Specificity and Regulation of Interaction between the P_{II} and AmtB₁ Proteins in *Rhodospirillum rubrum*[▽]

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The nitrogen regulatory protein P_{II} and the ammonia gas channel AmtB are both found in most prokaryotes. Interaction between these two proteins has been observed in several organisms and may regulate the activities of both proteins. The regulation of their interaction is only partially understood, and we show that in *Rhodospirillum rubrum* one P_{II} homolog, GlnJ, has higher affinity for an AmtB₁-containing membrane than the other two P_{II} homologs, GlnB and GlnK. This interaction strongly favors the nonuridylylated form of GlnJ and is disrupted by high levels of 2-ketoglutarate (2-KG) in the absence of ATP or low levels of 2-KG in the presence of ATP. ADP inhibits the destabilization of the GlnJ-AmtB₁ complex in the presence of ATP and 2-KG, supporting a role for P_{II} as an energy sensor measuring the ratio of ATP to ADP. In the presence of saturating levels of ATP, the estimated *K_d* of 2-KG for GlnJ bound to AmtB₁ is 340 μM, which is higher than that required for uridylylation of GlnJ in vitro, about 5 μM. This supports a model where multiple 2-KG and ATP molecules must bind a P_{II} trimer to stimulate release of P_{II} from AmtB₁, in contrast to the lower 2-KG requirement for productive uridylylation of P_{II} by GlnD.

The ammonium channel/rhesus (Amt/Rh) family of proteins is a widely distributed group of trimeric integral membrane proteins found in all domains of life that can function as gas channels of ammonia and perhaps carbon dioxide (13, 20, 29, 39, 43). A subset of this family, the AmtB proteins, is found in bacteria, archaea, some lower eukaryotes and plants. Homologs of *amtB* are often found in close proximity to genes encoding P_{II} homologs (46). P_{II} regulatory proteins are also found in most prokaryotes and some plant chloroplasts (3). P_{II} is a small, soluble, trimeric protein that regulates the functions of several other proteins involved in nitrogen metabolism (3, 4, 35, 36). AmtB appears to have two roles in the cell. The first function, to act as a channel for uncharged ammonia, has been explored physiologically, structurally, and computationally (28, 33, 44). The second function of AmtB is to interact with P_{II} and has only recently been described (6, 9, 21, 22, 53).

AmtB proteins have been shown to interact with homologs of P_{II} in several bacteria and in the archaeon *Methanococcus jannaschii* (8, 10, 17, 18, 44, 45, 47, 50, 53). The association of P_{II} with AmtB can physically block the ammonia gas channels of AmtB under conditions of nitrogen sufficiency in the cell. In addition, the AmtB-P_{II} complex is able to recruit at least one other protein to the membrane, dinitrogenase reductase-activating glycohydrolase (DRAG), in organisms capable of nitrogen fixation (22, 48). This membrane sequestration requires both an Amt protein and a P_{II} protein and results in the inability of DRAG to activate dinitrogenase reductase in vivo. Finally, AmtB is able to remove equimolar amounts of P_{II} from the cytoplasm, preventing P_{II} from interacting with at least some other proteins. Although membrane sequestration of P_{II}

has been shown to be important in recovering from nitrogen starvation in *Escherichia coli*, this function of AmtB is still poorly understood (6).

P_{II} homologs sense several small-molecule pools. The best-studied P_{II} protein is GlnB from *E. coli*, and insights into P_{II} protein function from this homolog apply to all P_{II} proteins studied to date. Carbon status and perhaps energy status are sensed directly by the binding of 2-ketoglutarate (2-KG) and ATP to each subunit of the P_{II} trimer (26). The binding of one 2-KG molecule or no molecules to P_{II} signals carbon deficiency. At higher 2-KG concentrations, the binding of second and third 2-KG molecules to P_{II} signals carbon sufficiency (26, 36). The binding of ATP lowers the *K_m* of P_{II} for 2-KG, which could act as a signal of energy sufficiency in the cell; however, previous studies have suggested that ATP may always present at saturating levels in *E. coli* (23). In contrast, cellular nitrogen status is indirectly sensed by the reversible modification of P_{II}, rather than the direct binding of nitrogen-rich metabolites. In *E. coli* and other bacteria such as *Rhodospirillum rubrum*, the bifunctional, uridylyltransferase/uridylyl-removing GlnD protein carries out uridylylation of P_{II}. In *E. coli* this function is regulated by glutamine levels: low levels of glutamine, signaling nitrogen deficiency, lead to uridylylation of P_{II} (P_{II}-UMP) by GlnD, while high levels of glutamine, signaling nitrogen sufficiency, lead to deuridylylation of P_{II}-UMP by GlnD (3, 35). GlnD appears to sense 2-KG as well as glutamine in *R. rubrum*, where high levels of 2-KG stimulate uridylylation, and high levels of glutamine stimulate deuridylylation, of P_{II} by GlnD (25).

The specificity and regulation of interaction between P_{II} and AmtB proteins are not fully understood. There is evidence that both GlnB and GlnK of *E. coli* can interact with the sole AmtB species present in that organism in vivo, and it is suggested that GlnK may interact more strongly with AmtB than does GlnB (9). Likewise, both P_{II} homologs of *Azospirillum brasilense* ap-

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pear able to associate with AmtB at the membrane in vivo (21). Only unmodified P_{II} has been found to interact with AmtB, and uridylylation of P_{II} has been shown to prevent the formation of the P_{II}-AmtB complex in *E. coli*, *A. brasilense*, and *R. rubrum* (11, 22, 53). Crystal structures of the *E. coli* complex reveal that the uridylyl group could sterically interfere with the surface of interaction between the two proteins (8, 17). However, not all P_{II} proteins are regulated by uridylylation. In *Streptomyces coelicolor* the same tyrosine residue uridylylated in enteric bacteria is instead adenylylated (19), and in cyanobacteria a conserved serine in P_{II} is phosphorylated in response to nitrogen limitation (16). In other organisms P_{II} might not be modified at all (49). Regardless of the decoration's identity, it is likely to inhibit interaction with an AmtB protein because of the location of the modified residue, but this hypothesis has not been tested.

