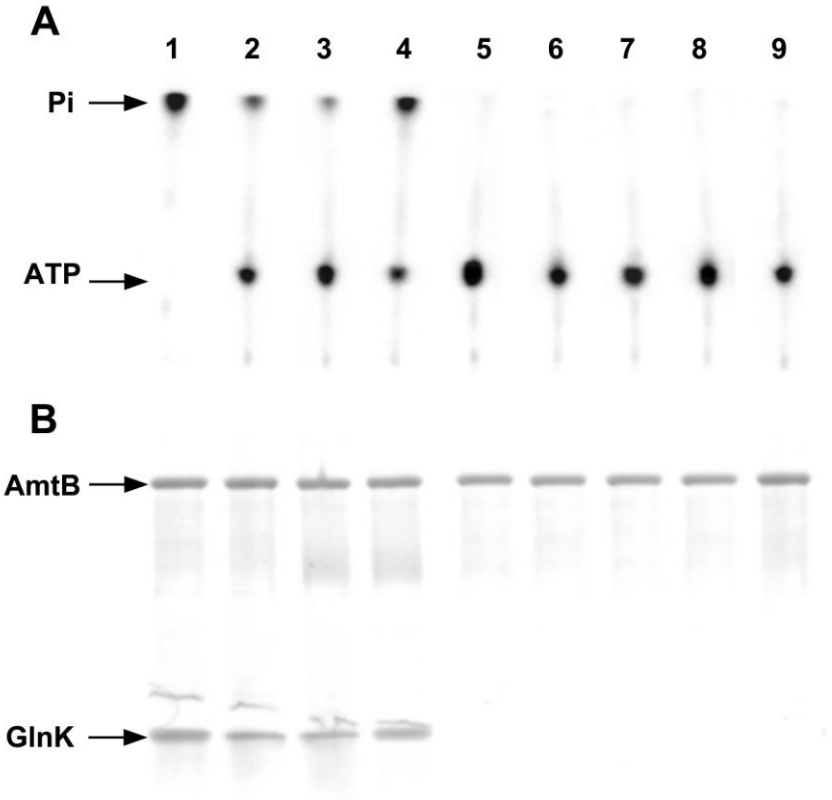

Figure 4

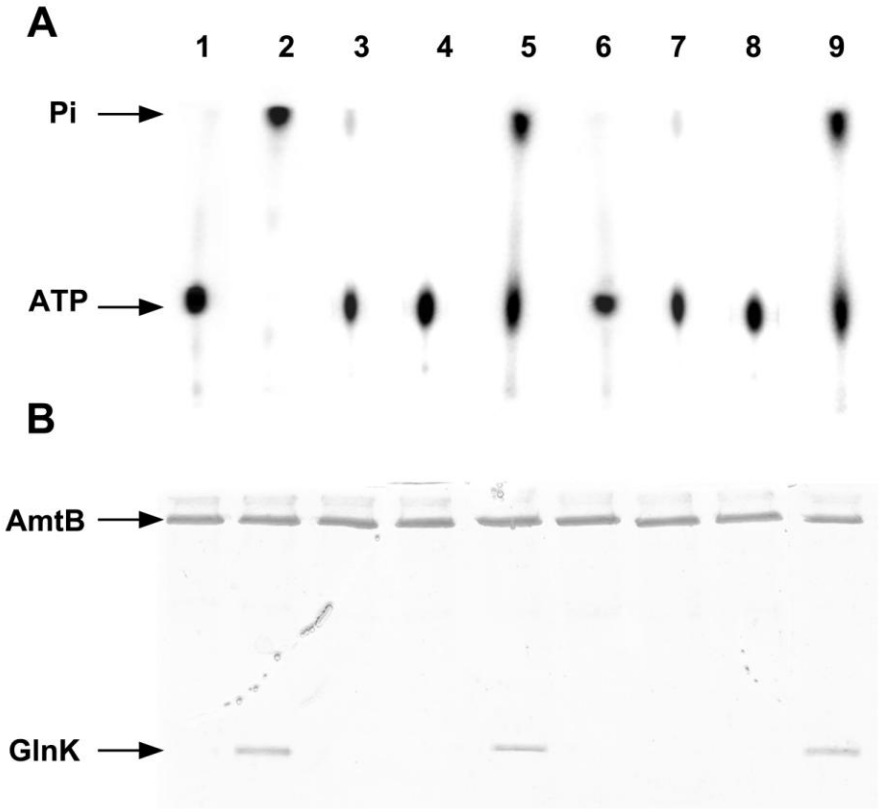

Figure 5

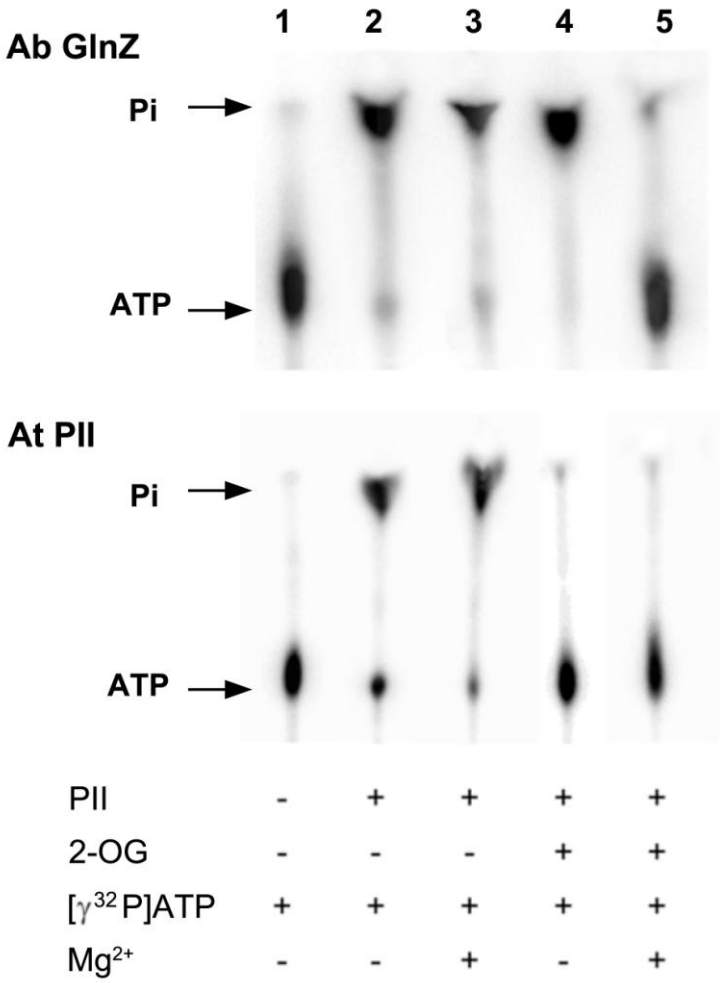

