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In Vitro Studies of the Uridylylation of the Three P_{II} Protein Paralogs from *Rhodospirillum rubrum*: the Transferase Activity of *R. rubrum* GlnD Is Regulated by α-Ketoglutarate and Divalent Cations but Not by Glutamine[▽]

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P_{II} proteins have been shown to be key players in the regulation of nitrogen fixation and ammonia assimilation in bacteria. The mode by which these proteins act as signals is by being in either a form modified by UMP or the unmodified form. The modification, as well as demodification, is catalyzed by a bifunctional enzyme encoded by the *glnD* gene. The regulation of this enzyme is thus of central importance. In *Rhodospirillum rubrum*, three P_{II} paralogs have been identified. In this study, we have used purified GlnD and P_{II} proteins from *R. rubrum*, and we show that for the uridylylation activity of *R. rubrum* GlnD, α-ketoglutarate is the main signal, whereas glutamine has no effect. This is in contrast to, e.g., the *Escherichia coli* system. Furthermore, we show that all three P_{II} proteins are uridylylated, although the efficiency is dependent on the cation present. This difference may be of importance in understanding the effects of the P_{II} proteins on the different target enzymes. Furthermore, we show that the deuridylylation reaction is greatly stimulated by glutamine and that Mn^{2+} is required.

In *Escherichia coli*, the pivotal role of sensing the nitrogen level within the cell is played by the bifunctional uridylyltransferase/uridylyl-removing enzyme, encoded by the *glnD* gene, through the interaction with glutamine as an indicator of the intracellular nitrogen status (12). The signal is then further transduced to the regulatory P_{II} proteins by uridylylation/deuridylylation catalyzed by GlnD. P_{II} proteins are among the most highly conserved signaling proteins in nature, with regulatory roles in both transcriptional and posttranslational processes (2, 23). In the *E. coli* system, GlnD catalyzes the uridylylation of P_{II} proteins at low glutamine concentrations, whereas at high concentrations of glutamine, the removal of UMP groups from the P_{II} proteins is stimulated (17). A number of targets for P_{II} proteins have been identified; in some cases the modified P_{II} interacts with its target(s), in some the unmodified form, and in some both forms (24). It seems, however, that in order to efficiently interact with a target, ATP and α-ketoglutarate must be bound to the P_{II} protein (17, 18). When considering the multiple targets for P_{II} proteins in their different uridylylation states, the role of GlnD in catalyzing the modification/demodification is of crucial importance.

A number of bacterial P_{II} proteins have been shown to be substrates for GlnD, but GlnB from *E. coli* is the most thoroughly studied (12, 17). In this bacterium, uridylylated GlnB does not interact with NtrB, the sensor of the two-component Ntr system. NtrB then catalyzes the phosphorylation of NtrC, the response regulator, that then acts as a transcriptional activator of operons involved in ammonium assimilation and nitrogen metabolism (3, 5, 13, 29). In contrast, under high-nitrogen conditions, GlnB is deuridylylated, and this form interacts with NtrB, promoting the phosphatase activity of NtrB, leading to dephosphorylation of NtrC. Another target enzyme for uridylylated GlnB is adenylyltransferase, encoded by *glnE*, a bifunctional enzyme catalyzing the adenylylation/deadenylylation of glutamine synthetase (9, 11, 14, 20, 31). Under low-nitrogen conditions, adenylyltransferase catalyzes the removal of AMP groups from glutamine synthetase, leading to a more-active enzyme. On the other hand, unmodified GlnB stimulates the adenylylation activity of GlnE and consequently the inactivation of glutamine synthetase.

Rhodospirillum rubrum is a photosynthetic purple free-living nitrogen-fixing bacterium, and as with other diazotrophs, the fixed nitrogen is further assimilated via the glutamine synthetase and glutamate synthase pathway (21, 22, 30). In *R. rubrum*, three P_{II} proteins have been identified, encoded by *glnB*, *glnK*, and *glnJ*, respectively (37). It was shown that in this bacterium, either GlnB or GlnJ is required for proper regulation of the dinitrogenase reductase ADP-ribosyl transferase (DRAT)/dinitrogenase reductase-activating glycohydrolase (DRAG) system, whose two enzymes are involved in posttranslational regulation of nitrogenase activity (26). Recently a mutation in *amtB1*, encoding a putative ammonia transporter, resulted in a strain impaired in DRAT/DRAG regulation (36, 40). The function of AmtB in this regulation is proposed to be to sequester unmodified GlnJ.

Furthermore, by mutational studies it has been shown that activation in *R. rubrum* of NifA, a transcriptional activator of nitrogen-fixing (*nif*) genes, requires the uridylylated form of GlnB and that neither GlnJ-UMP nor GlnK-UMP can substitute for GlnB-UMP (38).

Recently a number of mutations in *R. rubrum glnD* were constructed, and it was shown that the N-terminal region of

