

In Vivo Control of Glutamine Synthetase in the Facultative Phototrophic Bacterium *Rhodopseudomonas sphaeroides*

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In vivo control of glutamine synthetase (GS) in the facultative phototrophic bacterium *Rhodopseudomonas sphaeroides* was studied. The enzyme was partially purified from cells grown photosynthetically in ammonium-malate-medium. Its catalytic properties were characteristically changed by incubation with phosphodiesterase indicating an *in vivo* regulation by adenylation/deadenylation. Adenylation states of GS were measured as a function of variations in the nitrogen source and the light intensity during photosynthetic growth.

Introduction

Glutamine synthetase (EC 6.3.1.2) is one of the key enzymes in nitrogen metabolism. In gram-negative bacteria, its activity is regulated by covalent modification (adenylation/deadenylation). In addition to its catalytic function, the enzyme plays an important regulatory role in controlling the synthesis of various enzymes of N-metabolism in *Klebsiella aerogenes* [1]. Recently, evidence has been presented that, in the two facultative phototrophic bacteria, *Rhodopseudomonas capsulata* and *Rps. sphaeroides*, glutamine synthetase (GS) is also involved in the control of nitrogen fixation [2–4]. The GS of *Rps. capsulata* has already been shown to be modified by adenylation/deadenylation [5]. However, evidence for this covalent modification of GS in the second species, *Rps. sphaeroides*, was still lacking. In this communication we show that also the GS in *Rps. sphaeroides* is modulated *in vivo* by adenylation/deadenylation. Interestingly, the regulatory pattern of the *Rps. sphaeroides* enzyme differs from that of *Rps. capsulata* in that, in cultures with high external NH_4^+ -concentration, the GS is not fully adenylated and, secondly, that the state of GS-adenylation is markedly influenced by the light intensity during photosynthetic growth.

Materials and Methods

The present study was conducted with *Rps. sphaeroides* strain ATCC 17023 (= DSM 158). Stock

cultures were grown photosynthetically at 30 °C and 2500 lux light intensity in 50 ml-screw cap bottles using a malate- $(\text{NH}_4)_2\text{SO}_4$ -medium [6] supplemented with 0.05% (w/v) yeast extract. For growth experiments and enzyme assays, the bacteria were grown in defined media with malate, fructose or acetate as C-sources (0.4% [w/v] of each) and $(\text{NH}_4)_2\text{SO}_4$ (7.5 mM), Na-glutamate (15 mM) or glutamine (15 mM) as N-sources. For photosynthetic growth, illumination of the culture flasks was provided by 60 w tungsten lamps, or – if higher light intensities were required – by two 150 w “Attralux” lamps (Philips). Aerobic cultures were grown at 30 °C in Erlenmeyer flasks on a rotary shaker. Growth was monitored by measuring the turbidity at 660 nm in a “Spectronic 20” photometer (Bausch & Lomb, München). For the assay of glutamine synthetase activity (test system see below) in whole cells, the cultures were supplemented with 0.1 mg per ml of the detergent cetyltrimethyl-ammoniumbromide (CTAB). According to Bender *et al.* [7], the addition of CTAB prior to the collection of cells by centrifugation prevents rapid changes of the adenylation state of GS. To purify GS, cells were resuspended in 50 mM imidazole buffer, pH 7, containing 1 mM MnCl_2 . The cell suspension was then desintegrated by ultrasonic treatment. Unbroken cells and large cell debris were separated from the homogenates by centrifugation at 5000 rpm. Protein concentrations of the extracts were determined by the Lowry method [8].

GS (adenylated form) was partially purified from cell free extracts of NH_4^+ -grown cells (stationary phase) by firstly centrifuging the extracts for 20 min

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at $120\,000 \times g$ and then subjecting the GS containing supernatant to a fractionation with $(\text{NH}_4)_2\text{SO}_4$. The fraction precipitating between 65 and 80% saturation was collected by centrifugation at 10 000 rpm and dissolved in a small volume of 50 mM imidazole buffer, pH 7, plus 1 mM MnCl_2 . This procedure increased the specific GS activity (transferase test, see below) from about 0.2 to 12 $\mu\text{mol}/\text{min} \cdot \text{mg}$ protein.

GS activities were measured by using the γ -glutamyltransferase assay of Shapiro and Stadtman [9] as modified by Bender *et al.* [7]. In experiments designed to estimate the adenylation state of GS, CTAB-treated whole cells were used as enzyme source. The extent of inhibition by 60 mM MgCl_2 of the Mn^{2+} -dependent transferase activity measured at the "isoactivity point" of GS (see section "Results") was taken as an index of the adenylation state. The latter was calculated by using the equation

$$\bar{n} = 12 - 12 \left(\frac{\text{transferase activity with 60 mM MgCl}_2}{\text{transferase activity minus MgCl}_2} \right).$$

The "isoactivity point" of adenylylated and de-adenylylated GS (pH 6.9) was determined according to the method described in [7].