Figure S1

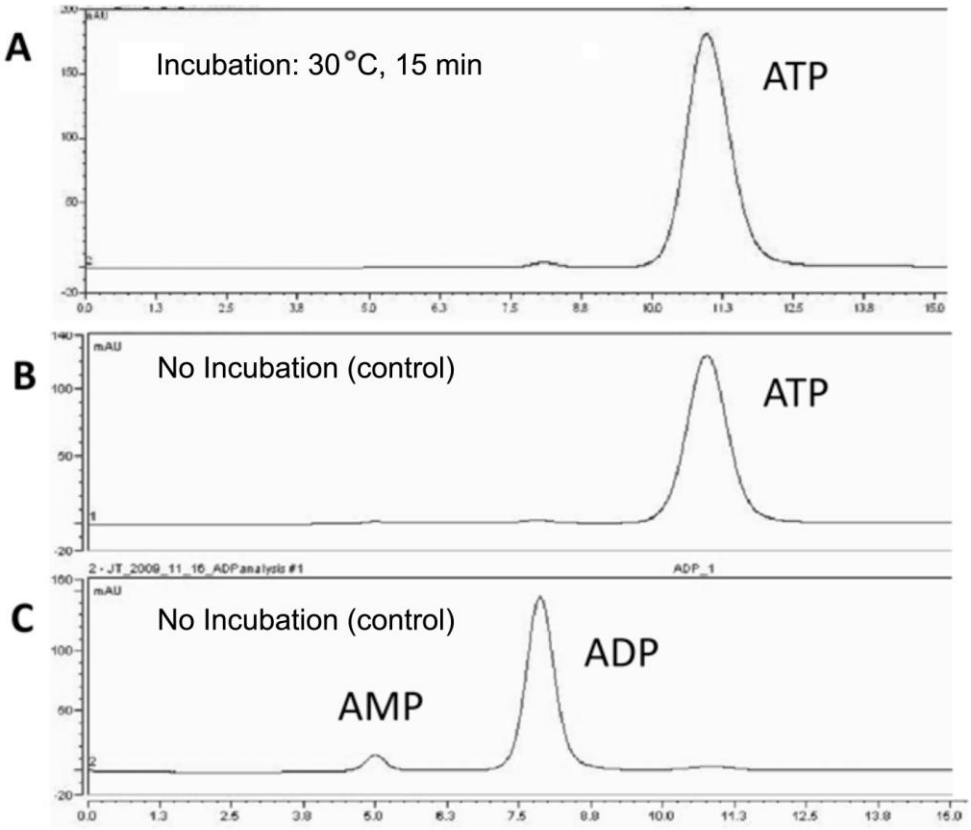
A
Incubation: 30°C, 15 min
ATP
B
No Incubation (control)
ATP
C
No Incubation (control)
AMP
ADP

Figure S2