Regulation of N₂ Fixation and Ammonia Assimilation in *Rhodopseudomonas sphaeroides* f. sp. *denitrificans*. Role of Glutamine

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Glutamine is shown to be the effector compound for the regulation of both nitrogenase and glutamine synthetase activities in *Rhodopseudomonas sphaeroides* f. sp. *denitrificans*. Glutamine synthetase and nitrogenase (components I and II) in this bacterium have similar properties to those from *Rhodopseudomonas capsulata*. Ammonia-shock treatment of washed cells, which resulted in an accumulation of [¹⁴C]glutamine from [¹⁴C]glutamate, was accompanied by an adenylylation of glutamine synthetase and an inhibition of nitrogenase. Treatment of cells with azaserine (which inhibits glutamate synthase) enhanced the accumulation of labelled glutamine from [¹⁴C]glutamate and ¹⁵NH₄Cl. Glutamine inhibited the nitrogenase activity of azaserine-treated cells to a greater extent than that of untreated cells.

INTRODUCTION

Purple non-sulphur photosynthetic bacteria utilize dinitrogen or ammonium salts as a nitrogen source for photosynthetic growth. Glutamine synthetase (EC 6.3.1.2) and glutamate synthase (EC 1.4.7.1) are key enzymes for the assimilation of ammonia in these bacteria (Tempest *et al.*, 1970; Nagatani *et al.*, 1971; Johansson & Gest, 1976). The addition of either ammonium ions or glutamine to a nitrogen fixing culture of photosynthetic bacteria produces a rapid inactivation of nitrogenase (Neilson & Nordlund, 1975; Hillmer & Gest, 1977*a, b*; Jones & Monty, 1979). This effect on nitrogenase activity has been termed a 'switch on/switch off' effect (Zumft & Castillo, 1978) and should be clearly distinguished from long term regulation at the genetic level. Ludden & Burris (1978) have reported that inactive nitrogenase from *Rhodospirillum rubrum* contains phosphate, ribose and adenine-like molecules attached to the Fe-protein of nitrogenase. It has also been found that a Mn²⁺-dependent enzyme (Nordlund & Eriksson, 1979; Ludden & Burris, 1979), recently purified from *Rhodospirillum rubrum* (Gotto & Yoch, 1982), removes the adenine moiety from the Fe-protein of nitrogenase (Ludden & Burris, 1979), producing the 'switch on' effect.

It is known that in diazotrophs glutamine synthetase is regulated by an adenylylation mechanism (Johansson & Gest, 1977; Alef *et al.*, 1981*a*). A high concentration of ammonia in the medium produces an adenylylation and inactivation of the enzyme. This effect is reversed when the ammonia has been utilized (Johansson & Gest, 1977; Alef *et al.*, 1981*a*).

Glutamine (Jones & Monty, 1979; Falk *et al.*, 1982; Haaker *et al.*, 1982; Michalski *et al.*, 1983) and/or glutamine synthetase (Johansson & Gest, 1977; Hilmer & Falbusch, 1979; Yoch & Cantu, 1980), rather than free ammonia, have been implicated in these regulatory mechanisms. Hillmer & Falbusch (1980) suggested that nitrogenase is under negative control of the adenylylated form of glutamine synthetase. Yoch (1980) and Alef *et al.* (1981*a*) concluded, however, that nitrogenase regulation is independent of the adenylylation state of this enzyme.

Abbreviations: Azaserine, *O*-diazooacetyl-L-serine; MSX, L-methionine-DL-sulphoximine.

Recently, we have shown (Michalski *et al.*, 1983) that either ammonia- or glutamine-shock treatment of toluene-permeabilized cells of *Rhodopseudomonas capsulata* preincubated with [^{14}C]ATP results in an incorporation of ^{14}C into the Fe-protein of nitrogenase and into glutamine synthetase, as well as the inactivation of both enzymes. The binding of the adenine derivative to the Fe-protein is not by a phosphate linkage.

In this paper we present evidence that in *Rhodopseudomonas sphaeroides* f. sp. *denitrificans* glutamine appears to be an important effector compound regulating nitrogenase activity, and that the main role of glutamine synthetase in this regulation is to produce glutamine.