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and/or relevant characteristics	Source or reference
E. coli One Shot TOP10	Host for pCR-Blunt-TOPO derivatives	Invitrogen
E. coli BL21(DE3)/pLysS	Host for overexpression of pET-15b derivatives; Ap^r Cm^r	Invitrogen
E. coli BL21 Star (DE3)	Source of *E. coli* GlnD activity	Invitrogen
E. coli RB 9040	Δ*glnD*; Host for overexpression of pGEXderivatives; Tc^r	5
R. rubrum S1	Wild type	
Plasmids		
pCR-Blunt II-TOPO	Blunt PCR cloning vector; Km^r	Invitrogen
pET-15b	His-tagged (N-terminal) overexpression vector; Ap^r	Novagen
pGEX-6P-2/3	GST-tagged (N-terminal) overexpression vectors; Ap^r	Amersham Biosciences
pMJET	pET-15b derivative giving recombinant GlnB; Ap^r	16
pET-GlnK	pET-15b derivative with *glnK* cloned between NdeI and BamHI, giving recombinant GlnK; Ap^r	Unpublished[a]
pET-GlnJ	pET-15b derivative with *glnJ* cloned between NdeI and BamHI, giving recombinant GlnJ; Ap^r	Unpublished[a]
pET-E.c GlnK	pET-15b derivative with *E. coli glnK* cloned between NdeI and XhoI, giving recombinant *E. coli* GlnK; Ap^r	This study
pGEX-GlnD	pGEX-6P-3 derivative with *glnD* cloned between BamHI and EcoRI, giving recombinant GlnD; Ap^r	This study
pGEX-GlnB	pGEX-6P-2 derivative with *glnB* cloned between BamHI and EcoRI, giving recombinant GlnB; Ap^r	This study
pGEX-GlnJ	pGEX-6P-2 derivative with *glnJ* cloned between BamHI and EcoRI, giving recombinant GlnJ; Ap^r	This study
pGEX-GlnK	pGEX-6P-2 derivative with *glnK* cloned between BamHI and EcoRI, giving recombinant GlnK; Ap^r	This study
pET-GlnB39	pET-15b derivative encoding recombinant GlnB Q39E; Ap^r	This study
pET-GlnJ39	pET-15b derivative encoding recombinant GlnJ Q39E; Ap^r	This study
pET-GlnK39	pET-15b derivative encoding recombinant GlnK Q39E; Ap^r	This study

[a] A. Jonsson, P. Teixeira, and S. Nordlund, unpublished.

GlnD is essential for uridylylation of GlnB and GlnJ, similar to the *E. coli* system (34, 39). In addition, the results in this study also suggest that NifA activation, DRAT/DRAG regulation, and NtrBC regulation require different levels of GlnD activity (39).

Although the regulation of GlnD has been studied with a number of bacteria, these studies have in most cases been performed at a physiological level or by mutational approaches (6–8, 10, 28, 32, 35).

In vitro studies of the uridylylation/deuridylylation activities of GlnD using purified components have to our knowledge been reported only for GlnD, GlnB, and GlnK from *E. coli* (4, 12, 17). In addition, purified GlnZ, a P_{II} protein from *Azospirillum brasilense*, and purified GlnB from *R. rubrum* have been shown to be substrates for *E. coli* GlnD (1, 41). The efficiency by which GlnD catalyzes uridylylation/deuridylylation of P_{II} proteins has been shown to depend on the ligands bound to the P_{II} protein and the divalent cation present (12). Different P_{II} proteins also show different characteristics, e.g., using *E. coli* proteins, deuridylylation of GlnK-UMP was reported to be slower than that of GlnB-UMP in the presence of magnesium ions (4).

Detailed studies at the molecular level have been described only for the *E. coli* system, but this may not be fully applicable to other bacteria, e.g., diazotrophs. Considering the different primary targets for the three P_{II} proteins in *R. rubrum*, it is of special interest to establish if the three P_{II} proteins show different characteristics with respect to uridylylation/deuridylylation catalyzed by GlnD. Here we report our studies of the requirements for GlnD to catalyze the uridylylation of the three P_{II} paralogs in *R. rubrum* with different cations. The effects of α-ketoglutarate, glutamine, and ATP have also been investigated for the uridylylation/deuridylylation activities, showing characteristics of GlnD not reported previously. All experiments were performed with purified *R. rubrum* GlnD and P_{II} proteins.

MATERIALS AND METHODS

Plasmid construction. Bacterial strains and plasmids used in this study are listed in Table 1. For cloning of *glnJ* and *glnK* into the pGEX-6P-2 and pET-15b plasmids and *glnD* into the pGEX-6P-3 plasmid, primers were designed with appropriate restriction sites indicated in Table 1. All genes were obtained by PCR using *Pfu* polymerase (Stratagene) with *R. rubrum* S1 DNA as a template, except for the cloning of *glnB* into pGEX-6P-2, where pMJET was used as a template. For cloning of *E. coli glnK* into pET-15b, *E. coli* DNA was used as a template; otherwise, the same principle as for the above constructs was applied. All PCR products were routinely subcloned into the pCR-Blunt II-TOPO vector (Invitrogen) and all constructs verified by sequencing. Standard molecular biology methods, essentially as described by Sambrook et al. (33), were used.

Cell growth and purification of *R. rubrum* proteins. Transformation of plasmid pGEX-GlnD into *E. coli* strain RB 9040 was done according to the method of Sambrook et al. (33). Cells were grown at 20 to 25°C in Luria-Bertani medium supplemented with ampicillin (50 μg/ml), tetracycline (15 μg/ml), and 10 mM glutamine. Cells were grown to an optical density at 600 nm (OD_{600}) of 1.1, followed by induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 1 h at 20 to 25°C. Cells were then harvested and frozen as pellets in liquid nitrogen. Pellets were resuspended in glutathione-*S*-transferase (GST)-binding buffer (pH 7.4) containing 142.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, complemented with 20 μg/ml DNase, one tablet of Complete Mini EDTA-free protease inhibitor/liter (Roche), 2 mM phenylmethylsulphonylfluoride, and 0.5 mg/ml lysozyme, followed by sonication (six times, 10 s each). The supernatant, after centrifugation at 40,000 × *g* for 20 min, was filtered through a 0.45-μm filter

and applied to a GSTrap column (5 ml; Amersham Biosciences), equilibrated in GST-binding buffer (without additions) at a flow rate of 0.2 ml/min. The column was washed with cleavage buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), and 200 mM glutamine. PreScission protease (160 U; Amersham Biosciences) was then added to the column, which was incubated at 4°C overnight, followed by 2 h at room temperature. After digestion, GlnD was eluted in cleavage buffer and assayed for GlnD activity.

