In vitro studies of the uridylylation of the three PII 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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Abstract

P$_{II}$ protein 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 $\alpha$-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.

Introduction

In *Escherichia coli* the pivotal role of sensing the nitrogen level within the cell is played by the bifunctional uridylyltransferase/uridylylremoving (UTase/UR) 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 both in transcriptional and in post-translational 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). There are a number of targets for P$_{II}$ proteins 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 $\alpha$-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 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 2-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 (ATase), encoded by *glnE*, a bifunctional enzyme catalyzing the adenylylation/deadenylylation of glutamine synthetase (9, 11, 14, 20, 31). Under low nitrogen conditions, ATase 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 in other diazotrophs, the fixed nitrogen is further assimilated via the glutamine synthetase and glutamate synthase pathway (21, 22, 30). In R. rubrum three PII proteins have been identified, encoded by glnB, glnK and glnJ respectively (37). It was shown that in this bacterium either GlnB or GlnJ are required for proper regulation of the DRAT/DRAG system, two enzymes involved in post-translational 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 of NifA in R. rubrum, 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 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 in 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 only been reported for GlnD, GlnB and GlnK from E. coli (4, 12, 17). In addition purified GlnZ, a PII 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 PII proteins has been shown to depend on the ligands bound to the PII protein and the divalent cation present (12). Different PII 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 only been reported for the *E. coli* system, but this may however not be fully applicable in other bacteria e.g. diazotrophs. Considering the different primary targets for the three PII proteins in *R. rubrum*, it is of special interest to establish if the three PII 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 PII 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 PII 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 template, except for the cloning of glnB into pGEX-6P-2, where pMJET was used as template. For cloning of E. coli glnK into pET 15b E. coli DNA was used as 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 were used (33).

Cell growth and purification of R. rubrum proteins

Transformation of plasmid pGEX-GlnD into E. coli strain RB 9040 was done according to Sambrook et al (33). Cells were grown at 20-25 °C in Luria Bertani medium (LB) supplemented with ampicillin (50 μg/ml), tetracycline (15 μg/ml) and 10 mM glutamine. Cells were grown to OD_{600} = 1.1 followed by induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 1 hour at 20-25 °C. Cells were then harvested and frozen as pellets in liquid nitrogen. Pellets were resuspended in GST-Binding buffer (pH 7.4) containing 142.7 mM KCl, 10 mM Na_{2}HPO_{4}, 1.8 mM KH_{2}PO_{4}, complemented with 20 μg/ml DNase, 1 tablet of Complete Mini EDTA-free Protease Inhibitor/L (Roche), 2 mM phenylmethylsulphonylfluoride (PMSF) and 0.5 mg/ml lysozyme followed by sonication for 6 X 10 sec. The supernatant after centrifugation at 40 000g 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 and incubated at 4 °C
overnight followed by 2 hours in room temperature. After digestion, GlnD was eluted in Cleavage buffer and assayed for GlnD activity.

All pET15b derivatives were transformed into *E. coli* BL21 (DE3) pLysS. Cultures were grown in Luria-Bertani medium at 37 °C, to OD$_{600}$=0.6 and then induced with 1 mM IPTG for 1 hr. 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, 20 mM Na$_2$PO$_4$, complemented with 20 µg/ml DNAse, 2 mM PMSF and 1 tablet of complete mini EDTA-free Protease Inhibitor (Roche)/l. The solution was then frozen in liquid nitrogen and thawed three times, followed by sonication 6 X 10 seconds, and then centrifuged 20 min at 40 000g. 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 and 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 OD$_{600}$=0.6 followed by induction with 1 mM IPTG for 2 hours at 20-25 °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) 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 for all PII proteins by standard PCR-mediated site-directed mutagenesis by using *Pfu* polymerase (Stratagene). The primers contained a 1-base pair mismatch, converting the codons for glutamine to 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 PII proteins was carried out at 30 °C in a reaction mixture (100 µl) containing 0.5 µM purified PII 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-32P]UTP, 25 mM MgCl2 and 250 µM α-ketoglutarate or 60 µM when 3 mM MnCl2 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 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 minutes and then run on a 18 % SDS-PAGE. After staining with Commassie Brilliant Blue followed by destaining, gels were dried and subjected to autoradiography on a phosphoimager for 1 hour and over-night. Incorporation of [α-32P]UMP into the proteins was then quantified and visualized using the Image Quant program.
Deuridylylation of GlnB and GlnJ

To determine deuridylylation of modified PII 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 PII protein and 1.3 µM GlnD were used. UTP and [α-32P]UTP were omitted. Samples were taken from the reaction mixtures after different time intervals and analyzed as in the uridylylation assay except that [32P]UMP remaining was quantified.