Finally, small molecules are known to have diverse regulatory effects on the P_{II}-AmtB complex, depending on the organism studied. In *E. coli* purified AmtB-GlnK complexes disassociate only in the presence of both ATP and 2-KG, while in *M. jannaschii* purified GlnK1-Amt1 complexes disassociate in the presence of low levels of ATP (11, 50). These may reflect real physiological differences in regulation in different organisms or may be complicated by the buffers and detergents used to purify the protein complexes. To date, there has been no in vitro study of the regulation of this interaction using AmtB in its native state as an integral membrane protein.

There are three P_{II} homologs, GlnB, GlnJ, and GlnK, in *R. rubrum* that interact with up to five other proteins involved in nitrogen regulation (52, 53, 54). All three P_{II} homologs can regulate the activity of GlnE (ATase), which reversibly adenylylates glutamine synthetase (52). Expressed from their native promoters, GlnB and GlnJ, but not GlnK, regulate the NtrBC two-component regulatory system, leading to expression of genes involved in nitrogen metabolism under conditions of nitrogen deficiency (52). GlnB and GlnJ also indirectly regulate nitrogen fixation by controlling the activities of two enzymes with opposing functions, dinitrogenase reductase ADP-ribosyl transferase (DRAT) and DRAG, which reversibly modify dinitrogenase reductase (52). Finally, only uridylylated GlnB is able to interact with and activate NifA, which is a transcriptional activator of genes involved in nitrogen fixation (52). Thus, the three P_{II} homologs in *R. rubrum* are not functionally interchangeable. The genes encoding the two GlnK-type P_{II} proteins, *glnJ* and *glnK*, each have *amtB* homologs immediately downstream. However, only the *amtB₁* gene downstream of *glnJ* plays important roles in the regulation of P_{II} in the cell (53). We therefore chose to examine whether specificity for a particular P_{II} existed in the *R. rubrum* AmtB₁ protein, as this could have substantial physiological consequences. Once such specificity was observed, we examined the role that small molecules have on the strength of interaction between a single P_{II}, GlnJ, and AmtB₁.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *R. rubrum* strains used in this study were UR2 (wild type), UR757 (Δ *glnB3 glnK1::aacC1*), and UR794 (*amtB₁::aacC1*) (27, 40, 53). *E. coli* strains used in this study were UQ4037 (pUX754) and UQ4038 (pUX755). Similar to the construction of an *R. rubrum* GlnB overexpression vector (pUX753), a 0.4-kb fragment of *R. rubrum glnK* or

glnJ was PCR amplified and cloned into pND706 at NdeI and EcoRI sites and then transformed into *E. coli* strain BK (Δ *glnB Δmdl-glnK::Kan*), yielding UQ4037 (pUX754) and UQ4038 (pUX755), respectively (32, 44). *R. rubrum* strains were grown in rich medium (SMN) or minimal malate glutamate medium (MG), as described previously (14, 30). To prepare larger volumes of cells for membrane harvesting, MG-grown cells were prepared by inoculating 100:1 from fully grown SMN cultures into MG medium and grown anaerobically under light to an optical density at 600 nm (OD₆₀₀) of about 0.9 (53). *E. coli* strains were grown in rich medium, Luria broth (LB) or LC (similar to LB medium but with NaCl reduced from 10 g/liter to 5 g/liter) (40).

Protein purification. GlnJ and GlnK were purified using a protocol similar to that used previously to purify GlnB (54). UQ4037 (for GlnJ) and UQ4038 (for GlnK) were grown overnight in 10 ml of 2× LB and inoculated into 600 ml of 2× LB medium, both supplemented with 100 μg/ml ampicillin and 25 μg/ml kanamycin. Cultures were grown at 30°C to an OD₆₀₀ of 0.8 to 1.1 and heat shocked at 44°C for an additional 4 to 5 h. Cells were concentrated by centrifugation at 10,800 × g for 10 min, resuspended in 50 mM Tris, pH 7.5, and broken by French press. Cell lysate was centrifuged as before, and then protein in the supernatant was precipitated with 45% (final) ammonium sulfate, centrifuged as before, and resuspended in 50 mM Tris, pH 7.5. GlnJ and GlnK were purified to greater than 75% purity by eluting the protein from a Q-Sepharose column with a gradient of 100 to 400 mM NaCl. GlnJ was further purified to over 95% purity on a G-100 size exclusion column. GlnK was further purified to over 90% purity on a hydroxyapatite column after elution with 10 mM KPO₄ buffer, pH 7.5. Proteins were resuspended in 50 mM Tris, 100 mM NaCl, and 5% glycerol, pH 7.4.

Membrane preparation. Cell harvesting, breakage by sonication, and separation of whole-cell clarified extract into cytoplasmic and membrane fractions using ultracentrifugation were performed as described previously (53). Separation of the P_{II}-AmtB₁-containing membrane into soluble and membrane-bound protein fractions was done in the following manner. Samples were centrifuged at 200,000 × g for 30 min at 4°C, and 80% of the total volume was removed as the supernatant fraction. The remaining supernatant was discarded, and the membrane pellet was resuspended in an equal volume of 50 mM NaPO₄ buffer, pH 6.8. The resuspended membrane was frozen at −80°C until use.

