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# Properties and Regulation of Glutamine Synthetase from *Rhodospirillum rubrum*

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**Glutamine synthetase from *Rhodospirillum rubrum* was purified and characterized with respect to its pH optimum and the effect of $Mg^{2+}$ on its active and inactive forms. Both adenine and phosphorus were incorporated into the inactive form of the enzyme, indicating covalent modification by AMP. The modification could not be removed by phosphodiesterase. Evidence for regulation of the enzyme by oxidation was obtained. Extracts from oxygen-treated cells had lower specific activities than did extracts from cells treated anaerobically. Glutamine synthetase activity was found to decrease in the dark in phototrophically grown cells; activity was recovered on re-illumination.**

Glutamine synthetase (GS) carries out the ATP-dependent synthesis of glutamine from glutamate and $NH_4^+$. The enzyme has been shown to be the primary $NH_4^+$ assimilatory enzyme under low-N growth conditions for gram-negative bacteria. The enzyme from *Escherichia coli* is regulated at the level of expression as well as by feedback inhibition and by the covalent modification of the enzyme by AMP (26). Turnover of *E. coli* GS may be enhanced by oxidation (16–18). Among the photosynthetic bacteria, GS has been purified from *Rhodospirillum rubrum* (25, 32) and *Rhodopseudomonas palustris* (2, 3), and a partial purification of the enzyme from *Rhodopseudomonas capsulata* has been reported (12). In the case of *R. palustris*, regulation of GS by adenylylation has been demonstrated (2); in *R. capsulata* and in *R. rubrum*, regulation by adenylylation has been proposed based on the differential inhibition of GS transferase activity of inactive (presumably adenylylated) GS by high levels of $Mg^{2+}$ (4, 12).

The pH dependence of GS from different sources has been found to vary, and the active and inactive forms of the enzyme do not necessarily exhibit the same pH optimum. The presence of 60 mM $Mg^{2+}$ also causes a shift in the pH optimum of GS. It is important that the pH and metal dependence of GS be determined for the enzyme from each source.

Several observations have led to the suggestion that GS in *R. rubrum* is regulated in some manner in addition to modification by AMP (8, 32). In this paper, evidence in support of additional regulatory modes is presented along with direct evidence for modification of the enzyme.

## MATERIALS AND METHODS

*R. rubrum* (ATCC 11170) was grown photoheterotrophically in 500-ml batches of Ormerod medium (23) with 27 mM glutamate as nitrogen source. In experiments with $^{32}P$, the phosphate concentration in the growth medium was lowered to 0.05 g/liter, and morpholinepropanesulfonic acid buffer (10 g/liter) was added to restore buffer capacity. When $H_3{}^{32}PO_4$ (1 mCi, carrier free) or [8-$^3$H]adenine (1 mCi) was used, the labeled compound was added 15 h before the cells were harvested.

GS was purified essentially as described by Soliman et al. (25), except that the cells were broken by osmotic shock (20). In some experiments the supernatant after the centrifugation (31,000 × *g*, 60 min) was used as the enzyme source. The activating enzyme for the Fe protein was purified by the method of Zumft and Nordlund (34). Where indicated, crude extracts of small volumes of cells were prepared by a grinding technique as previously described (14).

GS activity was measured in the γ-glutamyl transferase assay (27). The reaction mixture contained 40 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 40 mM imidazole, 20 mM glutamine, 0.4 mM $MnCl_2$, 0.4 mM ADP, 0.02 mM $KAsO_4$, and 0.02 mM $NH_4OH$ in a total volume of 1.0 ml. The pH of the assay was 7.6 unless otherwise indicated. For the estimation of the "adenylylation" state of the enzyme, the reaction was run in the same mixture, but with 60 mM $MgCl_2$ and pH 7.0. Reactions were run for 15 min at 30°C and stopped by the addition of 2 ml of a stop mix as described by Stadtman et al. (27). GS assays of crude extract supernatants of cell samples taken from darkened cultures (see Fig. 6) were performed as described above after extraction of the cells as previously described (14), except that methyl viologen was eliminated from the extraction buffer. γ-Glutamyl hydroxamate produced was estimated by absorbance at 540 nm. Subtilisin treatment of purified, radiolabeled GS was carried out at pH 8.0 for 6 h at room temperature. Nucleotide-bound peptide was isolated on a phenyl boronate column (4 by 0.5 cm) (Affigel 601, Bio-Rad Laboratories) equilibrated with 100 mM $NH_4$-formate buffer (pH 8.5). The nucleotide-peptide was eluted with 100 mM formic acid. The elution was monitored at 260 nm by a Gilson Holochrome column monitor.

