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In vivo modification of *Azotobacter chroococcum* glutamine synthetase

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A monospecific anti-(glutamine synthetase) antibody raised against glutamine synthetase of the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 immunoreacted with glutamine synthetase from the N_2-fixing heterotrophic bacterium *Azotobacter chroococcum*. In Western-blotting experiments this antibody recognized a single protein of a molecular mass of 59 kDa corresponding to glutamine synthetase subunit. This protein was *in vivo*-labelled in response to addition of ammonium, both [^{3}H]adenine and $H_3{}^{32}PO_4$ preincubation of the cells being equally effective. Nevertheless, the amount of glutamine synthetase present in *A. chroococcum* was independent of the available nitrogen source. Modified, inactive glutamine synthetase was re-activated by treatment with snake-venom phosphodiesterase but not by alkaline phosphatase. L-Methionine-DL-sulphoximine, an inhibitor of glutamine synthetase, prevented the enzyme from being covalently modified. We conclude that, in *A. chroococcum*, glutamine synthetase is adenylylated in response to ammonium and that for the modification to take place ammonium must be metabolized.

INTRODUCTION

The azotobacters are heterotrophic nitrogen-fixing aerobic bacteria that can use, besides molecular nitrogen, other inorganic nitrogen sources such as nitrate or nitrite. Ammonium, the product of the reduction of N_2 and nitrate, controls in its turn the activities of nitrogenase [1–3] and nitrate uptake [4], both energy-dependent events. In *Azotobacter chroococcum* [5] and *A. vinelandii* [6] ammonium is incorporated into carbon skeletons via the glutamine synthetase (GS)–glutamate synthase (GOGAT) route, which is thought to be solely responsible for the assimilation of ammonium. Since GS, that catalyses an ATP-requiring reaction, plays a pivotal role in nitrogen metabolism in many prokaryotes and eukaryotes, both the synthesis and activity of this enzyme have been considered to be strictly regulated in response to the available nitrogen source.

Regulation of GS by gene expression, feedback inhibition, and covalent modification has been studied extensively in Enterobacteria [7–9]. In *Escherichia coli*, GS is a dodecamer of 12 identical subunits, each of which can be regulated independently by reversible adenylylation rendering a less-active enzyme. The adenylylation–deadenylylation reactions are catalysed by adenylyltransferase, the activity of which is regulated by the regulatory protein P_{II}, which itself is regulated through reversible uridylylation carried out by an uridylyltransferase. The latter enzyme responds to the ratio of glutamine to α-oxoglutarate, shifting GS to the more adenylylated state as the ratio of glutamine to α-oxoglutarate increases and vice versa [10,11]. The adenylylation–deadenylylation system is operative in most Gram-negative [12–15] but not in gram-positive [16] bacteria, the methanogen *Methanobacterium ivanovi* [17] or cyanobacteria [18,19]. Besides this covalent modification, GS from both prokaryotic and eukaryotic organisms has been described to be ADP-ribosylated *in vitro*, and in some cases the reaction was accompanied by the loss of GS activity ([13] and references cited therein). The *in vivo* existence of this modification has not yet been reported, however.

Information on azotobacter GS is very scarce. The enzyme has been purified from *A. vinelandii* and shown to contain AMP bound covalently when isolated from ammonium-grown cells [20,21]. Recently it has been described that, in *A. vinelandii*, expression of *glnA*, the gene encoding glutamine synthetase, is not controlled by the available nitrogen source [22]. Here we report that GS of *A. chroococcum* did cross-react with antiserum raised against GS from the cyanobacterium *Synechocystis* sp. PCC 6803. We also show that, in *A. chroococcum*, GS is regulated by adenylylation–deadenylylation in response to the addition of ammonium to the growth medium, with ammonium determining the GS activity level rather than the enzyme synthesis. Finally, we report that in the presence of L-methionine-DL-sulphoximine (MSX), an inhibitor of GS, adenylylation of this enzyme was prevented.