Purification of uridylylated PII proteins

Either His-GlnB or His-GlnJ were incubated over-night 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 pre-charged with Ni2+ ions according to the same procedures as for purification of unmodified his-tagged PII proteins. Uridylylation efficiency was close to 100% as analyzed by 18 % SDS-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 PII 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. 50 µg of protein were 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% N₂/5% CO₂. Ammonium grown cells, N+, were supplemented with 28 mM NH₄Cl. After harvesting (OD₆₀₀≈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 MnCl₂, 1 mM DTT and 1 tablet of Complete Mini EDTA-free Protease Inhibitor/l (Roche). Cell breakage was performed in a Ribi-Cell fractionator followed by centrifugation at 2500g 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 1 tablet of Complete Mini EDTA-free Protease Inhibitor/L.

Electrophoresis and Western blotting

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

Results and Discussion

*Overexpression and purification of GlnD, GlnB, GlnK, and GlnJ from R. rubrum.*
The *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 glutathione-S-transferase (GST) in an *E. coli glnD* strain (RB9040), purified and verified by MALDI-TOF 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 20-22°C gave the best yield of purified GlnD (0.46 mg/l culture). When overexpressed with a 6×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 show that in all three *R. rubrum* PII paralogs there is a tyrosine at position 51, the residue being uridylylated in all bacterial PII proteins studied. The molecular mass of the purified PII proteins was estimated on 18% SDS-PAGE gels to be 12.4 kDa and 14.4 kDa when containing a 6×his tag, all in good agreement with the expected molecular masses. As we could not detect any difference in the uridylylation of untagged PII 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 Figure 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 PII 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 PII protein, enhancing the rate of uridylylation. 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 PII uridylylation in the R. rubrum system we examined the effect(s) in vitro using purified R. rubrum GlnD and PII proteins. To study these effect(s) we developed an assay to analyze incorporation of α-[³²-P]UMP into the PII proteins. Figure 1 shows a representative uridylylation experiment with GlnB in the presence of 3 mM MnCl₂ and 60 μM α-ketoglutarate at 0, 2, 5, 10 and 20 minutes followed by Western blot 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 MnCl₂, above and below this concentration, labeling was less (Figure 2A). No labeling of GlnB or GlnK could be detected when omitting either ATP, α-ketoglutarate or the divalent cations from the reaction mixture (data not shown). Surprisingly GlnJ was uridylylated even when ATP or α-ketoglutarate were omitted from the reaction mixture but not to the same extent as with both present (Figure 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 PII 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 however labeled, but to a much lower extent compared to 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, as 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 by 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 (three fold) and limiting concentrations of GlnB showed that GlnB was totally labeled after 15 minutes of incubation with excess concentrations of ATP and α-ketoglutarate. Additional 45 minutes of incubation did not increase the amount of labeling and Western blot 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 Figure 3. Similar values for α-ketoglutarate were also obtained for GlnJ and GlnK. Using these concentrations of α-ketoglutarate we compared the degree in uridylylation of the P\(_{II}\) proteins with either Mg\(^{2+}\) or Mn\(^{2+}\) in the assay. Results from a typical experiment are shown in Figure 4A for all three *R. rubrum* P\(_{II}\) paralogs. Figure 4B shows that the same amount of P\(_{II}\) proteins was 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 $\text{Mn}^{2+}$ always gave the highest degree of labeling. The results also show that both divalent cations can stimulate uridylylation, with more labeling of GlnB with $\text{Mg}^{2+}$ compared to $\text{Mn}^{2+}$ and the opposite for GlnJ and GlnK. The combination yielding the lowest level of labeled $P_{II}$ protein was GlnJ with $\text{Mg}^{2+}$.