Nitrogenase activity was determined by acetylene reduction as previously described (14). Samples for whole cell nitrogenase assays were removed from the culture via syringe. Cell samples from darkened cultures (Fig. 6) were assayed from a short (2-min) period in the light. Polyacrylamide gel electrophoresis was performed under nondenaturing conditions by the method of Davis (5) and in the presence of sodium dodecyl sulfate by the method of Laemmli (15). Protein concentration was determined by the method of Goa (9) after precipitation of protein with 5% trichloroacetic acid.

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TABLE 1. GS activity in extracts from cells grown under different conditions[a]

| N source during growth | $A_{600}$[b] | Nitrogenase activity of whole cells (nmol of $C_2H_2$/h per ml) | Glutamine synthetase activity (μmol/ min per mg) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Crude extract | | Supernatant | | Pellet | |
| | | | −Mg | +Mg | −Mg | +Mg | −Mg | +Mg |
| Glutamate, 27 mM | 2.7 | 2,121 | 1.1 | 1.83 | 9.9 | 10.9 | 1.7 | 2.9 |
| Glutamate, 27 mM, plus switch off | 3.4 | 2,310 (504)[c] | 0.2 | 0.15 | 1.7 | 1.2 | 0.1 | 0.09 |
| $NH_4Cl$, 20 mM | 2.9 | | 0.2 | 0.25 | 0.4 | 0.4 | 0.1 | 0.11 |

[a] Nitrogenase and GS activities were assayed as described in the text. The supernatant and the pellet are from the centrifugation at 31,000 × *g* per 60 min.
[b] $A_{600}$, Absorbancy at 600 nm.
[c] Activity 30 min after switch off by the addition of 4 mM $NH_4Cl$ plus 4 mM α-ketoglutarate.

Growth medium constituents, assay components, snake venom phosphodiesterase, and subtilisin were obtained from Sigma Chemical Co. $H_3{}^{32}PO_4$ and [8-$^3$H]adenine were from Amersham Corp. Phenyl boronate resin was obtained from Bio-Rad.

## RESULTS AND DISCUSSION

Previous reports have shown that addition of $NH_4{}^+$ to a nitrogen-fixing culture of *R. rubrum* grown with $N_2$ leads to an inactivation of GS (4, 7, 8, 32). Table 1 shows that when glutamate-grown cells are switched off by treatment with ammonia, nitrogenase activity decreases to 22%, and that the specific activity of GS in extracts from such cells is around 20% of that of the control. It is also shown that GS from switched-off cells is inhibited by 60 mM $MgCl_2$, but only to 25%, which is in contrast to the results obtained with the enzyme from *R. palustris* (2, 3). This phenomenon, i.e., a strong decrease in transferase activity in the absence of high concentrations of $MgCl_2$, has not been reported for GS from other photosynthetic bacteria, but the same effect was observed with GS from the gram-positive bacterium *Streptomyces cattleya* (28, 30). Evidence was also provided for adenylylation of the enzyme from *S. cattleya* being the reason for this loss of transferase activity (28).

The relative amount of GS activity that was found in the pellet was much higher in the extract from ammonia-grown cells (Table 1). This may reflect a difference in the binding to chromatophore membrane dependent on the state of the enzyme. Coprecipitation of GS with chromatophores has been reported and taken advantage of in the purification of the enzyme from *R. capsulata* (12). Yoch et al. (32) have proposed a regulatory role for chromatophores in the binding of GS.