All pET-15b derivatives were transformed into *E. coli* BL21(DE3)/pLysS. Cultures were grown in Luria-Bertani medium at 37°C to an OD_{600} of 0.6 and then induced with 1 mM IPTG for 1 h. Cell cultures were then harvested and frozen in liquid nitrogen as pellets. Cell pellets were resuspended in His-Binding buffer (pH 7.4) containing 500 mM NaCl, 10 mM imidazole, and 20 mM Na_2PO_4, complemented with 20 μg/ml DNase, 2 mM phenylmethylsulphonylfluoride, and one tablet of Complete Mini EDTA-free protease inhibitor (Roche)/liter. The solution was then frozen in liquid nitrogen and thawed three times, followed by sonication (six times, 10 s each), and then centrifuged for 20 min at 40,000 × *g*. The resulting supernatant was filtered through a 0.45-μm filter and applied to a HisTrap HP column (1 ml; Amersham Biosciences), equilibrated in His-binding buffer (without additions). Washing and elution of recombinant P_{II} proteins from the column were performed with His-binding buffer containing 200 or 500 mM imidazole, respectively. To obtain untagged and unmodified P_{II} proteins, all pGEX derivatives were transformed into *E. coli* strain RB 9040. The purification and growth was the same as for GlnD with the following exceptions. The cells were grown at 37°C to an OD_{600} of 0.6, followed by induction with 1 mM IPTG for 2 h at 20 to 25°C. The GST-binding buffer contained 140 mM NaCl and 2.7 mM KCl. The cleavage buffer contained 150 mM NaCl instead of 150 mM KCl, and a GSTrap column (1 ml; Amersham Biosciences) was used. All P_{II} proteins purified were applied to a desalting column, PD-10 (Amersham Biosciences), and equilibrated in 50 mM HEPES (pH 8.0), 200 mM KCl, and 10% glycerol.

Site-directed mutagenesis of GlnB, GlnJ, and GlnK. Upper and lower primers were designed for generating Q39E variants of all P_{II} proteins by standard PCR-mediated site-directed mutagenesis by using *Pfu* polymerase (Stratagene). The primers contained a 1-bp mismatch, converting the codon for glutamine 39 to that for glutamate. Templates used were pET-GlnK, pET-GlnJ, and pMJET. and the plasmids produced were named pET-GlnK39, pET-GlnJ39, and pET-GlnB39, respectively.

Uridylylation of GlnB, GlnK, and GlnJ. Uridylylation of all P_{II} proteins was carried out at 30°C in a reaction mixture (100 μl) containing 0.5 μM purified P_{II} protein, 0.13 μM GlnD, 50 mM HEPES (pH 7.6), 100 mM KCl, 2 mM ATP, 1 mM DTT, 0.3 mg/ml bovine serum albumin, 0.5 mM UTP supplemented with ≈0.5 μCi [α-^{32}P]UTP, 25 mM $MgCl_2$, and 250 μM α-ketoglutarate or 60 μM when 3 mM $MnCl_2$ was used in the assay. In the experiments with extracts of wild-type *R. rubrum*, 15 μM *R. rubrum* GlnB or *E. coli* GlnK together with extracts corresponding to 120 μg of protein were also added to the reaction mixture. Samples were taken from the reaction mixtures after different time intervals and mixed with sodium dodecyl sulfate (SDS) cocktail (130 mM TRIS, pH 6.8, 4.2% SDS, 20% [vol/vol] glycerol, 10% 2-mercaptoethanol, and 0.003% bromphenol blue), followed by boiling for 2 min, and then run on an 18% SDS-polyacrylamide gel. After staining with Coomassie Brilliant blue followed by destaining, gels were dried and subjected to autoradiography on a phosphorimager for 1 h and overnight. Incorporation of [α-^{32}P]UMP into the proteins was then quantified and visualized using the Image Quant program.

Deuridylylation of GlnB and GlnJ. To determine deuridylylation of modified P_{II} proteins, the reaction mixtures were the same as for uridylylation except that 500 μM α-ketoglutarate (or omitted when indicated), 10 mM glutamine (or omitted when indicated), 0.4 μM uridylylated P_{II} protein and 1.3 μM GlnD were used. UTP and [α-^{32}P]UTP were omitted. Samples were taken from the reaction mixtures after different time intervals and analyzed as in the uridylylation assay except that [^{32}P]UMP remaining was quantified.

Purification of uridylylated P_{II} proteins. Either His-GlnB or His-GlnJ were incubated overnight at 30°C with 0.13 μM GlnD together with the same effectors and substrates as mentioned above for uridylylation, in a total reaction volume of 5 ml. The samples were then applied to PD-10 columns equilibrated in 50 mM HEPES (pH 8.0), 200 mM KCl and 10% glycerol and purified using 1 ml HisTrap HP columns precharged with Ni^{2+} ions according to the same procedures as for purification of unmodified his-tagged P_{II} proteins. Uridylylation efficiency was close to 100% as analyzed by 18% SDS-polyacrylamide gel electrophoresis (PAGE).

Purification of *E. coli* GlnD activity. As a source for producing *E. coli* GlnD activity, *E. coli* BL21 Star (DE3) cells (without any plasmid) were used. Purification was essentially the same as for the His-tagged P_{II} proteins with the exception that cell breakage was done in a Ribi fractionator. *E. coli* GlnD activity was recovered by elution with 200 mM imidazole followed by desalting using a PD-10 column (Amersham Biosciences) into buffer containing 50 mM HEPES (pH 7.6). Fifty micrograms of protein was used in the assays.