Membrane extract from UR757 (*glnB glnK*) without bound P_{II} was prepared by incubating membranes for 1 h at 30°C in the presence of 10 mM 2-KG and 3 mM ATP, followed by two rounds of ultracentrifugation at 200,000 × g for 30 min at 4°C. All supernatant was removed, and the pellet was resuspended in an equal volume of 50 mM NaPO₄ buffer, pH 6.8, after each centrifugation to remove 2-KG and ATP from the final membrane preparation. No GlnJ was present in the membrane extract following such treatment, as determined by Western blotting using anti-GlnJ antibodies.

P_{II} disassociation and reassociation assays. Both disassociation and reassociation reactions took place in reaction buffer with a final concentration of 50 mM NaPO₄ buffer, pH 6.8, 100 mM NaCl, 25 mM MgCl₂, 1 mM MnCl₂, and 1 mM dithiothreitol, adapted from previous reports (9, 24, 54). ATP, ADP, and AMP-PNP [adenylyl 5'-(β,γ-imido)triphosphate tetralithium salt], at 95% or greater purity (Sigma-Aldrich, St. Louis, MO), and 2-KG were added to reactions. Membrane extract (250 μl), normalized to an OD₅₈₁ of 0.14, was added to 250 μl of 2× reaction buffer, and the mixture was mixed and incubated for 1 h at 30°C. Afterwards, the reaction mixtures were immediately placed on ice and then transferred to ultracentrifugation tubes for separation of soluble proteins from membranes and insoluble proteins by ultracentrifugation as described above. Incubation of reaction mixtures on ice prevented further changes in association between GlnJ and AmtB₁ (data not shown).

Immunoblotting of *R. rubrum* GlnB, GlnJ, and GlnK. Trichloroacetic acid precipitation was used to extract and concentrate protein from all protein samples (51). Proteins samples were loaded on low-cross-linker tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels as described previously (41, 53). Proteins were electrophoretically transferred to a nitrocellulose membrane and probed with polyclonal antibody against *R. rubrum* GlnJ or GlnB. Visualization was performed with either enhanced chemiluminescence detection reagent (Amersham Biosciences, Piscataway, NJ) and Kodak film for protein detection or with SuperSignal West Dura extended-duration substrate (Pierce, Rockford, IL) for quantitation using a Storm 860 phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Software. Storm scanner control, version 5.03 (Amersham Biosciences), was used to operate the Storm 860 phosphorimager, and ImageQuant, version 5.0, build 050 (Molecular Dynamics), was used to quantitate bands from Western blots. Data were then exported to Excel 2000 (Microsoft Corporation, Redmond, WA) and graphed and analyzed in KaleidaGraph, version 4.02 (Synergy Software, Reading, PA). Global adjustments to contrast and brightness of Western

blots were performed using Photoshop CS2 (Adobe Systems Incorporated, San Jose, CA).

RESULTS

The P_{II} homolog GlnJ associates with AmtB₁-containing membranes in vivo. Previously, we observed that some P_{II} was sequestered at the membrane in *R. rubrum* under conditions of nitrogen sufficiency and that membrane localization of P_{II} was absolutely dependent on the presence of AmtB₁ (53). However, the identity of the sequestered P_{II} was unclear. It has been predicted that the P_{II} homolog whose encoding gene is in close proximity to *amtB* will interact with AmtB (46). This hypothesis suggests that, since *glnJ* is in an operon with *amtB*₁ in *R. rubrum*, the proteins expressed by these genes are likely to interact. To test this possibility, we used a *glnB glnK* strain (UR757) in which GlnJ is the only P_{II} homolog produced. This strain also produced the AmtB homolog AmtB₁, but not the other AmtB homolog in *R. rubrum*, AmtB₂, since the *glnK::aacC1* insertion is polar on the downstream *amtB*₂ gene. We grew this strain in nitrogen-limiting MG medium to early exponential phase anaerobically in the presence of light; the *glnJ amtB*₁ operon is highly expressed under these culture conditions. However, GlnJ accumulates in a uridylylated form under nitrogen-limiting conditions. Therefore, the culture was then shifted to darkness for 20 min, which leads to partial deuridylylation of GlnJ. The cells were then separated into cytoplasmic and membrane fractions and analyzed by Western blotting. Equal volumes of each fraction, concentrated to the same degree, were loaded onto the tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel so that the relative amount of P_{II} protein in cell extracts was measured.

As expected, in Fig. 1 both an upper uridylylated GlnJ band and a lower nonuridylylated GlnJ band are visible in whole-cell extracts of the *glnB glnK* mutant UR757. Only nonuridylylated GlnJ is present in the membrane extract, while uridylylated GlnJ predominates in the cytoplasmic extract. This is in agreement with our earlier observation that nonuridylylated P_{II} in wild-type extracts preferentially interacts with an AmtB₁-containing membrane compared to uridylylated P_{II}, as well as prior observations for *E. coli* by others (9, 53). The proportion of total P_{II} in membrane extract versus cytoplasmic extract was also similar to the proportions we found in wild-type extracts. It is also consistent with recent structural model predictions that indicate that an uridylyl group on Tyr51 would sterically hinder interaction between P_{II} and AmtB in *E. coli* (8, 17).