The adenylylated and the unadenylylated forms of GS from *E. coli* show different pH dependence in the transferase assay, but an isoactivity pH is obtained (27). Furthermore, by measuring the activity at the isoactivity pH in the presence and absence of 60 mm $MgCl_2$, the degree of adenylylation of the *E. coli* enzyme can be estimated (27). The pH profiles of the inactive and the active forms of GS from *R. rubrum* are shown in Fig. 1. The activity of the enzyme from switched-off cells exhibit much lower specific activity in the whole pH range tested, and there is no isoactivity pH. The experiment was run in a buffer containing both HEPES and imidazole, but the same results were obtained with each buffer used alone. The pH optimum for the reaction in the presence of 60 mM $MgCl_2$ was more acidic than in the absence of $MgCl_2$, which is similar to what has been shown for the *E. coli* enzyme when assayed in imidazole buffer (27). The maximal activity in the presence of $MgCl_2$ was lower than without $MgCl_2$, which, in comparison with the *E. coli* enzyme, could mean that the *R. rubrum* GS is partially adenylylated. The pH profile of GS transferase activity has been studied in two other photosynthetic bacteria, *R. capsulata* (12) and *R. palustris* (3). The pH dependence of the enzyme from *R. capsulata* (12) is similar to that of the *E. coli* enzyme, and this was interpreted as meaning that the adenylylated and the unadenylylated forms have an isoactivity pH. However, the *R. palustris* enzyme shows no isoactivity pH, and the activity of the adenylylated form is somewhat lower in the pH range tested (2). Consequently, GS from *R. rubrum* shows a pH profile for the transferase activity that is significantly different from the enzyme from these two photosynthetic bacteria, but is similar to that of GS from *S. cattleya*, which as a pH optimum for both forms around 7.0 (30). Furthermore, the enzyme from ammonia-shocked cells of *S. cattleya* also has much lower activity at all pHs tested, even in the absence of $MgCl_2$, and no isoactivity pH was found (30).

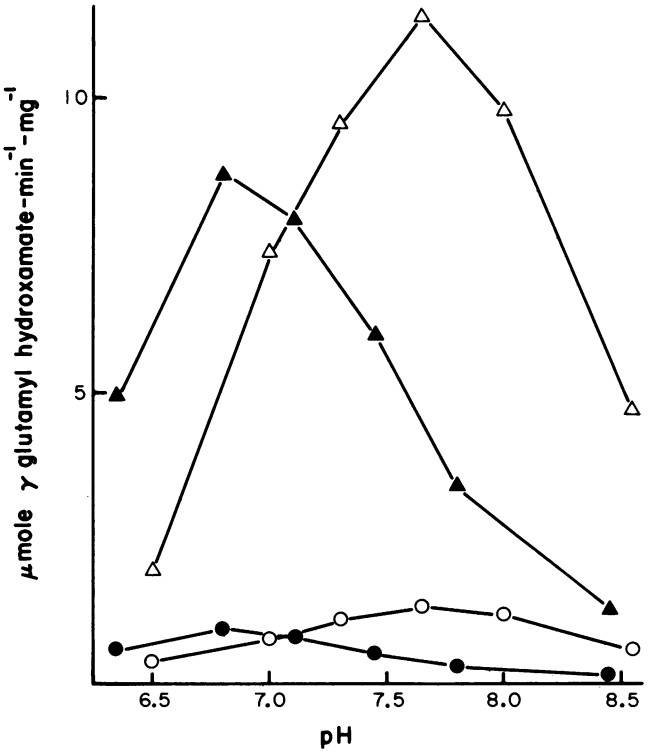


FIG. 1. pH profile of GS activity. Activity was measured as the formation of γ-glutamylhydroxamate as described in the text. Symbols: (△, ▲) supernatant (31,000 × *g*) from glutamate-grown cells; (○, ●) supernatant from switched-off glutamate-grown cells; (○, △) absence of $MgCl_2$; (●, ▲) presence of 60 mM $MgCl_2$.