METHODS

Bacterial strains and cultures. *Rhodopseudomonas sphaeroides* f. sp. *denitrificans* strain IL106 was kindly supplied by Dr T. Satoh, Department of Biology, Faculty of Science, Tokyo Metropolitan University, Tokyo 158, Japan. Cells were grown (5%, v/v, inoculum) in a liquid culture (Satoh *et al.*, 1976) with modifications as described previously (Kelley *et al.*, 1982). DL-Malate (21.4 mM) and NH_4Cl (concentration as indicated later) were included as carbon and nitrogen sources, respectively.

Wild type *Rhodopseudomonas capsulata* strain B10 was kindly supplied by the Photosynthetic Bacteria Group, University of Indiana, Bloomington, Indiana, USA. Bacteria were grown in a basal mineral medium (RCV) (Weaver *et al.*, 1975) supplemented with DL-lactate (30 mM) as carbon source and with NH_4Cl (concentration as indicated later) as nitrogen source.

Cells of both strains were grown photoheterotrophically at 30 °C under anaerobic conditions in completely filled screw-cap bottles, illuminated with 5000 lx (7.35 W m^{-2}). Cell growth was monitored by measuring OD_{660} . Dry weights were determined as described previously (Hillmer & Gest, 1977a).

Electrophoresis. Discontinuous electrophoresis was performed in the presence of 0.1% (w/v) SDS using either cylindrical or slab polyacrylamide gels according to Laemmli & Favre (1973). Gradient gel electrophoreses were run on Pharmacia PAA 4/30 gels (Margolis & Wrigley, 1975). Two-dimensional slab gel electrophoresis of proteins was carried out using a modification of the methods of O'Farrell (1975) and Iborra & Buchler (1976), as described by Hallenbeck *et al.* (1982b).

High-voltage paper electrophoresis of glutamine and glutamate was performed using a technique described previously (Sawhney & Nicholas, 1978). The samples of cell suspensions, incubated with either [^{14}C]glutamine or [^{14}C]glutamate, were extracted with 70% (v/v) ethanol for 30 min at room temperature. After centrifuging in an Eppendorf centrifuge type 5444 for 3 min, the supernatant fractions were spotted onto Whatman no. 1 filter paper. The amino acids were separated by high-voltage electrophoresis in 0.1 M-citrate buffer (pH 5.0) at 2 kV for 30 min in an apparatus designed by Tate (1968). After drying, the amino acids were located on the electrophoretograms by spraying with ninhydrin in acetone. Standards of glutamine and glutamate were run along with the samples. The areas corresponding to glutamine and glutamate were cut into 2 cm strips, placed into scintillation vials containing 5 ml of scintillation fluor (phase combining system, Amersham) and counted in a Packard Tri-Carb liquid scintillation spectrometer (model 460CD). The counting efficiency for ^{14}C was 70%. When cells were incubated with both ^{14}C -labelled amino acids and $^{15}\text{NH}_4\text{Cl}$ the areas of the electrophoretograms corresponding to the standards of glutamine and glutamate were cut into strips, digested in the presence of NH_4Cl (1 mg ^{14}N) and analysed for ^{15}N incorporation as described below.

L-[U- ^{14}C]Glutamine (8.9 GBq mmol $^{-1}$) and L-[U- ^{14}C]glutamic acid (10.4 GBq mmol $^{-1}$) were from Amersham. The radiochemical purity of both amino acids was tested by paper and thin-layer chromatography, as described by the producer in batch analysis sheets.

Experiments with [^{15}N]ammonia. Cells suspended in N-free culture medium (10 ml) were added to scintillation vials of 20 ml capacity, which had been pre-sparged with oxygen-free argon for 10 min. The vials were sealed with rubber septa and further gassed with argon (10 min), then shaken at 30 °C in a water bath illuminated from below with incandescent lamps (5000 lx). After 10 min preincubation, $^{15}\text{NH}_4\text{Cl}$ was added, to a final concentration of 2 mM (5.43 atom % excess). At various times 5 ml samples of the cell suspension were taken, washed with N-free culture medium and transferred to micro-Kjeldahl flasks for digestion (Kelley *et al.*, 1980). The samples were then analysed for ^{15}N incorporation (Sims & Cocking, 1958; Nicholas & Fisher, 1960; Khanna *et al.*, 1980) with a VG Micromass 602E spectrometer (Middlewich, Cheshire, UK).