MATERIALS AND METHODS

Materials

Coomassie Brilliant Blue R-250 was from Bio-Rad, Richmond, CA, U.S.A. Alkaline phosphatase and phosphodiesterase were from Boehringer-Mannheim, Mannheim, Germany. [^{3}H]Adenine (25.8 Ci/mol) and $H_3{}^{32}PO_4$ (8800 Ci/mmol) were purchased from du Pont–New England Nuclear, Dreieich, Germany. For determination of molecular mass, an electrophoresis calibration kit from Bio-Rad Laboratories, Hercules, CA, U.S.A., was used. All other chemicals were of analytical grade.

Organism and growth conditions

A. chroococcum A.T.C.C. 4412 (from the University of Valencia Collection, Valencia, Spain) was grown on nitrogen-free Burk's medium supplemented with 0.5% (w/v) sucrose as the sole energy and carbon source. When indicated, this medium was supplemented with NH_4Cl or KNO_3 at the concentration specified in each case. Growth conditions were as previously described [23].

Cell extracts

Cells were harvested by centrifugation at 10000 g for 10 min at 4 °C, washed in 50 mM Mops/KOH buffer, pH 7.5, and

Abbreviations used: GS, glutamine synthetase; GOGAT, glutamate synthase; MSX, L-methionine-DL-sulphoximine.
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resuspended at a concentration of 1 g of cells/3 ml of 50 mM Mops/KOH buffer, pH 7.5, containing 1 mM DL-dithiothreitol and 1 mM phenylmethanesulphonyl fluoride. Cells were disrupted by sonication (20 kHz; 75 W) for 5 min (in 30 s periods) with a Branson sonifier model B 12. The homogenate was centrifuged at 33000 *g* for 30 min at 4 °C, and the resulting supernatant constituted the cell extract.

Enzyme assays

GS activity was determined *in vitro* by the formation of γ-glutamylhydroxamate (transferase assay) in the presence of either Mn^{2+} (inactive form) or Mn^{2+} plus Mg^{2+} (active GS form) [21,24]. Nitrate reductase activity was determined *in vitro* with dithionite-reduced Methyl Viologen as the electron donor [25].

Immunoprecipitation of GS protein

The *A. chroococcum* GS protein was immunoprecipitated from cell extracts with antiserum directed against the *Synechocystis* sp. strain PCC 6803 GS (kindly provided by F. J. Florencio, from this Institute) as follows: 0.3 ml aliquots of extracts from *A. chroococcum* cells were mixed with increasing amounts of the crude rabbit (5 mg of protein/ml) antiserum. The immunoprecipitate was allowed to form overnight at 4 °C and then centrifuged at 10000 *g* for 10 min at 4 °C. The pellet was dissolved in 40 μl of sample buffer [26] for SDS/PAGE, and the supernatant saved for determining the remaining activities of GS and, where indicated, nitrate reductase.

Western blot (immunoblotting)

After SDS/PAGE, the proteins were transferred to nitrocellulose sheets as in [27], using a mini-Trans Blot electrophoretic transfer cell (Bio-Rad). To immunodetect proteins, the nitrocellulose filters were blocked overnight in 200 mM NaCl and 15 mM Tris/HCl, pH 7.4 (Tris/NaCl), containing 0.2% (w/v) sodium azide and 5% (w/v) dried skimmed milk. Anti-GS antibody (1:500) was added, and the mixture incubated with shaking overnight. The filters were washed four times with Tris/NaCl containing 0.05% (v/v) Tween 20, then peroxidase-conjugated anti-(rabbit IgG) serum (Sigma) was used as second antibody, and blots were developed as described by Kombrink et al. [28].

In vivo [^{3}H]adenine-labelling (^{32}P-labelling) experiments

A 2 ml culture was grown to mid-logarithmic phase [attenuance (D_{560}) of approx. 0.5] in nitrogen-free medium. Cells were harvested by centrifugation at 7000 *g* for 5 min at room temperature and resuspended in 1 ml of culture medium buffered with 50 mM Mops/KOH, pH 7.5, and without any potassium phosphate. Cells were incubated at 30 °C with continuous shaking (100 strokes·min^{-1}) for 10 min and then supplemented with either 19 μCi of [^{3}H]adenine or 2 μCi of [^{32}P]orthophosphoric acid. After further incubation for 2 h, 5 mM NH_4Cl was added, and the cells were harvested 1 h later. Sedimentation was improved by adding NaCl and EDTA to final concentrations of 100 mM and 10 mM respectively. The cells were resuspended in 40 μl of sample buffer [26] for electrophoresis and disrupted by freezing in liquid air followed by boiling for 3 min (repeated four times at least). The homogenate was centrifuged at 16500 *g* for 20 min and the supernatant constituted the labelled cell extract. Where indicated, immunoprecipitation of labelled proteins was carried out as described above for the unlabelled cell extract. When gels were loaded with proteins from cell extracts that were not immunoprecipitated, the GS protein was identified by Western-blot (immunoblotting) analysis.