Next, we sought to determine whether GlnJ is the primary P_{II} homolog found associated with the membrane in wild-type *R. rubrum*. Although GlnJ associated strongly with the membrane in vivo in the absence of GlnB and GlnK, there may be a mixture of P_{II} homologs at the membrane in wild-type cells. We grew UR2 (wild-type) cells photosynthetically but moved cells to darkness for 1 h before harvesting, allowing nearly complete deuridylylation of P_{II}. We then blotted equal volumes of cell extracts against anti-GlnB or anti-GlnJ antibodies (Fig. 1). Although there is substantial cross-reactivity between antibodies to different P_{II} homologs, anti-GlnB antibodies detected GlnB about 10-fold more strongly than GlnJ, while anti-GlnJ antibodies detected GlnJ about 3-fold better than GlnB (data not shown). When anti-GlnJ antibodies are used,

FIG. 1. Cellular localization of *R. rubrum* GlnJ (and GlnB) in cell extracts. Shown are Western blots of whole-cell, cytoplasmic, and membrane extracts of photosynthetically grown *R. rubrum* UR2 (wild type) and UR757 (*glnB glnK*) with antibodies against *R. rubrum* GlnB and GlnJ. Whole-cell extracts (W), cytoplasmic extracts (C), and membrane extracts (M) were loaded as indicated. (Upper two panels) Western blots of GlnJ in extracts of UR2 (top) and UR757 (bottom) extracts from cells 20 min after a shift from light to darkness. UR2 extracts contain all three P_{II} proteins, while UR757 extracts contain only GlnJ. Both P_{II}-UMP (upper band) and P_{II} (lower band) are present in whole-cell extract, while only unmodified P_{II} is present in membrane extract. (Lower two panels) Western blots of GlnJ (top) and GlnB (bottom) in UR2 extracts 1 h after a shift from light to darkness. Deuridylylation of P_{II} is almost complete. The majority of GlnJ signal is found in membrane extract, while more GlnB signal is present in cytoplasmic extract than in membrane extract. Both GlnB and GlnJ antibodies cross-react with other P_{II} proteins but are more sensitive to the P_{II} homolog they were raised against.

the majority of P_{II} signal is found in membrane extract, suggesting that most of the GlnJ is found at the membrane under these conditions. In contrast, with anti-GlnB antibodies there is slightly more P_{II} signal in the cytoplasmic extract than in membrane extract, suggesting that GlnB may be present in both cytoplasmic and membrane extracts. Thus, both GlnB and GlnJ can associate with AmtB₁ in the membrane under these conditions but GlnJ appears to be preferentially bound. To avoid complications from a potentially mixed P_{II} population, the following experiments on P_{II}-AmtB interaction used the *glnB glnK* strain as a source of purified AmtB₁-containing membrane, where GlnJ is the only P_{II} present in these extracts.

ATP and 2-KG affect the stability of GlnJ interaction with the AmtB₁-containing membrane. Proposed models of P_{II} interaction with AmtB suggest that all three sites of uridylylation, the Tyr51 residue of each monomer in the P_{II} trimer, would be inaccessible for modification by GlnD when GlnJ is bound to AmtB₁, and recent crystal structures of P_{II}-AmtB complexes support this hypothesis (2, 8, 17). Thus, although P_{II} uridylylation could prevent association between P_{II} and AmtB, the uridylylation process should not be able to cause complex disassociation. It seemed reasonable that small-molecule binding, not uridylylation, would be the primary regulator of interaction between these two proteins. This idea was supported by results obtained using purified *E. coli* GlnK-AmtB complexes, where 3.5 mM ATP and 1 mM 2-KG led to complex disassociation (11). However, these results were obtained with AmtB dissolved in detergent rather than in its native membrane environment. We therefore studied this issue in the physiologically relevant in vivo environment of purified membrane extracts

FIG. 2. Effects of small molecules on the stability of GlnJ-AmtB₁ interaction *in vitro*. Shown are Western blots of GlnJ on soluble and membrane-bound fractions of *R. rubrum* UR757 membrane extract. Small molecules were added to membrane extract containing AmtB₁-bound GlnJ at the concentrations listed, as described for the P_{II} association and disassociation assays in Materials and Methods. Soluble and membrane-bound proteins were separated by ultracentrifugation and concentrated by trichloroacetic acid precipitation to equal levels compared to the original membrane extract. Proteins from the supernatant (S) were loaded in the left lane of each panel, and membrane-associated proteins (M) were loaded in the right lane. High levels of 2-KG (10 mM) are sufficient to disrupt GlnJ-AmtB₁ interaction, while low levels of 2-KG (1 mM) are not, except in the presence of ATP. Substrate analogues ADP and oxaloacetic acid (OAA) do not substitute for ATP and 2-KG, respectively.

containing AmtB₁ and bound GlnJ; membrane extracts were prepared by sonication and therefore consisted primarily of inside-out vesicles exposing the GlnJ-AmtB₁ complex to solvent (42).

As seen in Fig. 2, when purified membrane extract from an *R. rubrum* *glnB glnK* mutant was incubated in buffer lacking exogenous ATP or 2-KG, almost all GlnJ remained associated with the membrane. In the presence of 3 mM ATP and 10 mM 2-KG, all GlnJ was released from the membrane and was found solely in the supernatant. To clarify whether ATP or 2-KG affected the stability of the GlnJ-AmtB₁ complex, either 3 mM ATP or 10 mM 2-KG was added separately. While 3 mM ATP alone had no effect, 10 mM 2-KG alone was able to efficiently remove GlnJ from the membrane. Increasing ATP levels up to 15 mM ATP had very little effect on the stability of GlnJ binding (data not shown). In contrast, in the absence of ATP lower levels of 2-KG (1 mM) caused only a small amount of GlnJ to disassociate (Fig. 2).