Experiments with $^{15}\text{N}_2$. Washed cells were prepared from nitrogen-starved cultures (grown in the presence of 2 mM- NH_4Cl) and then resuspended in N-free culture medium. Samples of cell suspension (10 ml) were added to the scintillation vials (20 ml capacity) fitted with septa, and then rigorously evacuated for 5 min with a two-stage vacuum pump. The vials were then gassed with 20% (v/v) $^{15}\text{N}_2$ (32.55 atom% excess) in helium and incubated in a shaking water bath at 30 °C under illumination, as outlined above. The cells were harvested and digested as described for the $^{15}\text{NH}_4\text{Cl}$ experiments.

($^{15}\text{NH}_4$) $_2\text{SO}_4$ (32.55 atom% excess) was purchased from L'Office National Industriel de L'Azote, Marseille, France. $^{15}\text{N}_2$ gas was produced from ($^{15}\text{NH}_4$) $_2\text{SO}_4$ in reaction with an alkaline solution of sodium hypobromite (Nicholas & Fisher, 1960).

Enzyme purification. Glutamine synthetases from *R. capsulata* and *R. sphaeroides* f. sp. *denitrificans* grown photoheterotrophically with 15 mM- NH_4Cl were purified according to Alef *et al.* (1981*b*) with modifications described by Michalski *et al.* (1983).

Nitrogenase from *R. sphaeroides* f. sp. *denitrificans* was partially purified from cells grown with 2 mM- NH_4Cl which contained active enzyme, using the method previously described for nitrogenase from *R. capsulata* (Hallenbeck *et al.*, 1982*a*). The activity of this enzyme was 53 nmol C_2H_4 produced min^{-1} (mg protein) $^{-1}$ as measured by the method of Hallenbeck *et al.* (1982*a*). The enzyme activity was not dependent upon Mn^{2+} .

Enzyme assays. Both the γ -glutamyl transferase and biosynthetic activities of glutamine synthetase were assayed according to the procedures of Shapiro & Stadtman (1970).

The adenylation state of glutamine synthetase was determined from the inhibition of the Mn^{2+} -dependent glutamyl transferase reaction by 60 mM- MgCl_2 . The isoactivity point of the adenylylated and deadenylylated forms of enzyme was 6.95. Deadenylation of purified glutamine synthetases was achieved in a reaction with snake venom phosphodiesterase (Sigma) using 0.05 activity unit of enzyme per mg glutamine synthetase. The reaction mixture was incubated in 0.125 M-Tris/HCl buffer, pH 8.5, for 15 min at 37 °C.

Glutamate synthase activity was measured in a Perkin-Elmer Lambda 5 spectrophotometer by monitoring the 2-oxoglutarate-dependent oxidation of NADPH at 340 nm (Meers *et al.*, 1970; Alef *et al.*, 1981*b*). This activity, which was not dependent on ammonia concentration in the culture medium, was approximately 60 nmol NADPH oxidized min^{-1} (mg enzyme) $^{-1}$.

Nitrogenase activity was determined by the acetylene reduction technique (Meyer *et al.*, 1978*a*). It was measured *in situ* in toluene-treated cells as described by Willison & Vignais (1982) and in intact cells by the method of Meyer *et al.* (1978*b*). Acetylene reduction was assayed by the gas chromatographic measurement of ethylene using a Packard chromatograph model 427.

Protein in both cell-free extracts and enzyme preparations was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

RESULTS

Enzymes of nitrogen assimilation

A modification of the procedure of Alef *et al.* (1981*b*) has been used for the purification of glutamine synthetases from *R. capsulata* and *R. sphaeroides* f. sp. *denitrificans*. The molecular weights of the glutamine synthetase subunits from both sources were found to be 58000 ± 1000 as determined by SDS-PAGE. The enzymes from both bacteria had, according to pore gradient PAGE, in the absence of SDS, the following molecular weight values: 57000 (minimal value for monomeric form) and 685000 (maximal value for undissociated enzyme). Glutamine synthetases from *R. capsulata* and *R. sphaeroides* f. sp. *denitrificans* were purified from cell cultures grown with a high concentration of NH_4Cl (15 mM). Both enzymes were found to be highly adenylylated, as determined either by the inhibition of their Mn^{2+} -dependent activities in the presence of Mg^{2+} or by treatment with snake venom phosphodiesterase (data not shown).