Analytical methods

Protein in cell extracts was estimated by the method of Markwell et al. [29] using BSA as standard. SDS/PAGE was performed according to Laemmli [26]. Protein markers were electrophoresed in parallel. The proteins were stained with Coomassie Brilliant Blue R-250. Fluorograms of *in vivo*-labelled proteins were obtained as described in [30].

RESULTS

Antibodies raised against GS from *Synechocystis* cross-react with *A. chroococcum* GS

Addition of increasing amounts of anti-GS antibody raised against the enzyme from the cyanobacterium *Synechocystis* to an *A. chroococcum* cell-free extract resulted in progressive disappearance of GS activity (Figure 1). Though not shown, it could be ascertained that the antiserum was equally effective in the immunoprecipitation of both active and inactive GS form. As illustrated in Figure 1, no effect of the antibody was observed on nitrate reductase activity, which was used as a negative control. Furthermore, the analysis of the immunoprecipitated material on SDS/PAGE revealed enrichment of a protein of approx. 59 kDa of molecular mass (Figure 2) that was not present in the antiserum alone (lane 7 in Figure 2). To our knowledge, the molecular mass of *A. chroococcum* GS has not been reported previously. The *A. vinelandii* GS, however, has been described as a dodecamer with monomer of molecular mass of either 56.5 [21], 53 [20] or 62 kDa, in the latter case when expressed in *Escherichia coli* [22]. On the basis of these data we concluded that the protein enriched in the immunoprecipitate corresponded to the GS monomer.

Taking into consideration that GS could be detected on SDS/PAGE, we next investigated whether or not this enzyme

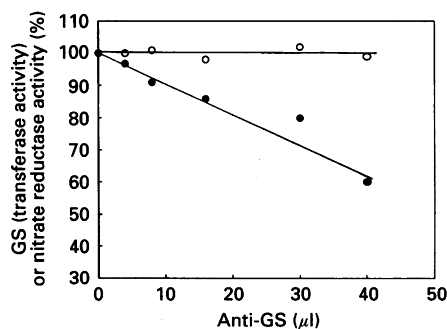

Figure 1 Immunotitration of *A. chroococcum* GS

Aliquots (300 μl) of cell extract (3 mg protein/ml) from 8 mM KNO_3-grown *A. chroococcum* cells were incubated with increasing volumes of the antibodies (5 mg/ml) raised against pure GS from *Synechococcus* sp. strain PCC 6301. The activities of glutamine synthetase (●) and nitrate reductase (○) remaining in supernatants were measured as described in the Materials and methods section. The level of 100% GS activity corresponds to 250 munits/mg of protein; 100% nitrate reductase activity of the control was 20 munits/mg of protein.

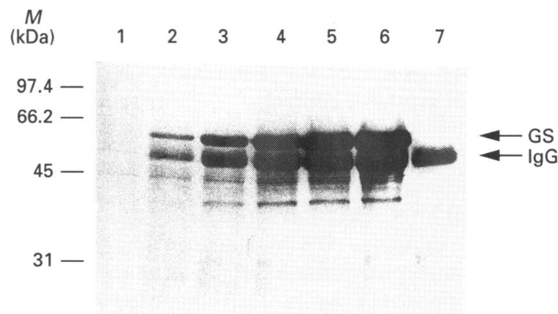

Figure 2 Effect of anti-GS antibody concentrations on immunoprecipitation of *A. chroococcum* GS

Samples described in Figure 1 were centrifuged and the corresponding immunoprecipitated proteins were electrophoresed in an SDS/12%-polyacrylamide gel. *A. chroococcum* GS was immunoprecipitated with 0, 4, 8, 16, 30 and 40 μl of anti-GS antibodies (lanes 1–6). Lane 7 corresponds to the antiserum alone (30 μl). Molecular masses (M) of protein standards are indicated.