The binding of ATP and 2-KG to P_{II} is known to be cooperative in *E. coli*, and this is likely to be true in *R. rubrum* as well (26, 36). Given this, we next tested a combination of 3 mM ATP and 1 mM 2-KG. If the presence of ATP increased the affinity of GlnJ for 2-KG, we would expect to see larger amounts of GlnJ released from the membrane than we see when 2-KG is added in the absence of ATP. Supporting this notion, addition of both ATP and 2-KG resulted in significant release of GlnJ from the membrane, while either ATP or 2-KG alone at the same concentration had little or no effect (Fig. 2). To confirm that this was a specific effect of ATP and 2-KG, substrate analogues ADP and oxaloacetic acid were added at similar concentrations. However, neither molecule was able to act as a substitute, indicating that the ability to disrupt the GlnJ-AmtB₁ complex is likely to be specific to ATP and 2-KG. The addition of up to 10 mM NH₄Cl did not significantly affect these results, suggesting there is no direct sensing of ammonia

by the P_{II}-AmtB₁ complex (data not shown). These results suggest that 2-KG binding to P_{II} may directly cause disruption of GlnJ-AmtB₁ interactions in *R. rubrum*, while ATP binding to P_{II} may act indirectly by lowering the amount of 2-KG required for destabilization.

The ratio of ATP to ADP affects the stability of the GlnJ-AmtB₁ complex. Recent crystal structures show ADP bound to P_{II} in the *E. coli* P_{II}-AmtB protein complex (17). Although the absolute level of ATP is thought to be the most important indicator of cellular energy status sensed by P_{II}, this result suggested that ADP levels could also be an important factor in controlling the interaction between GlnJ and AmtB₁ in *R. rubrum*. Specifically, we hypothesized that ADP might be able to compete with ATP for binding sites on P_{II}, thus lowering the effective concentration of available ATP. This would lead to an increase in the proportion of GlnJ bound to AmtB₁ in our *in vitro* assay using an isolated *R. rubrum* membrane. If this were true, it would suggest that P_{II} homologs may be able to sense the ratio of ATP to ADP as well as the absolute level of ATP in the cell as a measure of energy status.

We tested the stability of GlnJ binding to the AmtB₁-containing membrane as in the previous section (Fig. 3A). In the presence of 500 μ M 2-KG alone, there was little disassociation of GlnJ from the membrane compared to the 0 μ M 2-KG control sample. As expected, a large fraction of GlnJ disassociated when 500 μ M ATP was added to the reaction mixture in the presence of 500 μ M 2-KG. We then increased ADP from 0 mM to 2.5 mM while keeping 2-KG and ATP levels constant and assayed the amount of GlnJ found at the membrane. There was no difference in levels of GlnJ association with the membrane when 250 μ M ADP was added, but when 500 μ M, 1 mM, or 2.5 mM ADP was added, an increasing amount of GlnJ was bound to the AmtB₁-containing membrane. Finally, the presence of 2.5 mM ADP had no effect on GlnJ membrane association in the absence of ATP. This demonstrates that ADP is able to interfere with the ability of ATP to disrupt GlnJ-AmtB₁ interaction in the presence of 2-KG, presumably by direct competition for the three primary nucleotide binding sites on the P_{II} trimer.

Finally, we examined whether ATP hydrolysis was necessary for the release of GlnJ from AmtB₁ by adding equal amounts of either ATP or a nonhydrolyzable analog, AMP-PNP, to isolated membranes. We chose to test a high-2-KG condition, where a relatively small amount of ATP would be required for release of GlnJ, and a low-2-KG condition, where a large amount of ATP would cause release of GlnJ. After separation of soluble proteins from membrane-bound proteins, we compared the proportions of GlnJ remaining at the membrane under both conditions. As seen in Fig. 3B, AMP-PNP appears to be an effective if imperfect substitute for ATP in the release of GlnJ from AmtB₁-containing membranes. With high 2-KG (2 mM) present, almost all GlnJ was released when 100 μ M of either ATP or AMP-PNP was added in the presence of 2 mM 2-KG (Fig. 3B). Under the low-2-KG condition (500 μ M), ATP again caused nearly complete disassociation of GlnJ (Fig. 3A) while AMP-PNP caused partial disassociation (Fig. 3B), perhaps due to a slightly lower binding affinity of GlnJ for AMP-PNP compared to ATP, but this was not explored further. We conclude that, even if ATP hydrolysis by P_{II} can

FIG. 3. Effects of ADP/ATP ratio or AMP-PNP on GlnJ-AmtB₁ complex stability in vitro. Shown are Western blots developed with antibody against GlnJ on soluble (S) and membrane-bound (M) fractions of *R. rubrum* UR757 membrane extract. Small molecules added to each reaction are listed. Samples were treated as described for Fig. 2. (A) ADP stabilizes the GlnJ-AmtB₁ complex in the presence of ATP and 2-KG, with higher levels of ADP providing more stabilization. (B) AMP-PNP is able to substitute for ATP in destabilizing GlnJ-AmtB₁ interaction in the presence of 2-KG, indicating that ATP hydrolysis is not required for disassociation of the complex. Controls for the 0.5 mM 2-KG, 0.5 mM AMP-PNP condition are shown in panel A and were performed at the same time as reactions shown in panel B.

occur, it does not appear to be required for disruption of the GlnJ-AmtB₁ complex in *R. rubrum*.