The isoelectric point of the adenylylated form of glutamine synthetase from *R. sphaeroides* f. sp. *denitrificans*, determined by focusing on polyacrylamide slab gel with ampholines from pH 4.0 to 6.0, was found to be 5.3. This value is slightly lower than that for glutamine synthetase from *R. capsulata* (Michalski *et al.*, 1983), and higher than that for the *R. sphaeroides* enzyme (Engelhardt & Klemme, 1982).

Nitrogenase proteins were partially purified from washed cells of *R. sphaeroides* f. sp. *denitrificans* grown with a limiting amount of NH_4Cl . After gel electrophoresis of an active nitrogenase preparation, three easily visible protein bands were detected, corresponding to molecular weights of 60000, 53000 and 31000. The isoelectric points of these proteins were found to be 6.3, 5.8 and 5.5, respectively. These represent the MoFe-protein (two polypeptides) and the Fe-protein (one polypeptide) of nitrogenase, determined by reference to purified nitrogenase components from *R. capsulata* (Hallenbeck *et al.*, 1982*a*; Michalski *et al.*, 1983).

It was found by using one- and two-dimensional electrophoresis (not shown) that synthesis of the proteins of nitrogenase was repressed when the *R. sphaeroides* f. sp. *denitrificans* cells were grown with 15 mM- NH_4Cl .

Fig. 1. Effect of ammonia shock on (a) glutamate and glutamine contents, (b) the adenylylation state of glutamine synthetase and (c) *in vivo* nitrogenase activity in *R. sphaeroides* f. sp. *denitrificans*. (a) N-starved cells were supplemented with $[^{14}\text{C}]$ glutamate [2 mM, 10 μCi (370 kBq) per 15 mg dry wt]. Ammonia shock was produced by adding 15 mM- NH_4Cl (final concn) to the anaerobic cell suspension after a 30 min preincubation with $[^{14}\text{C}]$ glutamate. Radioactivity was determined after separation by high-voltage electrophoresis as described in Methods. $[^{14}\text{C}]$ Glutamate content: ■, shock treated; □, untreated. $[^{14}\text{C}]$ Glutamine content: ●, shock treated; ○, untreated. (b) The adenylylation state of glutamine synthetase (100% corresponds to completely adenylylated enzyme) was determined as in the inhibition of the γ -glutamyltransferase activity by 60 mM- Mg^{2+} in cell-free extracts at the isoactivity point of adenylylated and deadenylylated forms of enzyme. ▲, Shock treated; △, untreated. (c) *In vivo* nitrogenase activity was measured under identical conditions to (a) in separate vials by the C_2H_2 reduction assay. ●, Shock treated; ○, untreated.

Ammonia shock and glutamine accumulation

Cells grown with limiting NH_4Cl (2 mM) and then resuspended in N-free medium accumulated $[^{14}\text{C}]$ glutamate from the culture medium in the light. As shown in Fig. 1(a) glutamate reached a steady concentration inside the cells after 20–25 min incubation with $[^{14}\text{C}]$ glutamate. After the same time period, the rate of $[^{14}\text{C}]$ glutamine production by glutamine synthetase reached a steady state. The amount of $[^{14}\text{C}]$ glutamine detected in the cells was markedly lower than that of glutamate, presumably because of the rapid utilization of glutamine in further metabolic reactions, while the $[^{14}\text{C}]$ glutamate could be continuously accumulated from the medium. Under these conditions maximum activities of nitrogenase, glutamine synthetase (Fig. 1b, c) and glutamate synthase (not shown) were detected in the cells.

Ammonia shock, produced by adding NH_4Cl (final concn 15 mM) to the culture medium, was followed by a rapid accumulation (within 5 min) of $[^{14}\text{C}]$ glutamine (Fig. 1a) as well as by the adenylylation of glutamine synthetase (Fig. 1b). Nitrogenase was also completely inhibited (Fig. 1c). However, after further incubation, the concentration of $[^{14}\text{C}]$ glutamine and the adenylylation state of glutamine synthetase reverted to their original values. Simultaneously, a reversible increase of $[^{14}\text{C}]$ glutamate concentration was observed, probably resulting from a lower affinity of adenylylated glutamine synthetase for the substrate.