Results

The glutamate analogue, L-methionine-sulfoximine (MSO) irreversibly inactivates glutamine synthetases of eukaryotic and prokaryotic origin [10, 11]. Thus, it can be expected that MSO inhibits such cellular

activities which depend on GS activity. Examples in this respect are NH_4^+ -assimilation at low external ammonium salt concentration, assimilation of molecular nitrogen etc. As shown in Fig. 1, MSO at a concentration of 0.1 mM severely inhibits photosynthetic growth of *Rps. sphaeroides* even with high external NH_4^+ -concentration (15 mM). The growth inhibition by MSO is completely compensated by 15 mM glutamine or – similar to the results obtained with *Rps. capsulata* [12] – 15 mM glutamate. *In vitro*-tests with the partially purified GS of *Rps. sphaeroides* (for purification procedure, see section Materials and Methods) revealed that MSO at a concentration of 0.1 mM inactivated GS to about 99%. The inhibition of growth in NH_4^+ -medium by MSO and its reversal by glutamine are consistent with an obligatory role of GS in ammonium assimilation (even at high external concentrations of the latter). It has been postulated already in an earlier paper from this laboratory [6] that glutamate dehydrogenase (EC 1.4.1.2) of *Rps. sphaeroides*, due to its characteristic regulatory properties, does not function in ammonium assimilation in this organism. The severe growth inhibition by MSO further supports this notion. The finding that also glutamate compensates the inhibitory action of MSO might be due to protection of MSO-binding sites of GS.

Extracts from *Rps. sphaeroides* cells harvested from a photosynthetic malate-ammonium-culture (stationary phase) catalyze an ADP-dependent glutamyl-transfer with rates of about 0.2 $\mu\text{mol}/\text{min} \cdot \text{mg}$ protein. In such extracts, the ratio of transferase activity with 60 mM MgCl_2 to that without MgCl_2 is

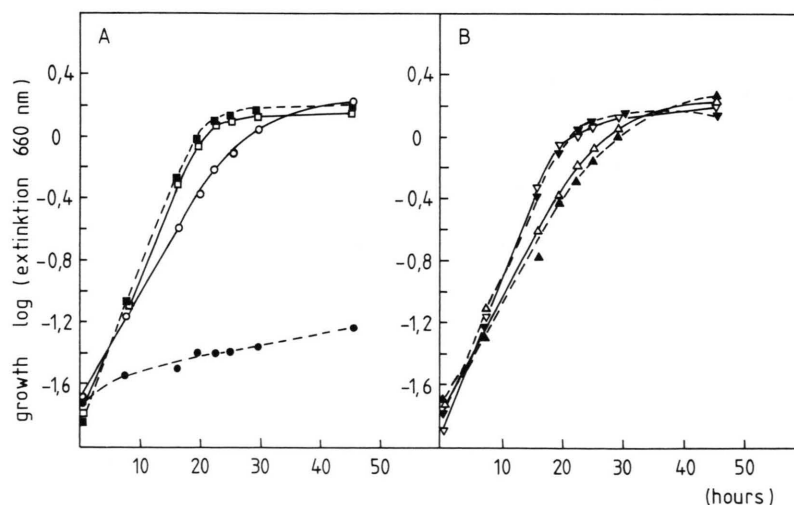


Fig. 1. Effect of L-methionine-sulfoximine (MSO) on photosynthetic growth (1600 lux) of *Rps. sphaeroides* in malate-media with different N-sources. A. ○, ●: 15 mM NH_4^+ ; □, ■: 15 mM NH_4^+ plus 15 mM glutamine. B. △, ▲: 15 mM NH_4^+ plus 15 mM glutamate; ▽, ▼: 15 mM NH_4^+ plus 15 mM glutamate plus 15 mM glutamine. Open symbols: Growth without MSO; filled symbols: growth with 0.1 mM MSO.

in the range of 0.04 to 0.1, suggesting the occurrence of an adenylylated GS in NH_4^+ -grown cells (see [7]). By treatment with phosphodiesterase (EC 3.1.4.1), adenylylated GS can be converted to the deadenylylated form [7]. Such changes in the adenylylation state of GS are reflected in changes of the MgCl_2 -sensitivity of the transferase activity.

The data presented in Fig. 2 show that, during an incubation of a partially purified GS from NH_4^+ -grown cells with phosphodiesterase, the activity ratio increases from about 0.05 to values close to 0.7 within 3 min. In accord with the data of ref. [7], we interpret this finding in that phosphodiesterase treatment of GS converts the enzyme from the adenylylated to the deadenylylated form.

Specific GS activities (assayed at the "isoactivity point" at pH 6.9) were measured in CTAB-treated *Rps. sphaeroides* cells grown with different C- and N-sources. The data in Table I show that, independent of the C-source, the lowest GS levels were measured in cultures with NH_4^+ as N-source. Thus, regulation of GS activity in *Rps. sphaeroides* not only occurs at the level of covalent modification, but also at the level of gene expression. The adenylylation

Table I. Glutamine synthetase levels and adenylylation states of glutamine synthetase in exponentially growing *Rhodospseudomonas sphaeroides* cells.