GlnB and GlnK bind the AmtB₁-containing membrane less efficiently than does GlnJ in vitro. Although we had shown that GlnJ is capable of interacting with AmtB₁ both in vivo and in vitro, it was unclear whether other P_{II} homologs in *R. rubrum* could bind AmtB₁ as strongly as GlnJ could. If the GlnJ-AmtB₁ complex could be reconstituted in vitro, we would be able to test the ability of GlnB or GlnK to also complex with AmtB₁ in vitro. We took advantage of our observation that addition of high levels of ATP and 2-KG caused complete release of GlnJ from isolated membranes to prepare AmtB₁-containing membranes stripped of GlnJ. Both GlnJ and small molecules were subsequently washed away with repeated ultracentrifugation steps, producing AmtB₁-containing membrane extract that we expected would be able to rebind GlnJ.

We first tested the ability of washed membranes to rebind GlnJ. Washed membranes treated with a twofold excess of purified GlnJ protein, compared to the original membrane extract, were able to bind approximately 60% of the GlnJ found in extract before treatment with ATP and 2-KG (data not shown). The decrease in GlnJ binding capacity of washed membranes was probably due to membrane loss during the washing and resuspension steps or imperfect resuspension of the membrane after ultracentrifugation, which might mask some AmtB₁ inside membrane particles. However, the important observation remained that a membrane stripped of GlnJ could rebind GlnJ in our buffer conditions. This allowed us to test the ability of the AmtB₁-containing membrane to individually bind each of the three P_{II} homologs in *R. rubrum*.

Data shown in Fig. 4 are from representative experiments, all using the same preparation of membrane extract from wild-type cells; different preparations differed slightly in GlnJ binding capacity but showed the same trends. Different amounts (0.21 μ g to 10 μ g) of *R. rubrum* GlnJ were added to washed membranes, corresponding to a final P_{II} concentration of about 11 to 540 nM. The maximal amount of added GlnJ, 10 μ g, was 2.5-fold more than the amount of GlnJ found in the isolated membrane and about 4-fold more than the maximal amount of rebound GlnJ. The amounts of GlnJ recovered from soluble and membrane-bound fractions were quantitated by Western blotting. The amount of GlnJ associated with the membrane increased as more GlnJ was added to the membrane extract but approached saturation of the membrane's binding capacity at about 2.5 μ g total GlnJ, a concentration of about 135 nM GlnJ (Fig. 4A).

When either purified GlnB or GlnK was added to membrane extract, the results were very different (Fig. 4B and C). Very little GlnB or GlnK was associated with the membrane, though in each case, the detected amount was higher than that in membrane samples from a strain lacking AmtB₁ (data not shown). At higher levels of added GlnB or GlnK there was no associated increase in membrane binding, unlike the GlnJ results. This suggests that, while both GlnB and GlnK are able to associate specifically with AmtB₁, this interaction is less stable than the GlnJ-AmtB₁ interaction and is lost during our sample processing. This supports the notion that in wild-type *R. rubrum* AmtB₁ primarily interacts with GlnJ.

ATP and 2-KG act synergistically to promote release of GlnJ from the AmtB₁-containing membrane. The strong, relatively stable interaction between GlnJ and AmtB₁ in membrane extracts allowed us to further explore the effects of small molecules on the stability of this complex. Specifically, we examined a broad range of both ATP and 2-KG concentrations to estimate the disassociation constants for each small molecule in the presence or absence of the other. After treatment with small molecules, Western blotting quantitated the proportions of GlnJ found associated with the membrane and free in solution. These values were then normalized to the maximal proportion of GlnJ found at the membrane under any condition, as a small amount of GlnJ, about 5%, disassociated from the membrane during treatment under any condition.

We first examined the effects of 2-KG on the stability of the GlnJ-AmtB₁ complex (Fig. 5A). In the absence of ATP, levels of 2-KG under 1 mM had no significant effect on complex stability. Increasing levels of 2-KG resulted in more release of

FIG. 4. In vitro comparison of specificities between AmtB₁ and the P_{II} homologs. Shown is a quantitative analysis of P_{II} rebinding to the AmtB₁-containing membrane. P_{II} remaining in solution (circles) and in the membrane pellet (squares) was measured by quantitative Western blotting. (A) Increasing amounts of GlnB are added to a fixed amount of AmtB₁-containing membrane stripped of P_{II}. (B) GlnJ titration. (C) GlnK titration. GlnB antibodies were used to probe GlnB, while GlnJ antibodies were used to probe GlnJ and GlnK. Data points are from a representative experiment using the same preparation of membrane extract. Protein was concentrated or diluted such that final amounts of P_{II} loaded fell within the linear range of protein as determined by using pure protein standards. Repetitions of several different data points suggest an error of less than $\pm 10\%$.

GlnJ; however, even 15 mM 2-KG failed to cause complete disassociation of GlnJ. The K_d of GlnJ-AmtB₁ for 2-KG in the absence of ATP was calculated to be $5,000 \pm 430 \mu\text{M}$ by curve fitting a standard disassociation equation to the data with the

FIG. 5. 2-KG and ATP disrupt GlnJ-AmtB₁ interaction in a dose-dependent fashion. Shown is a quantitative analysis of GlnJ binding to the AmtB₁-containing membrane at increasing ATP or 2-KG concentrations. (A) Various amounts of ATP were added to membrane extract isolated from UR757 while 2-KG levels were kept constant at 0 μM or 500 μM . (B) 2-KG was added in various amounts to membrane extract while ATP levels were kept constant at 0 mM or 3 mM. (C) The AmtB₁-containing membrane was stripped of GlnJ by incubation at high ATP and 2-KG and resuspended in buffer lacking ATP and 2-KG. Various amounts of 2-KG and either 0 mM or 3 mM ATP were added before adding 2 μg GlnJ. The amounts of GlnJ in solution and at the membrane were quantified by Western blotting. Error bars represent the standard deviations of multiple independent samples; data points without error bars are based on a single sample.

software program KaleidaGraph. Next, we examined the effects of 2-KG in the presence of 3 mM ATP. This concentration of ATP was chosen as a physiologically relevant level that may be present under conditions of energy sufficiency (34).