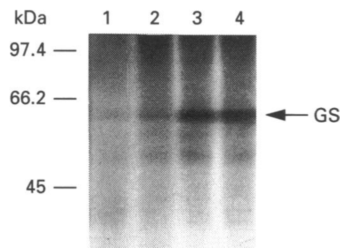

Figure 3 *In vivo* ^{32}P labelling of ammonium-inactivated GS from *A. chroococcum*

^{32}P labelling of combined nitrogen-free-grown cells was carried out as described in the Materials and methods section. The cell extracts thus obtained (50 μg of protein) were electrophoresed in an SDS/10%-polyacrylamide gel and the gel subjected to autoradiography. Lanes: 1, cell extract of non-ammonium-treated cells (containing active GS); 2, 3 and 4, cell extracts from cells treated with 0.5 mM NH_4Cl for 30, 60 and 90 min respectively (containing inactive GS). The molecular masses (*M*) of protein standards are indicated.

was subject to covalent modification in response to ammonium in *A. chroococcum*.

In vivo labelling of *A. chroococcum* GS

When diazotrophically grown *A. chroococcum* cells were preincubated in the presence of $H_3{}^{32}PO_4$ and then subjected to an ammonium shock (5 mM NH_4Cl), the 59 kDa protein previously identified as GS subunit was labelled, this labelling increasing with time of incubation in the presence of ammonium (Figure 3). This result indicated that GS was modified as a consequence of the ammonium shock by a group containing phosphate.

It is well established, as stated above, that GS from most Gram-negative bacteria is regulated by an adenylylation–deadenylylation system and that, in this case, the modified enzyme is radiolabelled *in vivo* in the presence of ^{32}P. To check whether this regulatory process is operative in *A. chroococcum*, two approaches were made. First, when cells were pulse-labelled with [^{3}H]adenine, the enzyme was radiolabelled in response to

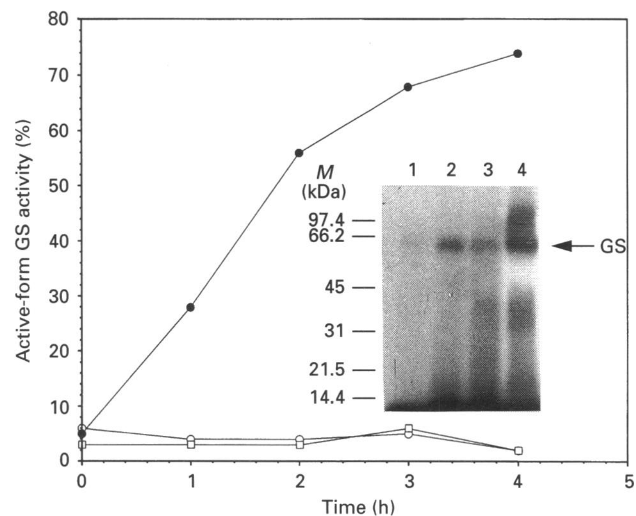

Figure 4 Effect of alkaline phosphatase and phosphodiesterase on glutamine synthetase inactivated by ammonium

Diazotrophically grown *A. chroococcum* cells were treated with 5 mM NH_4Cl for 1 h to inactivate GS and then used to obtain the corresponding cell extract. The extracts were incubated at 37 °C with 30 units/mg protein alkaline phosphatase (○), 1 unit/mg of protein phosphodiesterase (●) or without any addition (□), and the Mg^{2+}-dependent *in vitro* γ-glutamyltransferase activity (active form) was determined at the indicated times. To follow the release of ^{32}P from radiolabelled GS (inset), the extracts from ammonium-treated cells were immunoprecipitated after 1 h incubation with either phosphodiesterase (lane 3), alkaline phosphatase (lane 4) or no addition (lane 2). Lane 1 represents the immunoprecipitate of the extract from non-ammonium-treated cells; 100% of GS activity corresponds to 300 munits/mg of protein. In the inset *M* is molecular mass.

ammonium addition in a similar way to that described in Figure 3 (see below), indicating that the modifying group also contained adenine. Secondly, treatment of an enzyme preparation from ammonium-grown cells, and hence of low activity, with snake-venom phosphodiesterase promoted the re-activation of GS activity (Figure 4). A similar treatment with alkaline phosphatase did not mediate any re-activation of GS. Analysis by SDS/PAGE and autoradiography of the enzyme preparation after re-activation with phosphodiesterase and then immunoprecipitated demonstrated removal of the modifying group, as shown in Figure 4 (inset). Taken together these results strongly support that, in *A. chroococcum*, GS is regulated by an ammonium-induced adenylylation–deadenylylation.