Preparation of wild-type *R. rubrum* extracts. *R. rubrum* strain S1 was grown in the minimal medium described by Ormerod, with the omission of glutamate (27). For growth under nitrogen-fixing conditions, referred to as N−, cultures were gassed with a mixture of 95% N_2/5% CO_2. Ammonium-grown cells, N+, were supplemented with 28 mM NH_4Cl. After harvesting (OD_{600} of ≈1.5), the cells were frozen as pellets in liquid nitrogen. The pellets were thawed in buffer containing 100 mM HEPES (pH 7.6), 1 mM $MnCl_2$, 1 mM DTT, and one tablet of Complete Mini EDTA-free protease inhibitor/liter (Roche). Cell breakage was performed in a Ribi cell fractionator followed by centrifugation at 2,500 × *g* to remove cell debris and unbroken cells. The extracts were then applied to PD-10 columns equilibrated in buffer containing 100 mM HEPES (pH 7.6), 1 mM DTT, and one tablet of Complete Mini EDTA-free protease inhibitor/liter.

Electrophoresis and Western blotting. Samples for SDS-PAGE (18%) followed by Western blotting were mainly the same as for the autoradiographic procedures with some exceptions. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane and further incubated with antibodies against *R. rubrum* GlnB or GlnJ. The ECL (Amersham Biosciences) was used for detection. Samples for Western blotting were loaded after at least 3 weeks to avoid interference of radioactivity from the labeled [α-^{32}P]UMP-P_{II} proteins.

RESULTS AND DISCUSSION

Overexpression and purification of GlnD, GlnB, GlnK, and GlnJ from *R. rubrum*. *R. rubrum* GlnD is a 936-amino-acid protein with a molecular mass of about 105 kDa, as deduced from the gene sequence (http://genome.ornl.gov/microbial/rrub/). The *glnD* gene was cloned and overexpressed as a fusion protein with GST in an *E. coli* Δ*glnD* strain (RB 9040), purified, and verified by matrix-assisted laser desorption ionization–time-of-flight analysis. The final product had a molecular mass of around 100 kDa as resolved by 8% SDS-PAGE (data not shown). Growing the cells at 20 to 22°C gave the best yield of purified GlnD (0.46 mg/liter culture). When overexpressed with a six-His tag at the N-terminal end instead of the GST tag, most of the GlnD fraction formed inclusion bodies. Attempts to dissolve the inclusion bodies were unsuccessful.

Sequence analysis showed that in all three *R. rubrum* P_{II} paralogs, there is a tyrosine at position 51, the residue being uridylylated in all bacterial P_{II} proteins studied. The molecular masses of the purified P_{II} proteins were estimated on 18% SDS-PAGE gels to be 12.4 kDa and 14.4 kDa when containing a six-His tag, all in good agreement with the expected molecular masses. Since we could not detect any difference in the uridylylation of untagged P_{II} proteins and the His-tagged version in our assays (data not shown), we used the His-tagged proteins in all experiments (except in the experiment shown in Fig. 9) for convenience.

Uridylylation—the effect of divalent cations. In *E. coli*, ammonium assimilation and thus nitrogen metabolism are controlled by the concentrations of α-ketoglutarate and glutamine (25). Both have effects on the uridylylation/deuridylylation reactions catalyzed by GlnD (12). Mutational studies performed with *E. coli* GlnD have shown that both activities of GlnD reside within the same domain (25). α-Ketoglutarate is believed to exert its effect by binding to the P_{II} protein, whereas the effect of glutamine is caused by interaction with GlnD (12). An increase in the cellular concentration of α-ketoglutarate reflects a decrease in the availability of fixed nitrogen, and under these conditions, three α-ketoglutarate molecules are bound to the P_{II} protein, enhancing the rate of uridylylation.

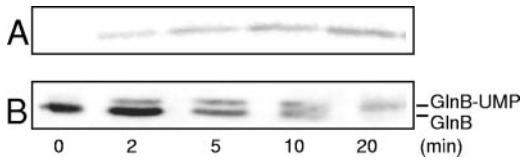

FIG. 1. Uridylylation of *R. rubrum* GlnB. GlnB (0.5 μM) was incubated in a uridylylation reaction mixture with 0.13 μM *R. rubrum* GlnD, 3 mM $MnCl_2$, 60 μM α-ketoglutarate, 2 mM ATP, and 0.5 mM UTP, supplemented with [α-^{32}P]UTP. Samples were withdrawn from the reaction mixtures after 0, 2, 5, 10, and 20 min of incubation and stopped by addition of SDS cocktail. The samples were subjected to SDS-PAGE and visualized by autoradiography in panel A or Western blotting in panel B, immunoblotted with antibodies against *R. rubrum* GlnB. In panel B, the lower band corresponds to unmodified GlnB and the upper to the modified form of GlnB.

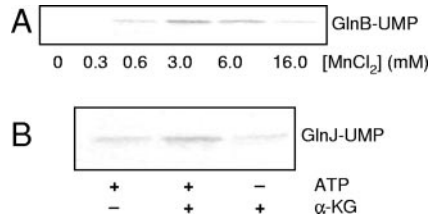

FIG. 2. Effect of $MnCl_2$, ATP, and α-ketoglutarate (α-KG) on uridylylation. (A) Uridylylation of GlnB (0.5 μM) with 0, 0.3, 0.6, 3.0, 6.0, or 16 mM $MnCl_2$ together with 2 mM ATP, 60 μM α-ketoglutarate, 0.13 μM GlnD, and 0.5 mM UTP supplemented with [α-^{32}P]UTP. (B) Effect of the absence of ATP or α-ketoglutarate on uridylylation of GlnJ (0.5 μM). The assays also contained 3 mM $MnCl_2$, 0.13 μM GlnD, and 0.5 mM UTP supplemented with [α-^{32}P]UTP. Uridylylation was assayed by incorporation of [α-^{32}P]UMP as described in Materials and Methods. Samples were withdrawn from the reaction mixtures after 20 min of incubation and stopped by addition of SDS cocktail.