Fig. 2. Incorporation of ^{14}C and ^{15}N into the cell glutamine (●, with and ○, without azaserine, 0.5 mM) and glutamate (▲, with and △, without azaserine, 0.5 mM) after preincubation of the cell suspension ($0.8 \text{ mg dry weight ml}^{-1}$) with [^{14}C]glutamine [2 mM , $10 \mu\text{Ci}$ (370 kBq) per 16 mg dry wt] and $^{15}\text{NH}_4\text{Cl}$ (2 mM) (a, b) or [^{14}C]glutamate [2 mM , $10 \mu\text{Ci}$ (370 kBq) per 16 mg dry wt] and $^{15}\text{NH}_4\text{Cl}$ (2 mM) (c, d). Note that each of the ^{14}C -labelled amino acids tested was added to an N-starved cell suspension together with $^{15}\text{NH}_4\text{Cl}$. The amount of both isotopes in glutamine and glutamate was determined after separating the amino acids from cell extracts by high-voltage electrophoresis as described in Methods.

Effect of azaserine on the accumulation of glutamine

Azaserine, a specific inhibitor of glutamate synthase (Stewart *et al.*, 1981), was taken up by washed cells within approximately 30 min, resulting in a complete inactivation of the enzyme. As shown in Fig. 2(a, b) the incubation of N-starved, azaserine-pretreated cells with [^{14}C]glutamine and $^{15}\text{NH}_4\text{Cl}$ (both 2 mM) resulted in a marked increase of ^{14}C - and ^{15}N -labelling of the glutamine pool compared with untreated cells. In this case, however, ^{15}N -labelling of glutamine was probably associated with the production of glutamine from endogenous unlabelled glutamate and $^{15}\text{NH}_4\text{Cl}$. The incorporation of $^{15}\text{NH}_4\text{Cl}$ into glutamine occurred only during the first 60 min of incubation, since at this 60 min stage glutamine synthetase was found to be 60% adenylylated.

When azaserine-pretreated cells were supplied with [^{14}C]glutamate and $^{15}\text{NH}_4\text{Cl}$ (both 2 mM) the simultaneous labelling of the glutamine pool with both isotopes was observed (Fig. 2c, d). The concentration of glutamine produced reached values of $9.8\text{--}11.2$ and $9.5\text{--}10.1 \text{ nmol (mg dry weight)}^{-1}$ within the first 30–60 min, as calculated from ^{14}C and ^{15}N assays, respectively. Again, the incorporation of the isotopes into glutamine decreased during a further 60 min incubation because of the adenylylation of glutamine synthetase.

Incorporation of ^{15}N -labelled compounds into cell-nitrogen

The effects of MSX and azaserine, the respective inhibitors of glutamine synthetase and glutamate synthase (Stewart *et al.*, 1981), as well as the effects of glutamate, glutamine and glycine on the incorporation of $^{15}\text{NH}_4$ into cell nitrogen are shown in Fig. 3. When the N-starved, washed cells were preincubated with either 0.5 mM -MSX or 0.5 mM -azaserine for 30 min, no incorporation of $^{15}\text{NH}_4$ was observed during a further 2.5 h period (Fig. 3a) and both glutamine synthetase and glutamate synthase were inactive. The preincubation of washed cells with glutamine (4 mM) and glycine (4 mM) inhibited $^{15}\text{NH}_4\text{Cl}$ incorporation by 70% and 25%, respectively (Fig. 3b). Glutamine synthetase was found to be about 60% adenylylated after

Fig. 3

Fig. 4

Fig. 3. Incorporation of $^{15}\text{NH}_4^+$ (2 mM) into cell nitrogen after preincubation (30 min) of cell suspensions ($0.6 \text{ mg dry wt ml}^{-1}$) with (a) 0.5 mM-MSX ($\square$), or 0.5 mM-azaserine ($\triangle$) and (b) 10 mM-glutamate ($\circ$), 20 mM-glutamate ($\bullet$), 4 mM-glutamine ($\blacktriangle$) or 4 mM-glycine ($\blacksquare$). Preincubation and further incubation were performed anaerobically in the light, at 30°C , for the times indicated. Incorporation of ^{15}N was determined as described in Methods.