A stimulating effect of $\text{Mn}^{2+}$ compared to $\text{Mg}^{2+}$ has also been observed in the *E. coli* system with GlnB, but this was regarded as non-physiological (12). Our data may not reflect the physiological situation, but we believe that $\text{Mn}^{2+}$ does have a role in uridylylation and most certainly in the deuridylylation (see below). Further studies are required to distinguish whether the effect of $\text{Mg}^{2+}$ and $\text{Mn}^{2+}$ is related to interactions of the cations directly with GlnD, 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 efficiency of the $P_{II}$ proteins depending on which divalent cation used in the assay is not obvious. Also the physiological role of $\text{Mn}^{2+}$ is not established although there are $\text{Mn}^{2+}$ dependent enzymes in *R. rubrum*, e.g. GS 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 as well as Zhang et al (39) have also shown that GlnJ is uridylylated when the ammonium ions added are metabolized. This would indicate that either $\text{Mn}^{2+}$ is present in sufficient concentrations or that in vivo unknown factors have an effect on the activities of GlnD with GlnJ as substrate.
Uridylylation – the effect of glutamine

In contrast to the *E. coli* system we could not see any inhibitory effect of glutamine on uridylylation catalyzed by purified *R. rubrum* GlnD (Figure 5A) (12, 17). We considered that some component(s) necessary had been lost during the purification process of the recombinant protein and therefore examined uridylylation in extracts from wild-type *R. rubrum* grown under either N+ or N- conditions. There was however no effect of glutamine under these assay conditions neither with N+ extracts (Figure 5C) nor 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 & Methods), uridylylation of GlnB from *R. rubrum* was inhibited by addition of glutamine (Figure 5B). The same results were obtained with *R. rubrum* GlnK, 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.

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 Ni²⁺ 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 using KCl instead of NaCl in the buffer system the \textit{R. rubrum} GlnD preparations were active in deuridylylation, in agreement with previous reports showing that GlnD from \textit{E. coli} is more stable in buffers containing KCl (17).

Figure 6 shows a representative deuridylylation experiment with GlnB-UMP as substrate. In Figure 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 compared to uridylylation. The same results were obtained for $\alpha$-[\textsuperscript{32}P]UMP-GlnJ. As seen in Figure 7 glutamine seems to give the greatest stimulating response for deuridylylation (Figure 7, compare column pairs 1 and 4). When titrating the glutamine concentration required to detect deuridylylation of GlnJ-UMP, 1 mM was sufficient (Figure 8), although 10 mM were used in the deuridylylation assays. The same results was obtained with $\alpha$-[\textsuperscript{32}P]UMP-GlnB. No deuridylylation after 40 minutes of incubation of either GlnB-UMP or GlnJ-UMP was observed when omitting all of ATP, $\alpha$-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 compared to the \textit{E. coli} system where deuridylylation also can occur with Mg$^{2+}$ alone, although with lower efficiency than with Mn$^{2+}$ (12).

Figure 7 shows that some deuridylylation activity of GlnD could be detected when only ATP and $\alpha$-ketoglutarate were added to the assay without glutamine (Figure 7, column pairs 2 and 3), but the activity was three times higher when glutamine was
included (Figure 7, column pair 1). Our data suggests 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 similar characteristics as the *R. rubrum* system (12).

We also wanted to study the effect of unmodified P$_U$ protein on the deuridylylation reaction. We therefore produced His tagged GlnB-UMP that migrates detectably slower than untagged GlnB-UMP on the SDS-PAGE system used. As shown in Figure 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 (Figure 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 (Figure 9, lanes 3 and 4), and that uridylylation can proceed even in the presence of glutamine (Figure 9, Lanes 1 and 2). The results in Figure 9 (lane 1) show substantial uridylylation already after 5 minutes that is faster than under the conditions used in Figure 1. This is due to the addition of ten times more GlnD in the deuridylylation assay.

In *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 \textit{E. coli} GlnD (25). Furthermore, our results indicate a preference of GlnD for the unmodified GlnB compared to GlnB-UMP as deuridylylation only occurred in the absence of GlnB.