On the other hand, glutamine acts as an inhibitor of the uridylylation reaction and stimulates deuridylylation (12, 18).

To determine the role of α-ketoglutarate, divalent cations, and glutamine in the regulation of P_{II} uridylylation in the *R. rubrum* system, we examined the effect(s) in vitro using purified *R. rubrum* GlnD and P_{II} proteins. To study these effect(s), we developed an assay to analyze incorporation of [α-^{32}P]UMP into the P_{II} proteins. Figure 1 shows a representative uridylylation experiment with GlnB in the presence of 3 mM $MnCl_2$ and 60 μM α-ketoglutarate at 0, 2, 5, 10, and 20 min, followed by Western blotting, showing a shift over time in the migration of GlnB. The same results were obtained with GlnJ and GlnK. The highest degree of uridylylation was obtained at 3 mM $MnCl_2$; above and below this concentration, labeling was less (Fig. 2A). No labeling of GlnB or GlnK could be detected when either ATP, α-ketoglutarate, or the divalent cations were omitted from the reaction mixture (data not shown). Surprisingly, GlnJ was uridylylated even when ATP or α-ketoglutarate was omitted from the reaction mixture but not to the same extent as with both present (Fig. 2B). However, when both ATP and α-ketoglutarate were omitted from the reaction mixture, no labeling of GlnJ was obtained (data not shown).

In an *E. coli* GlnB variant in which glutamine 39 was changed to glutamate, the uridylylation efficiency was lower (15). It was suggested that this effect was due to an inability of the variant to correctly bind α-ketoglutarate or ATP or effects on the interaction between GlnD and the mutated P_{II} proteins. In order to further study the effect of α-ketoglutarate on the *R. rubrum* proteins, we generated Q39E variants of all three paralogs. When assaying uridylylation with the Q39E variant of GlnB or GlnK, no incorporation of [α-^{32}P]UMP at any concentration of α-ketoglutarate used was observed. The GlnJ Q39E variant was labeled, however, but to a much lesser extent than native GlnJ (data not shown). Taken together, this indicates that the effect of α-ketoglutarate on *R. rubrum* GlnJ is different from that on GlnB or GlnK, since in the absence of either α-ketoglutarate or ATP, the native form of GlnJ was still uridylylated and the effect of the Q39E mutation on GlnJ was less than on the other paralogs.

A previous report showed that Mg^{2+} and Mn^{2+} clearly have different effects on the rate at which *E. coli* GlnD catalyzes uridylylation (12). We investigated the influence of Mg^{2+} and Mn^{2+} in the *R. rubrum* in vitro system. Initial titration experiments with these cations using excess amounts of GlnD (threefold) and limiting concentrations of GlnB showed that GlnB was totally labeled after 15 min of incubation with excess concentrations of ATP and α-ketoglutarate. An additional 45 min of incubation did not increase the amount of labeling, and Western blotting also showed that all GlnB was modified (data not shown). When titrating the α-ketoglutarate concentration for uridylylation of GlnB in the presence of either Mg^{2+} or Mn^{2+}, maximal modification was obtained with 250 μM and 60 μM α-ketoglutarate, respectively, as shown in Fig. 3. Similar

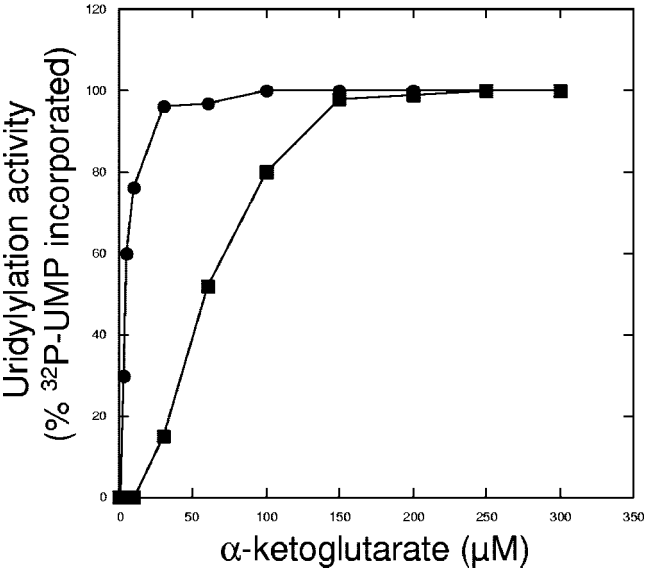

FIG. 3. The effect of α-ketoglutarate on uridylylation of GlnB with either $MgCl_2$ or $MnCl_2$. The assay contained 0.5 μM GlnB, 2 mM ATP, 0.13 μM GlnD, and 0.5 mM UTP supplemented with [α-^{32}P]UTP. Twenty-five millimolar $MgCl_2$ (■) or 3 mM $MnCl_2$ (●). Samples were withdrawn from the reaction mixtures after 20 min of incubation and stopped by addition of SDS cocktail. The incorporation of [α-^{32}P]UMP at 300 μM α-ketoglutarate was set to 100% for both $MgCl_2$ and $MnCl_2$. No more labeling was detected at α-ketoglutarate concentrations higher than 300 μM. The samples were subjected to SDS-PAGE and visualized by autoradiography. The amount of labeled, uridylylated GlnB was quantified using the Image Quant program. The data shown are representative of at least three independent experiments for both $MgCl_2$ and $MnCl_2$.