Fig. 4. Inhibition of $^{15}\text{N}_2$ incorporation into ammonia (a) by ammonia shock, 4 mM- NH_4Cl added at 0 min ($\triangle$) or at 45 min ($\blacktriangle$); or 15 mM- NH_4Cl added at 0 min; (b) by 4 mM-glutamine with ($\diamond$) or without ($\blacksquare$) 0.5 mM-azaserine. ^{15}N -incorporation in the presence of 0.5 mM-MSX ($\circ$) or 2 mM-MSX ($\bullet$) when glutamine synthetase was completely inhibited is also shown. Incubations, always in the presence of MSX (0.5 mM), were performed anaerobically, in light (5000 lx) at 30°C in 20 ml vials containing 10 ml (approx. 6 mg dry wt) of N-starved cell suspension. The original activity of nitrogenase was $0.9 \mu\text{mol C}_2\text{H}_4 \text{ h}^{-1} (\text{mg dry wt})^{-1}$, determined by the *in vivo* assay.

glutamine treatment and 20% inhibited when glycine was used (not shown). These results are in agreement with previous reports (Woolfolk *et al.*, 1966; Alef *et al.*, 1981b; Michalski *et al.*, 1983) on the modulation of glutamine synthetase activity by these two amino acids. Neither $^{15}\text{NH}_4\text{Cl}$ incorporation nor enzyme activities were affected when the cells were preincubated with glutamate (even at 10 mM, Fig. 3b).

In the next experiments (Fig. 4), the effects of ammonia, glutamine and azaserine on the incorporation of $^{15}\text{N}_2$ in the presence of MSX were studied. The preincubation of washed cells with MSX (0.2 mM or 0.5 mM, which completely inhibited glutamine synthetase activity) did not affect the $^{15}\text{N}_2$ fixation into $^{15}\text{NH}_4^+$, and the incorporation of $^{15}\text{N}_2$ remained linear during a 2.5 h incubation (Fig. 4a, b). $^{15}\text{N}_2$ was incorporated far less rapidly into the cell ammonia when 4 mM- NH_4Cl was added to the cell suspensions (Fig. 4a), and ammonia shock (15 mM) resulted in a complete inhibition of $^{15}\text{N}_2$ fixation. When glutamine (4 mM) was used instead of ammonia, $^{15}\text{N}_2$ incorporation was inhibited by 70% in the presence of azaserine and 100% in its absence (Fig. 4b). In all cases, when $^{15}\text{N}_2$ incorporation was inhibited, the nitrogenase was similarly affected (data not shown). Thus, the decrease in the rate of $^{15}\text{N}_2$ incorporation brought about by ammonia and glutamine seems to be due to their regulatory effects on nitrogenase.

DISCUSSION

Nitrogenase, glutamine synthetase and glutamate synthase are key enzymes in the assimilation of inorganic nitrogen (N_2 and ammonium) into photosynthetically grown cells of *R.*

sphaeroides f. sp. *denitrificans*. It is also known that some of the N_2 produced by denitrification can be utilized via nitrogenase and incorporated into cell nitrogen (Dunstan *et al.*, 1982). The physical and regulatory properties of nitrogenase, as well as glutamine synthetase, purified from this bacterium are very similar to those reported for other photosynthetic bacteria (Nordlund *et al.*, 1977; Alef *et al.*, 1981*b*; Hallenbeck *et al.*, 1982*a, b*; Michalski *et al.*, 1983). In agreement with earlier reports (Hillmer & Gest, 1977; Zumft & Castillo, 1978; Hallenbeck *et al.*, 1982*a*; Michalski *et al.*, 1983) it was found that the addition of ammonia to nitrogen-starved cultures of *R. sphaeroides* f. sp. *denitrificans* resulted in an immediate, reversible inhibition of the nitrogenase in living cells (Fig. 1*c*).

Our findings, however, are not in complete agreement with recently published data of Engelhardt & Klemme (1982) for glutamine synthetase. They found that the molecular weight of each subunit was 50 000 and that of the native enzyme was 600 000 for *R. sphaeroides*, compared with 58 000 and 685 000 for the enzymes from *R. capsulata* (Michalski *et al.*, 1983) and *R. sphaeroides* f. sp. *denitrificans* reported herein. Moreover, the molecular weight, as well as the isoelectric point, of the glutamine synthetase from *R. sphaeroides* varied according to the adenylylation state of the enzyme (Engelhardt & Klemme, 1982). In our experiments, however, the isoelectric points of both glutamine synthetases were not markedly dependent on the adenylylation state of enzyme (changes of only ± 0.2 pH unit).