In summary we have shown that GlnD from \textit{R. rubrum} exhibits characteristics that differ from those of GlnD from \textit{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 \textit{R. rubrum} in response to changes in the nitrogen status in the cell. From our results it would seem that the concentration of $\alpha$-ketoglutarate plays a central role as this metabolite is required for and stimulates uridylylation. Interestingly our studies on GlnE from \textit{R. rubrum} show that glutamine does not have a direct effect on this enzyme, but that $\alpha$-ketoglutarate is an important effector although exerting its function on the P$_{\text{II}}$ protein (Jonsson et al unpublished). Addition of ammonium ions to nitrogen fixing \textit{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 $\alpha$-ketoglutarate concentration. Another interesting feature of \textit{R. rubrum} GlnD is the difference in rate by which the \textit{R. rubrum} P$_{\text{II}}$ proteins are uridylylated with different cations that may have implications on their different regulatory roles in \textit{R. rubrum}. In conclusion we believe that the \textit{R. rubrum} system with the three P$_{\text{II}}$ 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 \textit{R. rubrum} being a diazotroph, a phototroph or both, will require extended in vitro studies in other bacteria. It is however striking that in \textit{R. rubrum} both nitrogenase activity and
ammonium assimilation are regulated at the metabolic level by mechanisms that are dependent on the presence of PII proteins thus assigning a central role for GlnD.

Acknowledgements

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References


**TABLE 1.** Bacterial strains and plasmids used in this study.

*Jonsson, Teixeira and Nordlund, unpublished*

<table>
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<th>Strains</th>
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Figure legends

Figure 1. Uridylylation of *R. rubrum* GlnB. GlnB (0.5 μM) was incubated in uridylylation reaction mixture with 0.13 μM *R. rubrum* GlnD, 3 mM MnCl₂, 60 μM α-ketoglutarate, 2 mM ATP and 0.5 mM UTP supplemented with α-[³²P]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 and Western blot in panel B immunoblotted with antibodies against *R. rubrum* GlnB. In B, the lower band corresponds to unmodified GlnB and the upper to the modified form of GlnB.

Figure 2. Effect of MnCl₂, ATP and α-ketoglutarate on uridylylation. A Uridylylation of GlnB (0.5 μM) with 0, 0.3, 0.6, 3.0, 6.0 and 16 mM MnCl₂ together with 2 mM ATP, 60 μM α-ketoglutarate, 0.13 μM GlnD and 0.5 mM UTP supplemented with α-[³²P]UTP. B Effect of the absence of ATP or α-ketoglutarate on uridylylation of GlnJ (0.5 μM). The assays also contained 3 mM MnCl₂, 0.13 μM GlnD and 0.5 mM UTP supplemented with α-[³²P]UTP. Uridylylation was assayed by incorporation of α-[³²P]UMP as described in Materials & Methods. Samples were withdrawn from the reaction mixtures after 20 minutes of incubation and stopped by addition of SDS cocktail.

Figure 3. The effect of α-ketoglutarate on uridylylation of GlnB with either MgCl₂ or MnCl₂. The assay contained 0.5 μM GlnB, 2 mM ATP, 0.13 μM GlnD, and 0.5 mM UTP supplemented with α-[³²P]UTP. 25 mM MgCl₂ (■) or 3 mM MnCl₂ (●).
Samples were withdrawn from the reaction mixtures after 20 min of incubation and stopped by addition of SDS cocktail. The incorporation of $\alpha$-[\(^{32}\)P]UMP at 300 $\mu$M $\alpha$-ketoglutarate was set to 100% for both MgCl\(_2\) and MnCl\(_2\). No more labeling was detected at $\alpha$-ketoglutarate concentrations higher than 300 $\mu$M. 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 a representative for at least 3 independent experiments for both MgCl\(_2\) and MnCl\(_2\).

**Figure 4.** Effect on uridylylation by Mg\(^{2+}\) and Mn\(^{2+}\). The three P\(_{II}\) paralogs (0.5 $\mu$M) were incubated with either 250 $\mu$M $\alpha$-ketoglutarate and 25 mM Mg\(^{2+}\) (Lanes 1, 3 and 5), or 60 $\mu$M $\alpha$-ketoglutarate and 3 mM Mn\(^{2+}\) (Lanes 2, 4 and 6). For both divalent cations used 2 mM ATP, 0.13 $\mu$M GlnD and 0.5 mM UTP supplemented with $\alpha$-[\(^{32}\)P]UTP were included in the reaction mixture. Samples were withdrawn from the reaction mixtures after 20 minutes of incubation and stopped by addition of SDS cocktail. **A** Autoradiogram showing incorporation of $\alpha$-[\(^{32}\)P]UMP into GlnB lanes 1 and 2; GlnJ lanes 3 and 4; GlnK lanes 5 and 6. **B** Commassie stained SDS-PAGE showing the levels of P\(_{II}\) proteins loaded in A. **C** Histogram showing the difference in incorporation of $\alpha$-[\(^{32}\)P]UMP between the *R. rubrum* P\(_{II}\) proteins with Mg\(^{2+}\) (dark) or Mn\(^{2+}\) (grey) 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.