Prevention by MSX of GS modification

MSX is an extensively used inhibitor of GS activity. In *A. chroococcum* cells that had been preincubated with MSX, ammonium was without effect on either nitrogenase activity [2] or nitrate uptake [4], the conclusion being drawn that ammonium must be metabolized to exert its inhibitory effect on these processes. Figure 5 illustrates an experiment on [^{3}H]adenine-labelling of GS with *A. chroococcum* cells subject to an ammonium shock in the absence (lane 2) and in the presence of MSX (lane 3). In the control treatment (lane 1), the cells were incubated in the presence of radiolabelled adenine alone. As can be seen, cells treated with ammonium showed very high radioactive labelling, as stated above, and MSX prevented GS from being covalently modified. Though not shown it was found that MSX removed the [^{3}H]adenine labelling from GS when added once $NH_4{}^+$ inhibition of GS had taken place. These results therefore suggest that for GS adenylylation to take place

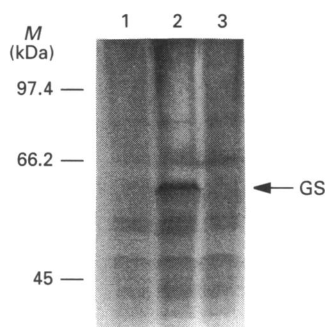

Figure 5 Prevention by MSX of *in vivo* [^{3}H]adenine-labelling of GS during ammonium inactivation

[^{3}H]Adenine labelling of combined nitrogen-free-grown cells was carried out as described in the Materials and methods section. The cell extracts thus obtained (50 μg of protein) were electrophoresed in an SDS/10%-polyacrylamide gel and the fluorogram was carried out. Lanes: 1, extract from non-ammonium-treated cells (containing active GS); lane 2, cell extract from bacteria treated with 5 mM NH_4Cl for 60 min (containing inactive GS); lane 3, cell extract from bacteria treated with 5 mM MSX 30 min prior to the 5 mM NH_4Cl addition. The molecular masses (*M*) of protein standards are indicated.

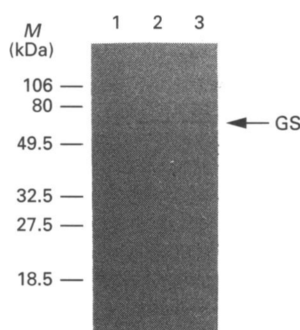

Figure 6 Western-blot analysis of *A. chroococcum* cell extracts using anti-GS antibodies

Cell extracts were obtained from 24 h-old cells grown in combined nitrogen-free medium (lane 1) and in media containing either 8 mM KNO_3 (lane 2) or 10 mM NH_4Cl (lane 3). Aliquots (60 μg of protein) of cell extracts were electrophoresed in an SDS/12%-polyacrylamide gel, and the Western blot was carried out as described in the Materials and methods section.

ammonium has to be assimilated, which is in agreement with the proposal that in Enterobacteria GS adenylylation–deadenylylation depends ultimately on the glutamine/α-oxoglutarate ratio.

Effect of the nitrogen source on GS protein content

We investigated next whether, in *A. chroococcum*, the level of GS protein synthesis was regulated by ammonium also. To check this possibility cells that had been grown under N_2-fixing conditions, which it is known to exhibit a GS biosynthetic Mg^{2+}/Mn^{2+} activity ratio higher than in combined nitrogen-containing medium, indicative of a low degree of adenylylation, were transferred to media with nitrate or ammonium as the nitrogen source, or again to N_2-fixing conditions. Cells were then allowed to multiply for 24 h, approx. 10-fold the generation time, and GS protein measured by Western blotting. Figure 6 depicts that the amount of GS protein in the corresponding cell extracts was practically the same independently of the available nitrogen source. Therefore, in *A. chroococcum*, regulation of GS activity overrides protein synthesis control.