ATP dramatically lowered the levels of 2-KG required for destabilization of the GlnJ-AmtB_I complex. Furthermore, complete disassociation of GlnJ was seen only in the presence of ATP. The K_d of the GlnJ-AmtB_I complex for 2-KG in the presence of 3 mM ATP was approximately $340 \pm 30 \mu\text{M}$. This synergistic effect of 2-KG and ATP is also observed in the model of cooperative binding found for *E. coli* and *Synechococcus* sp. P_{II} proteins (15, 26, 35). *R. rubrum* P_{II} also demonstrates cooperative binding of ATP and 2-KG in in vitro modification experiments using *R. rubrum* GlnD and GlnB proteins (data not shown).

Next, we titrated increasing amounts of ATP in the absence of 2-KG or in the presence of 500 μM 2-KG, a level of 2-KG that caused only a small amount of GlnJ release in the absence of ATP (Fig. 5B). In the absence of 2-KG, over 90% of GlnJ remained associated with the membrane even at 15 mM ATP, the highest level of ATP tested. This indicates that either ATP alone is unable to interact with the GlnJ-AmtB_I complex or ATP binding does not significantly affect the stability of the complex. However, in the presence of 500 μM 2-KG, addition of ATP promoted complex disassociation. Levels of ATP below 100 μM had little effect on stability, and the K_d of the GlnJ-AmtB_I complex for ATP in the presence of 500 μM 2-KG was $630 \pm 65 \mu\text{M}$. However, this is not a saturating level of 2-KG; for example, our data in Fig. 3B indicate that, in the presence of 2 mM 2-KG and absence of ADP, even as little as 100 μM ATP was sufficient to release most GlnJ from AmtB_I.

Finally, we tested the effect of small molecules on the ability of the GlnJ-AmtB_I complex to reform in vitro. The results, shown in Fig. 5C, are almost identical to those in Fig. 5B. This was expected, as both reaction mixtures were incubated for an extended period of time and should have reached equilibrium between bound and unbound GlnJ in the samples. This is also a confirmation that the reconstituted P_{II}-AmtB_I complexes behave similarly to those isolated from *R. rubrum*.

DISCUSSION

Most prokaryotes have one or more P_{II} homologs, as well as at least one AmtB homolog, yet the question of specificity between these two families of proteins has not been examined in any depth. Our results indicate that in *R. rubrum* the P_{II} homolog GlnJ, which is transcribed from the *glnJ amtB_I* operon, interacts more strongly with AmtB_I than do the other two P_{II} homologs present, GlnB and GlnK. This is direct evidence supporting the notion that, if the genes encoding the P_{II} and AmtB proteins are in close proximity to each other, the proteins can interact in vivo (46). However, both GlnB and GlnK are able to interact with AmtB_I in the absence of GlnJ in *R. rubrum*. This verifies our previous experiments that indirectly suggested that interaction between GlnB and AmtB_I also occurs in vivo and that this interaction can have physiological significance (52, 53). These results are consistent with our previous observations that in *R. rubrum* AmtB_I can interact with all three P_{II} homologs in vivo, but with various degrees of specificity (53).

Why would there be specificity in interaction between P_{II} and AmtB proteins? A known difference between P_{II} homologs in *R. rubrum* is that only GlnB is able to activate NifA, which activates transcription of genes involved in nitrogen fix-

ation; GlnJ and GlnK are unable to activate NifA. In addition, while the *glnJ amtB_I* operon is expressed only under nitrogen-limiting conditions, *glnB* is expressed constitutively, although expression is also upregulated under nitrogen-limiting conditions (3, 54). We have found that, when *R. rubrum* experiences an increase in nitrogen levels in the cell, GlnJ may be the preferred P_{II} homolog sequestered to the membrane. This would leave a relatively large proportion of GlnB free in the cytoplasm, which might then reactivate NifA upon subsequent decrease in nitrogen levels. Therefore, one possible reason for specificity between P_{II} and AmtB is to regulate which functions the remaining free P_{II} is capable of performing. Additionally, if the level of 2-KG required for uridylation of GlnB by *R. rubrum* GlnD is less than that required for disassociation of GlnJ from AmtB_I, then GlnJ would be modified after GlnB in vivo; this would again allow a specific uridylylated P_{II} to function despite the presence of multiple P_{II} proteins in the cell. However, the minimal level of 2-KG required for efficient modification of *R. rubrum* P_{II} in vivo is not yet known (25).

In organisms such as *E. coli*, the two P_{II} homologs GlnB and GlnK appear to be functionally interchangeable when expressed at equal levels (4). In such a case, P_{II} binding to AmtB still has the effective consequence of transiently and reversibly sequestering the majority of cytoplasmic P_{II} to the membrane. This would enable the cell to more rapidly recover from a transient increase in nitrogen status by quickly increasing the concentration of active P_{II} in the cell (6). While GlnK is preferentially bound to AmtB at the membrane over GlnB, there is no clear requirement for this specificity of interaction between GlnK and AmtB in *E. coli* (9).

The small-molecule requirements we observed for release of GlnJ from AmtB_I in *R. rubrum* are similar to those observed for *E. coli*: both ATP and 2-KG are required for GlnJ release when only 1 mM of 2-KG is added to high levels of ATP (11). However, 2-KG alone led to complex disassociation at millimolar concentrations, and high ATP levels neither stabilized nor destabilized the complex, whereas ATP was required for complex stability in *E. coli*. Higher levels of 2-KG were not tested in *E. coli*, so it is unclear if 2-KG alone would have effects on complex stability in this organism (11). In contrast, ATP alone was sufficient to disrupt the P_{II}-AmtB complex in *Bacillus subtilis* and the presence of 2-KG had almost no effect on complex stability (18). Low levels of ATP (100 μM) in the absence of 2-KG were also sufficient to disrupt a purified GlnK1-Amt1 complex from *M. jannaschii* (50).