**Figure 5.** The effect of glutamine on uridylylation. In A, B and C, 3 mM MnCl\(_2\), 2 mM ATP, 60 $\mu$M $\alpha$-ketoglutarate and 0.5 mM UTP supplemented with $\alpha$-[\(^{32}\)P]UTP were included in the reaction mixture. **A** 0.5 $\mu$M GlnB with 0.13 $\mu$M purified *R.
rubrum GlnD. B 15 µM GlnB with 50 µg E. coli GlnD activity. C 15 µM 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 and 20 minutes of incubation and stopped by addition of SDS-cocktail.

**Figure 6.** Deuridylylation activity of purified R. rubrum GlnD. The reaction mixture contained 0.4 µM GlnB-UMP, 1.3 µM GlnD, 3 mM MnCl₂, 500 µM α-ketoglutarate, 2 mM ATP and 10 mM glutamine. The activities were estimated as the decrease in labeled α-[³²P]UMP-GlnB. Samples were withdrawn from the reaction mixtures after 0, 10 and 40 minutes and stopped by addition of SDS-cocktail. The samples were subjected to SDS-PAGE and visualized by autoradiography (A) and Western blot (B) immunoblotted with antibodies against R. rubrum GlnB.

**Figure 7.** Stimulation of the deuridylylation activity of purified R. rubrum GlnD. The data is from at least three independent experiments showing percentage α-[³²P]UMP removed after 40 minutes of incubation for both GlnB-UMP (dark) and GlnJ-UMP (grey). For every pair of columns 0.4 µM GlnB-UMP/GlnJ-UMP, 1.3 µM GlnD, and 3 mM MnCl₂ 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 is from at least three independent experiments showing % α-[³²P]UMP removed after 40 minutes of incubation for both GlnB-UMP and GlnJ-UMP.
**Figure 8.** Effect of glutamine on deuridylylation activity of purified *R. rubrum* GlnD.

The uridylylremoving activity was estimated as a decrease in labeled $\alpha$-$[^{32}P]$UMP-GlnJ after 40 minutes of incubation. The reaction mixture contained 0.4 µM GlnJ-UMP, 1.3 µM GlnD, 3 mM MnCl$_2$, 500 µM $\alpha$-ketoglutarate, 2 mM ATP and 0 to 10 mM glutamine. Samples were withdrawn from the reaction mixtures after time 0 (Lanes 1, 3, 5 and 7) and 40 minutes (Lane 2, 4, 6 and 8). The reactions were stopped by addition of SDS-cocktail.

**Figure 9.** Effect of GlnB on deuridylylation. The reaction mixtures contained 1.3 µM GlnD, 3 mM MnCl$_2$, 500 µM $\alpha$-ketoglutarate, 2 mM ATP, 10 mM glutamine and 0.5 mM UTP supplemented with $\alpha$-$[^{32}P]$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 minutes (lanes 1 and 3) and after 60 minutes (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 $\alpha$-$[^{32}P]$UTP also included in the assay. The samples were subjected to SDS-PAGE and visualized by autoradiography.
Figure 1
Figure 2
Figure 3

![Graph showing the relationship between uridylylation activity and \( \alpha \)-ketoglutarate concentration. The x-axis represents \( \alpha \)-ketoglutarate concentration (\( \mu \)M) ranging from 0 to 300, and the y-axis represents uridylylation activity (% \( ^{32} \)P-UMP incorporated) ranging from 0 to 120.]
Figure 4

A

B

C

GlnB  GlnJ  GlnK

1  2  3  4  5  6

UTase activity (arbitrary units)

GlnB  GlnJ  GlnK

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Figure 5

[Diagram showing A, B, and C with lanes 1 and 2 labeled with GlnB-UMP, -gln, and +gln]
Figure 6

A

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<tr>
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GlnB-UMP

GlnB

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Figure 7

![Bar graph showing GlnD activity (% of wild-type removed)]

- ATP: +, -, +, +
- α-KG: +, -, +
- gln: +, +, +, -

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Figure 8