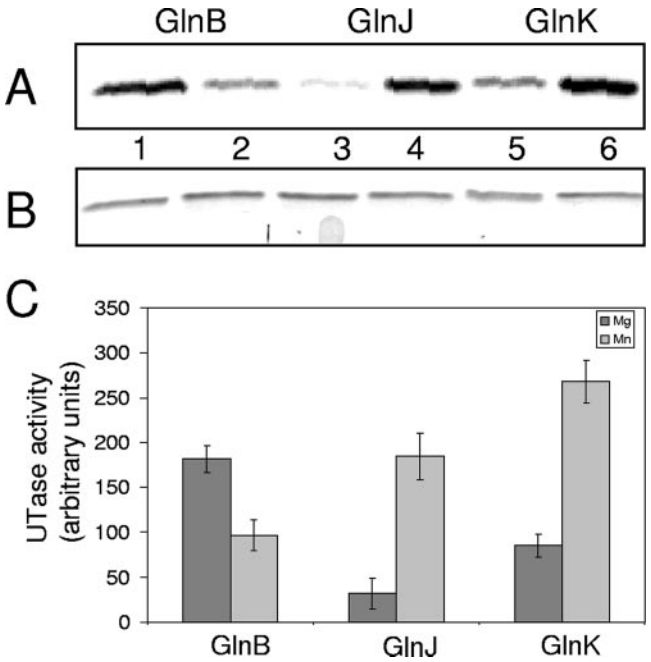

FIG. 4. Effect on uridylylation of Mg^{2+} or Mn^{2+}. The three P_{II} paralogs (0.5 μM) were incubated with either 250 μM α-ketoglutarate and 25 mM Mg^{2+} (lanes 1, 3, and 5) or 60 μM α-ketoglutarate and 3 mM Mn^{2+} (lanes 2, 4, and 6). For both divalent cations used, 2 mM ATP, 0.13 μM GlnD, and 0.5 mM UTP, supplemented with [α-^{32}P]UTP, were included in the reaction mixture. Samples were withdrawn from the reaction mixtures after 20 min of incubation and stopped by addition of SDS cocktail. (A) Autoradiogram showing incorporation of [α-^{32}P]UMP into GlnB (lanes 1 and 2), GlnJ (lanes 3 and 4), or GlnK (lanes 5 and 6). (B) Coomassie-stained SDS-PAGE showing the levels of P_{II} proteins loaded in panel A. (C) Histogram showing the difference in incorporation of [α-^{32}P]UMP between the *R. rubrum* P_{II} proteins with Mg^{2+} (dark) or Mn^{2+} (gray) in the uridylylation reaction. The amount of labeled, uridylylated GlnB, GlnJ, or GlnK was quantified using the Image Quant program. The data shown are from at least three independent experiments. UTase, uridylyltransferase.

values for α-ketoglutarate were also obtained for GlnJ and GlnK. Using these concentrations of α-ketoglutarate, we compared the degree of uridylylation of the P_{II} proteins with either Mg^{2+} or Mn^{2+} in the assay. Results from a typical experiment are shown in Fig. 4A for all three *R. rubrum* P_{II} paralogs. Figure 4B shows that the same amounts of P_{II} proteins were loaded in the lanes. It should be noted that the SDS-PAGE system used in this experiment does not allow separation of modified and unmodified P_{II}. Figure 4C depicts results of quantification of the labeled P_{II} proteins. For every set of quantification experiments performed, the combination of GlnK with Mn^{2+} always gave the highest degree of labeling. The results also show that both divalent cations can stimulate uridylylation, with more labeling of GlnB with Mg^{2+} than with Mn^{2+} and the opposite for GlnJ and GlnK. The combination yielding the lowest level of labeled P_{II} protein was GlnJ with Mg^{2+}.

A stimulating effect of Mn^{2+}, compared to results with Mg^{2+}, has also been observed for the *E. coli* system with GlnB, but this was regarded as nonphysiological (12). Our data may not reflect the physiological situation, but we believe that Mn^{2+} does have a role in uridylylation and most certainly in

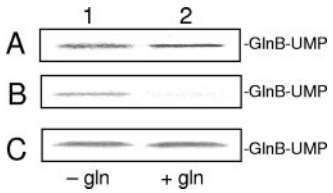

FIG. 5. The effect of glutamine on uridylylation. In panels A, B, and C, 3 mM $MnCl_2$, 2 mM ATP, 60 μM α-ketoglutarate, and 0.5 mM UTP supplemented with [α-^{32}P]UTP were included in the reaction mixture. (A) GlnB (0.5 μM) with 0.13 μM purified *R. rubrum* GlnD. (B) GlnB (15 μM) with 50 μg *E. coli* GlnD activity. (C) Fifteen micromolar GlnB with *R. rubrum* N+ extract (120 μg protein). Lane 1, no glutamine added; lane 2, 10 mM glutamine added. Samples were withdrawn after 0 or 20 min of incubation and stopped by addition of SDS cocktail.

deuridylylation (see below). Further studies are required to distinguish whether the effect of Mg^{2+} and Mn^{2+} is related to interactions of the cations directly with GlnD or with the P_{II} proteins or if they have an effect in the binding between P_{II} proteins and GlnD.

The physiologically relevant explanation for the difference in uridylylation efficiencies of the P_{II} proteins depending on which divalent cation used in the assay is not obvious. Also, the physiological role of Mn^{2+} is not established, although there are Mn^{2+}-dependent enzymes in *R. rubrum*, e.g., glutamine synthetase and DRAG. However, results in our laboratory show that GlnJ-UMP in fact is deuridylylated in vivo in response to addition of, e.g., ammonium ions to the culture (data not shown). We and Zhang et al. (39) have also shown that GlnJ is uridylylated when the ammonium ions added are metabolized. This would indicate either that Mn^{2+} is present in sufficient concentrations or that in vivo unknown factors have an effect on the activities of GlnD with GlnJ as a substrate.