The role of glutamine synthetase (Hillmer & Falbusch, 1973; Johansson & Gest, 1977; Yoch, 1981) and/or glutamine (Jones & Monty, 1979; Falk *et al.*, 1982; Haaker *et al.*, 1982; Michalski *et al.*, 1983; Arp & Zumft, 1983) in the inactivation of nitrogenase is still unknown. Recently, we have shown (Michalski *et al.*, 1983) that after either ammonia or glutamine shock of toluene-treated *R. capsulata* cells preincubated with [^{14}C]ATP, both the glutamine synthetase and the Fe-protein of nitrogenase were inactivated and labelled with ^{14}C . Furthermore, it was established that the binding of the adenine moiety to the Fe-protein was not by a phosphate linkage and that the enzymic modification of nitrogenase and glutamine synthetase occurred by different mechanisms (Michalski *et al.*, 1983). On repeating this experiment with cells of *R. sphaeroides* f. sp. *denitrificans* (not shown) we obtained similar results, including a pronounced effect of glutamine shock on the process of inactivation of both glutamine synthetase and nitrogenase. Thus, in *R. sphaeroides* f. sp. *denitrificans*, glutamine is involved in the regulation of nitrogenase. Arp & Zumft (1983) have recently reported that glutamine, but not ammonia, effected nitrogenase inactivation in *R. palustris*, in the absence of glutamine synthetase activity.

The ammonia shock produced by the addition of 15 mM- NH_4Cl to the washed cells of *R. sphaeroides* f. sp. *denitrificans* preincubated with [^{14}C]glutamate for 30 min resulted in a rapid, reversible accumulation of ^{14}C -labelled glutamine in the cells, and simultaneous adenylylation of glutamine synthetase, as well as inactivation of nitrogenase (Fig. 1*a, b, c*). Similarly, when cells pretreated with azaserine (glutamate synthase was completely inhibited) were supplied with both [^{14}C]glutamate and $^{15}NH_4Cl$, simultaneous labelling of the enriched glutamine pool with both isotopes occurred (Fig. 2), and again glutamine synthetase and nitrogenase were partially inhibited (data not shown). These results indicate that the primary response of *R. sphaeroides* f. sp. *denitrificans* to ammonia shock is the overproduction of glutamine, which, in turn, is regulated by the adenylylation state of glutamine synthetase. Indeed, the data for $^{15}NH_4^+$ incorporation into cell nitrogen (Fig. 3) indicate that the activity of glutamine synthetase ($^{15}NH_4$ incorporation rate) depends on the intracellular glutamine level, i.e. the enzyme was found to be inactive when azaserine was used to inhibit glutamate production from glutamine by glutamate synthase (Figs 2 and 3). Moreover, we found that the nitrogenase activity in *R. sphaeroides* f. sp. *denitrificans* is sensitive to the glutamine level in the cells. The incorporation of $^{15}N_2$ into $^{15}NH_4^+$ (the nitrogenase activity), was partially inhibited by either ammonia or glutamine, but the inhibition was complete when azaserine was added (Fig. 4).

It has been previously reported for the non-photosynthetic bacteria *Klebsiella pneumoniae* and *Clostridium pasteurianum* (Kleiner, 1976, 1979) that the change in the extracellular ammonium level was paralleled by the intracellular glutamine concentration, which, in turn, regulated glutamine synthetase activity. It has been also suggested that glutamine may act as co-repressor of nitrogenase synthesis. Our results indicate, however, that glutamine is an important effector

compound for short-term nitrogenase activity regulation, but it is possible that glutamine may not affect nitrogenase activity directly. It has been postulated that the 'switch off' effect could be due to some other unidentified compound which responds to the intracellular concentration of glutamine (Falk *et al.*, 1982; Haaker *et al.*, 1983), possibly in combination with changes in the energy state of the cell. The main role of glutamine synthetase in this regulation however appears to be the production of glutamine.

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