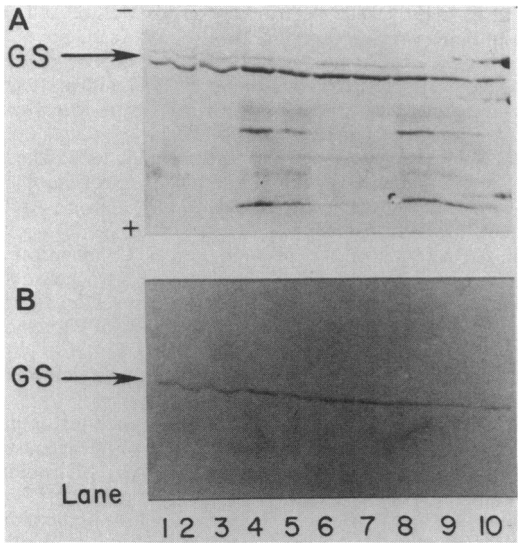


FIG. 2. Effect of snake venom phosphodiesterase of $^{32}P$-labeled *R. rubrum* GS. (A) Coomassie blue-stained sodium dodecyl sulfate gel of GS purified from *R. rubrum* grown in the presence of $^{32}P$. Lanes: 1, purified GS; 2, purified GS; 3, time 0 of snake venom phosphodiesterase treatment; 4–10, time course of snake venom phosphodiesterase treatment (5, 10, 15, 20, 30, 60, and 100 min, respectively). (B) autoradiogram of gel shown in A; lanes are as indicated above.

The biosynthetic reaction of *R. rubrum* GS was not studied as extensively as the transferase reaction; however, it was determined that the ratio of transferase to biosynthetic activity was 136 with active, purified GS.

To further study the reason for the loss of activity, GS was isolated from *R. rubrum* cells to which $H_3{}^{32}PO_4$ or [8-$^3$H]adenine was added. Figure 2 shows a sodium dodecyl sulfate gel and its autoradiogram of the inactive form, i.e., isolated from switched-off cells grown in the presence of $H_3{}^{32}PO_4$. Figure 3 shows a densitometer tracing of a fluorogram of a gel of GS isolated from cells grown in the presence of [8-$^3$H]adenine. In both cases, GS was specifically labeled, which indicates that this form of the enzyme contains a nucleotide. Attempts were made to remove the labeled

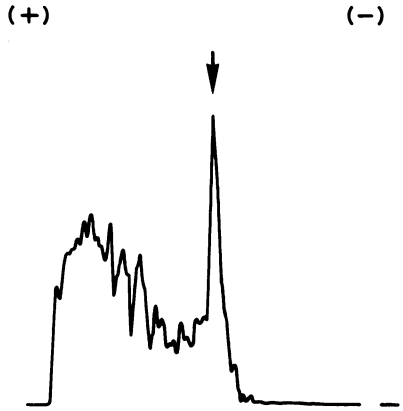


FIG. 3. Incorporation of [8-$^3$H]adenine into GS from *R. rubrum*. The fluorogram of sodium dodecyl sulfate gel of GS purified from *R. rubrum* grown in the presence of [8-$^3$H]adenine was scanned on a Zeineh densitometer. The arrow indicates the position of GS on the gel as determined by protein staining.

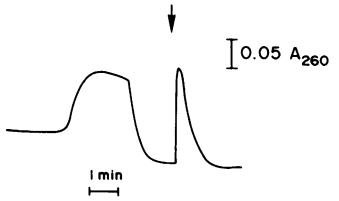


FIG. 4. Elution profile of subtilisin-treated GS on boronate column. The arrow indicates where the pH was lowered by changing to 100 mM formate buffer (pH 2.0). $A_{260}$, Absorbancy at 260 nm.

compound by treatment with snake venom phosphodiesterase, but there was no effect (Fig. 2). In addition, this treatment did not lead to any increase in activity, either in the absence or presence of $MgCl_2$ (data not shown). Since the inactive form of the Fe protein of *R. rubrum* nitrogenase can be activated by an activating enzyme in the presence of ATP and a divalent metal ion (19, 22), treatment of inactive GS with activating enzyme was attempted. Activating enzyme was unable to activate GS. Furthermore, inactive GS from *R. rubrum* could not be activated by heating the enzyme; this is in contrast to the *R. rubrum* Fe protein, which can be activated by heating (6).