Uridylylation—the effect of glutamine. In contrast to results with the *E. coli* system, we could not see any inhibitory effect of glutamine on uridylylation catalyzed by purified *R. rubrum* GlnD (Fig. 5A) (12, 17). We considered that some component(s) necessary had been lost during the purification process of the recombinant protein, and therefore, we examined uridylylation in extracts from wild-type *R. rubrum* grown under either N+ or N− conditions. There was no effect of glutamine under these assay conditions, however, either with N+ extracts (Fig. 5C) or with extracts from N− cells (data not shown). To exclude the possibility that the observed effect was on the *R. rubrum* P_{II} proteins, we used *E. coli* GlnK in the assay with both *R. rubrum* extracts and purified *R. rubrum* GlnD. No inhibition of uridylylation could be observed (data not shown). Interestingly, when using a partially purified *E. coli* GlnD fraction (see Materials and Methods), uridylylation of GlnB from *R. rubrum* was inhibited by addition of glutamine (Fig. 5B). The same results were obtained with *R. rubrum* GlnK and GlnJ and *E. coli* GlnK (data not shown). Taken together, these results suggest that the lack of response to glutamine is due to the characteristics of *R. rubrum* GlnD, and it raises the question of how the deuridylylation activity is regulated. From our results it would seem that the major effector of this activity is α-ketoglutarate.

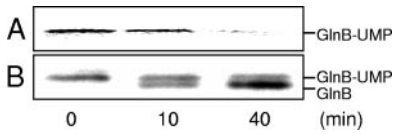

FIG. 6. Deuridylylation activity of purified *R. rubrum* GlnD. The reaction mixture contained 0.4 μM GlnB-UMP, 1.3 μM GlnD, 3 mM $MnCl_2$, 500 μM α-ketoglutarate, 2 mM ATP, and 10 mM glutamine. The activities were estimated as the decrease in labeled [α-^{32}P]UMP-GlnB. Samples were withdrawn from the reaction mixtures after 0, 10, or 40 min and stopped by addition of SDS cocktail. The samples were subjected to SDS-PAGE and visualized by autoradiography (A) or Western blotting (B), immunoblotted with antibodies against *R. rubrum* GlnB.

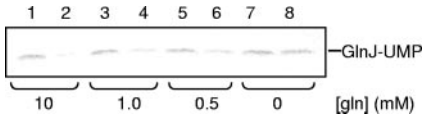

FIG. 8. Effect of glutamine on deuridylylation activity of purified *R. rubrum* GlnD. The uridylyl-removing activity was estimated as a decrease in labeled [α-^{32}P]UMP-GlnJ after 40 min of incubation. The reaction mixture contained 0.4 μM GlnJ-UMP, 1.3 μM GlnD, 3 mM $MnCl_2$, 500 μM α-ketoglutarate, 2 mM ATP, and 0 to 10 mM glutamine. Samples were withdrawn from reaction mixtures after time zero (lanes 1, 3, 5, and 7) or 40 min (lanes 2, 4, 6, and 8). Reactions were stopped by addition of SDS cocktail.

Deuridylylation of *R. rubrum* P_{II} proteins. In *E. coli*, glutamine not only inhibits uridylylation but also stimulates the deuridylylation of GlnB and GlnK with purified components, albeit at a lower rate for GlnK (4, 12). In order to study the effect of glutamine on deuridylylation catalyzed by *R. rubrum* GlnD, GlnB and GlnJ were labeled with [α-^{32}P]UMP, followed by purification on a Ni^{2+} chelating affinity column, and were then used as substrates. Initially we purified GlnD with NaCl in the buffer system used for GST-tagged proteins as recommended by the manufacturer (Amersham Biosciences), but we could not detect any deuridylylation activity of GlnD. However, when KCl was used instead of NaCl in the buffer system, the *R. rubrum* GlnD preparations were active in deuridylylation, in agreement with previous reports showing that GlnD from *E. coli* is more stable in buffers containing KCl (17).

Figure 6 shows a representative deuridylylation experiment with GlnB-UMP as a substrate. In Fig. 6A, a decrease in the radioactivity corresponding to removal of the UMP group from GlnB is clearly seen. Figure 6B shows a Western blot of the same samples. It should be noted that 10 times more GlnD was required in the reaction mixture to detect deuridylylation than was required for uridylylation. The same results were obtained for [α-^{32}P]UMP-GlnJ. As seen in Fig. 7, glutamine seems to give the greatest stimulating response for deuridylylation (Fig. 7, compare column pairs 1 and 4). When titrating the glutamine concentration required to detect deuridylylation of GlnJ-UMP, 1 mM was sufficient (Fig. 8), although 10 mM was used in the deuridylylation assays. The same results were obtained with [α-^{32}P]UMP-GlnB. No deuridylylation after 40 min of incubation of either GlnB-UMP or GlnJ-UMP was observed when omitting all of ATP, α-ketoglutarate, and glutamine from the assay (data not shown). Furthermore, there was no deuridylylation in the absence of added Mn^{2+} with or without Mg^{2+} (data not shown). This is a notable difference from the *E. coli* system, where deuridylylation also can occur with Mg^{2+} alone, although with lower efficiency than with Mn^{2+} (12).