Culture condition	C-source	N-source	Glutamine synthetase activity [$\mu\text{mol}/\text{min} \cdot \text{mg protein}$]	Adenylylation state of glutamine synthetase [\bar{n}]
Anaerobic, light (8000 lux)	malate	NH_4^+	0.19–0.22	5–6
	fructose	NH_4^+	0.2–0.3	5–6
	acetate	NH_4^+	0.17–0.20	5–6
	malate	glutamate	0.7–0.9	0–0.6
Anaerobic, light (500 lux)	malate	NH_4^+	0.2	7–9
	malate	glutamate	0.7	6–8
Anaerobic, light (6000 lux)	malate	NH_4^+	0.2	10–11.5
stationary phase				
Aerobic, dark	malate	NH_4^+	0.2	2–3

GS activities (γ -glutamyl transferase activity) were measured in CTAB-treated whole cells according to the method described in [7]. Adenylylation states were calculated by using the ratio of transferase activity with MgCl_2 to that without as index (see section Materials and Methods) with \bar{n} giving the average number of adenylylated subunits.

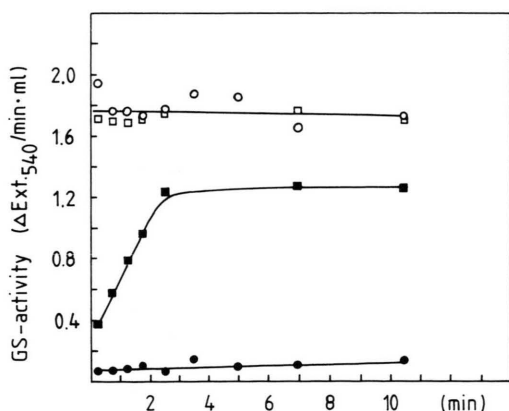


Fig. 2. Effect of phosphodiesterase (EC 3.1.4.1) treatment of *Rps. sphaeroides* glutamine synthetase (GS) on Mg^{2+} -sensitivity of GS-catalyzed transferase reaction. GS partially purified from malate- NH_4^+ -cultures was used for the experiment. 3 units of GS (1 unit is the enzyme activity catalyzing the formation of $1 \mu\text{mol } \gamma$ -glutamylhydroxamate per min) per ml were incubated with 1.6 units snake venom phosphodiesterase (Boehringer, Mannheim) in 50 mM imidazole buffer, pH 7, plus 1 mM MnCl_2 at 30°C . At the times indicated, 0.1 ml-samples were removed from the mixture and assayed for transferase activity (37°C , pH 6.9) in the presence and absence of 60 mM MgCl_2 . ○, without Mg^{2+} , no phosphodiesterase (PD) treatment; ●, plus Mg^{2+} , no PD treatment; □, without Mg^{2+} , PD treatment; ■, plus Mg^{2+} , PD treatment.

states of GS in exponentially growing *Rps. sphaeroides* cells are practically independent of the C-source, but dependent on the N-source and the overall energy supply of the culture (Table I). In NH_4^+ - as well as in glutamate-cultures, low light intensities (200 to 500 lux) favour a high adenylylation state ($\bar{n} = 6-9$). Only at high light intensities (> 5000 lux), *i.e.* optimal energy supply of the cultures, N-source-dependent differences in GS-adenylylation states ($\bar{n} = 5-6$ for NH_4^+ -cultures; $\bar{n} = 0-0.6$ for glutamate cultures) are obtained.

Discussion

Like glutamine synthetases in most gram-negative bacteria, including that of the facultative phototrophic bacterium, *Rps. capsulata* [5], the enzyme of *Rps. sphaeroides* was shown to be covalently modified by adenylylation/deadenylylation. The regulatory pattern of GS in the latter organism is similar to that described for the enzyme in *Rps. capsulata*. This is of interest in so far as nitrogenase activities in both organisms seem to be closely connected with GS activity. Hillmer and Fahlbusch [2] proposed that

GS in *Rps. capsulata* might control N_2 -fixation by direct interaction with the nitrogenase enzyme-complex. On the other hand, Jones and Monty [4] suggested that glutamine (*i. e.* the product of the GS-catalyzed reaction) was involved in nitrogenase repression in *Rps. sphaeroides*. Our present studies have shown that the degree of GS-adenylylation in NH_4^+ -cultures of *Rps. sphaeroides* is not as high as in *Rps. capsulata* (see ref. [5]). For GS of the latter species, adenylylation states of $\bar{n} = 10-11$ were measured in NH_4^+ -grown cells. In addition, these values were independent of the light intensity during growth [5]. These differences in the regulatory pattern of GS might be important in a reevaluation of the role of GS in nitrogenase regulation. If the GS-mediated control of nitrogenase activity and/or bio-

synthesis would depend on the adenylylation state of GS, the response of nitrogenase activity (measured, for example, as the ability of cells to photoproduce molecular hydrogen) to variations in such cultural condition that have been shown to affect the adenylylation state of GS, would be rather different in *Rps. capsulata* and *Rps. sphaeroides*.

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