Both allosteric effectors of P_{II}, ATP and 2-KG, affected the stability of the GlnJ-AmtB_I complex in *R. rubrum*. Overall, the K_d values obtained for both ATP and 2-KG were significantly higher than values others have observed for binding of one ATP or one 2-KG molecule in *E. coli* GlnB (24). In vitro experiments with purified *R. rubrum* P_{II} homologs and purified *E. coli* GlnD suggest that the K_d of *R. rubrum* P_{II} for 2-KG is similar to that of *E. coli* (our unpublished results). The large increase in measured binding constants for 2-KG and ATP could be attributed to two possibilities: either multiple molecules of 2-KG and ATP must bind the GlnJ-AmtB_I complex for it to disassociate or the K_d of GlnJ for 2-KG is dramatically altered when GlnJ is bound to AmtB_I. Our results favor the former model, as the calculated binding constants were similar whether GlnJ was already bound to the AmtB_I-containing

membrane or whether it was added to a membrane stripped of P_{II} . However, while we predict that the binding of one 2-KG molecule has little effect on GlnJ-AmtB₁ complex stability, it is still unclear whether only two or all three binding sites for 2-KG on GlnJ must be filled to allow release of GlnJ from AmtB₁. It has been suggested that an intermediate state of 2-KG binding has physiological relevance in *E. coli*, where P_{II} with three ATP molecules and one 2-KG molecule bound is likely to interact with NtrB (23, 26, 35). In addition, the ability of high concentrations of 2-KG and ATP to prevent interaction between P_{II} and AmtB proteins is the simplest explanation for the recent observation for *Rhodobacter capsulatus* that fully unmodified GlnK fails to be sequestered in the membrane in a *glnB* mutant despite the presence of AmtB protein (47).

Our results indicate that competition between ADP and ATP for binding sites on P_{II} can be a relevant factor in regulating interaction between P_{II} and AmtB. Other proteins have also been shown to be regulated by the ratio of ATP to ADP such as SpoIIAB in *Bacillus subtilis*, where one of two potential protein targets is bound according to which nucleotide is in the majority (1). In our system, high levels of ADP are able to stabilize the GlnJ-AmtB₁ complex in the presence of ATP and 2-KG, which would otherwise destabilize the complex. Sensing the ATP to ADP ratio allows yet another means to exert subtle control over the degree of response to energy depletion and nitrogen deficiency. ADP has also recently been found to influence the ability of *E. coli* P_{II} to interact with receptors GlnE and NtrB and the signal transduction protein GlnD; in each case, ADP appears to signal energy limitation and limits the degree of response by P_{II} to nitrogen limitation (P. Jiang and A. J. Ninfa, personal communication).

Although effects on the stability of the AmtB₁-GlnJ complex of *R. rubrum* were seen only when the ratio of ADP to ATP was greater than 1 in our experiments, these data still support the idea of energy charge being an important regulatory input of P_{II} protein function. Adenylate energy charge is determined by formula $([ATP] + 1/2[ADP])/([ATP] + [ADP] + [AMP])$ and has commonly been estimated to be near 0.85 during logarithmic growth in bacteria, implying there is much less ADP than ATP in these cells (7). However, many reports of energy charge give a ratio of ADP to ATP that is significantly higher. Specifically, in *R. rubrum* under conditions identical to those used during collection of cells for determining the localization of P_{II} to the membrane, the estimated energy charge is only about 0.66 and the ratio of ADP to ATP is approximately 1 within the limits of experimental error (38). Also, adenylate energy charge has been measured at significantly lower levels in both log and stationary-phase growth conditions in multiple bacterial species, where the ratio of ADP to ATP is as much as 3 (5, 12, 31, 37). Given that nonlogarithmic growth conditions are more likely to be relevant in the wild, we predict that energy sensing by P_{II} is an important function of this protein.

Uridylylation of GlnJ in *R. rubrum* provides an additional level of regulation of interaction between GlnJ and AmtB₁, above and beyond small-molecule concentrations. Only non-uridylylated P_{II} interacts with AmtB, and recent crystal structures show that modification of P_{II} would sterically block interaction. Therefore, there are two effective concentrations of P_{II} in the cell: total P_{II} and that fraction of P_{II} that is unmodified and therefore available for interaction with AmtB₁. Even

if the concentrations of small molecules in the cell are permissive of interaction between P_{II} and AmtB, there necessarily is a delay while P_{II} modifications are removed before interaction can take place.

In summary, we have demonstrated that AmtB₁ of *R. rubrum* shows some specificity for binding the P_{II} homolog GlnJ over the other two P_{II} homologs present, GlnB and GlnK, and that only nonuridylylated GlnJ interacts strongly with AmtB₁. The binding of ATP and 2-KG to GlnJ specifically disrupts the interaction between GlnJ and AmtB₁, but ATP hydrolysis is not required for this process. The level of 2-KG required suggests that multiple 2-KG molecules may need to bind GlnJ for complex disassociation in *R. rubrum*. Finally, ADP stabilizes the GlnJ-AmtB₁ complex in the presence of ATP and 2-KG, suggesting that the ratio of ADP to ATP may be sensed by P_{II} as a measure of cellular energy status for at least some P_{II} functions.

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