Although the inactive form of GS from *R. rubrum* may be modified with AMP, our results show that in contrast to what has been shown for other photosynthetic bacteria, the standard criteria for adenylylation, i.e., inhibition by 60 mM $MgCl_2$, activation, and removal of AMP by snake venom phosphodiesterase, are not valid for the *R. rubrum* enzyme.

A peptide containing the bound nucleotide was isolated by chromatography of subtilisin-treated, purified GS on a phenyl boronate column. Figure 4 shows the elution profile of this column. Radioactivity due to $^{32}P$ and [8-$^3$H]adenine was present in the peak eluted with formate. The UV spectrum of the peak is shown in Fig. 5; the spectrum is characteristic of adenine nucleotides. As with native protein, phosphodiesterase was incapable of removing the nucleotide from the peptide. That the phosphodiesterase was active was confirmed with NAD as a substrate.

Nitrogenase activity in photosynthetic bacteria is affected by nitrogen supply (1, 11, 13, 21, 24, 29, 33), and a role for GS in mediating the switch off of nitrogenase activity in photosynthetic bacteria has been proposed (7, 10, 31) and challenged (1, 29). However, GS in *R. capsulata* is inactivated in the dark (12). Because the Fe protein of nitrogenase in *R. rubrum* also becomes inactive by modification in the dark (14), the activity of GS from the cultures incubated in the dark was measured. Figure 6 shows that GS activity does decrease in the dark and recover when the culture is re-illuminated.


FIG. 5. UV spectrum of isolated peptide-bound nucleotide. The spectrum was recorded on a Cary 14 spectrophotometer.

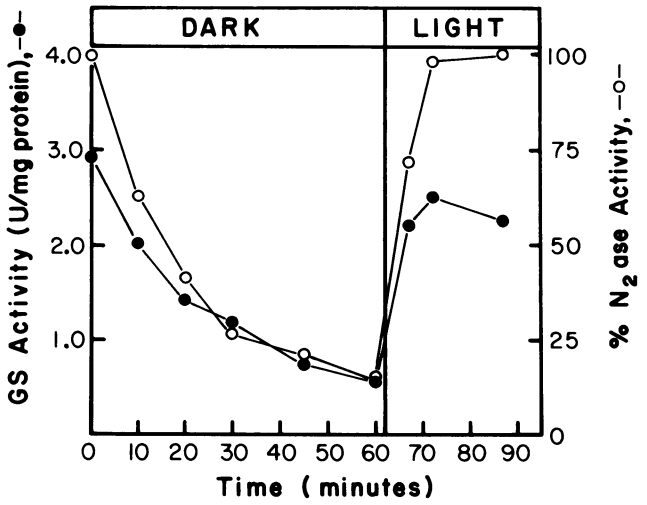


FIG. 6. Effect of dark treatment on nitrogenase activity and GS activity in extracts of treated cells. Nitrogenase activity (○) and GS activity (●) were monitored as described in the text.

GS is not an oxygen labile enzyme; however, nitrogenase is extremely oxygen labile. When comparisons are made between GS activity in extracts versus nitrogenase activity in extracts, the GS is often extracted aerobically, whereas the nitrogenase extract is prepared anaerobically. We wanted to determine whether anaerobic extraction might have an effect on GS activity in extracts. Six extractions were performed, three anaerobically and three aerobically. The GS specific activity in the anaerobically prepared extracts (average specific activity of 7.4 U/mg of protein per min) are double the specific activity of aerobically prepared extracts (average specific activity of 3.16 U/mg of protein per min). This experiment has been repeated several times with different batches of cells, always with the same result. Apparently some conversion of GS to an inactive form occurs during treatment of the cells with air. In these experiments, cells were broken by osmotic shock and GS was measured by transferase assay in the absence of $Mg^{2+}$.

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