DISCUSSION

At the structural level, GS from most bacteria is a dodecamer with identical subunits. Electron-microscopic studies and computerized image processing have shown that GS is formed from two superimposed hexagons, each composed of six subunits arranged radially with respect to the central hole [31,32]. It is not surprising, then, that antigenic cross-reactivity is detected between *A. chroococcum* GS and the antiserum directed against the enzyme from *Synechocystis*. It is to be noted, however, that the antiserum cross-reacts less efficiently with *A. chroococcum* GS than with *Calothrix*, another cyanobacterium, enzyme [33]. Tronick et al. [34] showed that *E. coli* GS antiserum showed antigenic homology with the GSs of a number of Gram-negative bacteria, but no antigenic cross-reactivity was detected with the GSs of Gram-positive bacteria or eukaryotic organisms. The only exception to this was the cross-reaction observed between *E. coli* GS antiserum and the Gram-positive *Streptomyces* GS. Later on, Orr et al. [35] held a different view on the basis of the amino acid composition, N-terminal sequences and predicted conformation potentials of the GSs from the filamentous cyanobacterium *Anabaena* sp. strain 7120, the Gram-negative bacterium *E. coli*, and the gram-positive bacterium *Bacillus subtilis*. Since they observed limited sequence similarity among all three enzymes, they proposed that antigenic cross-reactivity does not necessarily depend upon the Gram reaction or state of adenylylation. The results presented here, showing cross-reactivity of *A. chroococcum* GS with antibody raised against the cyanobacterium GS, strongly support this hypothesis, because the adenylylation–deadenylylation regulatory system is lacking in *Synechocystis* [19].

Ammonium assimilation in the azotobacters is thought to take place exclusively through the GS-GOGAT route [5,6], and inhibition of either GS or GOGAT has been widely used in investigations into the ammonium regulation of nitrogenase activity or nitrate uptake, as stated above. On the basis of studies on the effect of snake-venom phosphodiesterase treatment on the enzyme isolated from cells grown on a nitrogen-rich medium, and the *in vitro* incorporation of radioactivity from [^{14}C]ATP into deadenylylated enzyme in the presence of either crude extract from *A. vinelandii* or adenylyltransferase from *E. coli*, it was concluded that *A. vinelandii* GS is regulated by adenylylation–deadenylylation [21]. Experiments on *in vivo* incorporation of radioactivity from either [^{32}P]orthophosphate or [^{3}H]adenine, both components of the modifying group, and the use of an antibody raised against GS from a cyanobacterium, allow us now to show the covalent modification of *Azotobacter* GS, following an ammonium shock, by the combined method of radiolabelling and immunoprecipitation. Though our results cannot rule out the possibility that GS might be further re-activated by an enzyme that removes ADP-ribose, re-activation of modified *A. chroococcum* GS with snake-venom phosphodiesterase strongly suggest that GS adenylylation is of prime importance in the covalent modification of GS. As mentioned above, an ADP-ribosyltransferase activity capable of modifying *Rhodospirillum rubrum* GS *in vitro* has been described recently [13].

The internal C/N balance plays an essential role in the control of the inorganic-nitrogen metabolism of most bacteria. In this respect, the central role performed by GS is unquestionable.

Actually, the adenylylation of GS is itself subject to such C/N balance, as shown by the fact that inhibition of GS by MSX prevented the enzyme from adenylylation after ammonium shock. Results presented here suggest that, in *A. chroococcum*, the central role of GS in the control of nitrogen metabolism relies only on modulation of GS activity. Thus Western-blot analysis of crude extracts from cells grown on different nitrogen sources did not reveal any significant influence of the nitrogen source on the amount of GS protein. In this connection it has been reported, as mentioned above, that the level of mRNA for *glnA*, the gene coding for GS, is not affected by the nitrogen source in *A. vinelandii* either [22]. By contrast, the GS protein levels in ammonium-grown cyanobacteria are approx. 50% of those found in cells grown on nitrate [33] or under N_2-fixing conditions [36].

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