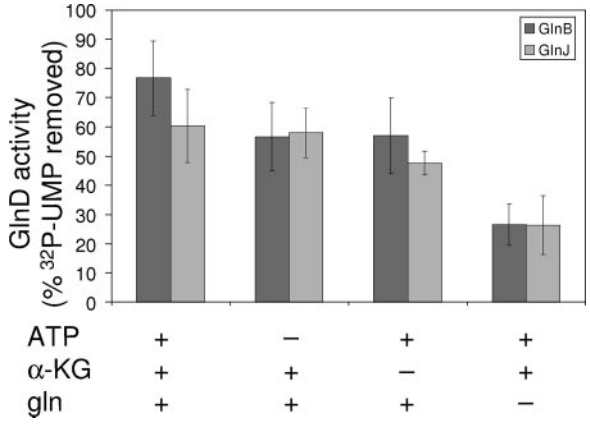

FIG. 7. Stimulation of the deuridylylation activity of purified *R. rubrum* GlnD. The data are from at least three independent experiments showing percentages of [α-^{32}P]UMP removed after 40 min of incubation for both GlnB-UMP (dark) and GlnJ-UMP (gray). For every pair of columns, 0.4 μM GlnB-UMP/GlnJ-UMP, 1.3 μM GlnD, and 3 mM $MnCl_2$ were added to the deuridylylation mixture. The first pair of columns shows deuridylylation with 500 μM α-ketoglutarate, 2 mM ATP, and 10 mM glutamine. The second pair shows deuridylylation without 2 mM ATP, and the third without 500 μM α-ketoglutarate. The last pair of columns shows deuridylylation without 10 mM glutamine. The data are from at least three independent experiments showing percent [α-^{32}P]UMP removed after 40 min of incubation for both GlnB-UMP and GlnJ-UMP.

Figure 7 shows that some deuridylylation activity of GlnD could be detected when only ATP and α-ketoglutarate were added to the assay without glutamine (Fig. 7, column pairs 2 and 3), but the activity was three times higher when glutamine was included (Fig. 7, column pair 1). Our data suggest that when the in vivo glutamine levels increase, deuridylylation of GlnB-UMP and GlnJ-UMP is stimulated almost irrespective of the α-ketoglutarate and ATP concentrations. In the *E. coli* system, α-ketoglutarate, ATP, and glutamine are all required for the deuridylylation activity when Mg^{2+} is used in the assay. On the other hand, when Mn^{2+} is the cation used, only glutamine is required, showing characteristics similar to those of the *R. rubrum* system (12).

We also wanted to study the effect of unmodified P_{II} protein on the deuridylylation reaction. We therefore produced His-tagged GlnB-UMP, which migrates detectably slower than untagged GlnB-UMP in the SDS-PAGE system used. As shown in Fig. 9 (lanes 1 and 2), GlnB does inhibit deuridylylation of His-GlnB-UMP, and it is also uridylylated. When GlnB was omitted from the reaction mixture, His-GlnB-UMP was demodified (Fig. 9, lanes 3 and 4). These results indicate a higher affinity for unmodified GlnB under the conditions used. It should be noted that UTP and glutamine were included in all reaction mixtures, showing that deuridylylation can occur in the presence of UTP (Fig. 9, lanes 3 and 4) and that uridylylation can proceed even in the presence of glutamine (Fig. 9, Lanes 1 and 2). The results in Fig. 9 (lane 1) show substantial uridylylation already after 5 min that is faster than under the

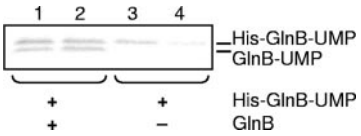

FIG. 9. Effect of GlnB on deuridylylation. Reaction mixtures contained 1.3 μM GlnD, 3 mM $MnCl_2$, 500 μM α-ketoglutarate, 2 mM ATP, 10 mM glutamine, and 0.5 mM UTP supplemented with [α-^{32}P]UTP. Lanes 1 and 2 also contained 0.4 μM GlnB and 0.4 μM His-GlnB-UMP, while only His-GlnB-UMP was added in lanes 3 and 4. Samples were withdrawn from the reaction mixtures after 5 min (lanes 1 and 3) or after 60 min (lanes 2 and 4). Note that both experiments were performed under deuridylylation conditions (1.3 μM GlnD and 10 mM glutamine) but with 0.5 mM UTP supplemented with [α-^{32}P]UTP also included in the assay. Samples were subjected to SDS-PAGE and visualized by autoradiography.

conditions used in Fig. 1. This is due to the addition of 10 times more GlnD in the deuridylylation assay.

With *E. coli*, similar experiments also showed inhibition of deuridylylation when GlnB was added to the deuridylylation reaction (12). On the other hand, UTP strongly inhibited the deuridylylation reaction, but this is not the case for the *R. rubrum* system. We believe that the activities of GlnD occur within the same domain and at overlapping active sites, as has also been suggested for *E. coli* GlnD (25). Furthermore, our results indicate a preference of GlnD for the unmodified GlnB over GlnB-UMP, since deuridylylation occurred only in the absence of GlnB.

In summary, we have shown that GlnD from *R. rubrum* exhibits characteristics that differ from those of GlnD from *E. coli*, the most interesting being that glutamine does not inhibit the uridylylation activity. This raises the question of how the uridylylation/deuridylylation activities are regulated in *R. rubrum* in response to changes in the nitrogen status in the cell. From our results, it would seem that the concentration of α-ketoglutarate plays a central role, since this metabolite is required for and stimulates uridylylation. Interestingly, our studies on GlnE from *R. rubrum* show that glutamine does not have a direct effect on this enzyme but that α-ketoglutarate is an important effector, although exerting its function on the P_{II} protein (Jonsson et al., unpublished). Addition of ammonium ions to nitrogen-fixing *R. rubrum* has been shown to cause a transient increase in the cellular glutamine concentration (19), but there are no studies on the concomitant effect on the α-ketoglutarate concentration. Another interesting feature of *R. rubrum* GlnD is the difference in the rates at which the *R. rubrum* P_{II} proteins are uridylylated with different cations, which may have implications for their different regulatory roles in *R. rubrum*. In conclusion, we believe that the *R. rubrum* system with the three P_{II} proteins and the metabolic regulation of nitrogen fixation offers an interesting variation of regulatory features with respect to nitrogen metabolism in bacteria. To determine if the differences are related to *R. rubrum* being a diazotroph, a phototroph, or both will require extended in vitro studies with other bacteria. It is striking, however, that in *R. rubrum* both nitrogenase activity and ammonium assimilation are regulated at the metabolic level by mechanisms that are dependent on the presence of P_{II} proteins, thus assigning a central role for GlnD.

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