Regulation of Nitrogenase Synthesis in Intact Cells of *Rhodospirillum rubrum*: Inactivation of Nitrogen Fixation by Ammonia, L-glutamine and L-asparagine

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SUMMARY

The synthesis of nitrogenase by intact cells of *Rhodospirillum rubrum* was repressed in N-free media supplemented with L-glutamine or L-asparagine, but was unaffected by the presence of L-glutamate, L-aspartate or L-histidine. Specific activities attained by cultures in supplemented media maintained under Ar-CO₂ were 2 to 3 times higher than those in N-free medium under N₂-CO₂. A loss in total activity occurred both in cultures growing with N₂ after maximum activity had been reached, and in cultures maintained under Ar when the gas phase was changed to N₂. There was a rapid loss in nitrogen-fixing activity when low concentrations of NH₄⁺, L-glutamine or L-asparagine were added to cultures with high activities, but this could be recovered in the absence of demonstrable protein synthesis. During growth, the degree of inactivation brought about by 0.5 mM-inactivator increased to 80 to 90 %, and NH₄⁺ excreted into the medium reached a maximum concentration towards the end of exponential growth.

INTRODUCTION

Nitrogen fixation is widely distributed among prokaryotic micro-organisms (Postgate, 1974); nitrogenase has been purified from several of them (Stewart, 1973; Postgate, 1974; Kleiner & Chen, 1974), and its regulation studied in representatives of most groups: *Azotobacter vinelandii* (Strandberg & Wilson, 1968; Shah, Davis & Brill, 1972) and *A. chroococcum* (Drozd, Tubb & Postgate, 1972); *Klebsiella pneumoniae* (Parejko & Wilson, 1970; Tubb & Postgate, 1973); *Clostridium pasteurianum* (Daesch & Mortenson, 1972); *Rhodospirillum rubrum* (Munson & Burris, 1969); *Anabaena* spp. (Neilson, Rippka & Kunisawa, 1971; Kulasoorya, Lang & Fay, 1972).

In all of these organisms, it was found that nitrogenase synthesis was repressed in cultures growing at the expense of NH₄⁺ and it was synthesized only under conditions of nitrogen limitation. Further, since high activities of the enzyme were found in cultures maintained under atmospheres of He or Ar and lacking N₂, it was generally concluded that the synthesis of nitrogenase is not induced by N₂ but rather derepressed by the absence of combined nitrogen. These experiments support the hypothesis that NH₄⁺ or a closely related compound regulates the synthesis of nitrogenase. Recent evidence (Streicher *et al.* 1974; Tubb, 1974) has pointed to a central role for glutamine synthetase.

Although nitrogen fixation has been demonstrated in several photosynthetic bacteria (Lindstrom, Tove & Wilson, 1950; Zakhvateva, Malafeeva & Kondrat'eva, 1970) and active cell-free preparations have been obtained from *R. rubrum* (Munson & Burris, 1969; Biggins, Kelly & Postgate, 1971; Nordlund & Baltscheffsky, 1973) and *Chromatium*

(Winter & Arnon, 1969; Evans, Telfer & Smith, 1973), the control of nitrogenase synthesis has not been extensively studied.

This investigation is concerned with the regulation of the synthesis and the inactivation of the N_2 -fixing system in intact cells of *R. rubrum*.

We measured acetylene-reducing activity under anaerobic conditions and assumed that during derepression neither substrate nor light is limiting. With this assumption, the rate of acetylene reduction is a measure of the amount of nitrogenase present. In intact bacteria, nitrogen fixation requires (in addition to the presence of nitrogenase) the effective functioning of the ATP-generating system and a source of electrons. In experiments which involved loss of N_2 -fixing capacity, these several processes were not separated and we use the expression 'inactivation of nitrogen fixation' to cover all of the reactions involved, without any implications as to their mechanisms.

METHODS

Organisms and culture conditions. *Rhodospirillum rubrum* strain S1 was grown anaerobically in the light at 28 °C in the medium of Bose, Gest & Ormerod (1961) either with ammonium sulphate in filled bottles, or in a medium lacking combined N under an atmosphere of N_2 - CO_2 (98:2). *Rhodopseudomonas palustris* (DSM126) was grown similarly in the medium recommended by N. Pfennig (personal communication) containing (g/l distilled water): K_2HPO_4 , 0.3; KH_2PO_4 , 0.2; $MgSO_4 \cdot 7H_2O$, 0.4; NaCl, 0.4; $CaCl_2 \cdot 2H_2O$, 0.075; disodium succinate, 2.0; yeast extract, 1.0; ferric citrate, 0.005. Trace elements were provided by adding 10 ml of a solution containing (mg/l): $ZnSO_4 \cdot 7H_2O$, 10; $MnCl_2 \cdot 4H_2O$, 3; H_3BO_3 , 30; $CoCl_2 \cdot 6H_2O$, 20; $CuCl_2 \cdot 2H_2O$, 1; $NiCl_2 \cdot 6H_2O$, 2; $Na_2MoO_4 \cdot 2H_2O$, 30. The pH was adjusted to 6.8 and combined nitrogen, where necessary, provided by NH_4Cl (0.5 g/l).

For studies of enzyme synthesis, cells were grown with NH_4^+ , harvested aseptically by centrifuging for 10 min at 650 g, washed with sterile N-free medium, and resuspended in sterile medium either lacking combined N or supplemented with one of the amino acids at a concentration of 10 mM. Solutions (20 ml) of L-glutamine (100 mM) and L-asparagine (100 mM) were made up immediately before use, sterilized by filtration and added aseptically to the medium. The other amino acids were added to N-free medium, the pH adjusted to 6.9 and the medium sterilized by autoclaving. Experiments were carried out in 250 ml bottles containing 200 ml medium; each bottle had a rubber stopper, through which passed a gas inlet tube with a sterile cotton-wool filter and a narrow capillary tube, through which samples could be withdrawn by means of a syringe and 15 cm hypodermic needle. Cultures were gassed either with N_2 - CO_2 (98:2) or Ar- CO_2 (98:2).

Nitrogenase assay in intact cells. Reproducible results could be obtained by collecting the samples in syringes flushed ten times with N_2 , and by flushing the bottles used for assay for 15 min before and 5 min after adding the sample. Initially we obtained erratic results for enzyme activity. This was caused by the introduction of trace amounts of oxygen before assay. In experiments on enzyme inactivation, errors due to variable oxygen inhibition during repetitive sampling were eliminated by removal of a 10 ml sample and adding 1 ml portions to each assay bottle. Since all of the experiments recorded here were carried out independently at least twice, with only minor quantitative differences in the results, we feel that artefacts due to interference from oxygen were successfully eliminated.

The assay was carried out in 8 ml bottles containing 1.25 ml N-free medium to which 2 M-sodium malate pH 6.9 (0.1 ml) was added. Samples (0.5 to 1.0 ml) were introduced anaerobically, acetylene (1 ml) was added and the bottles were incubated for 20 min at 30 °C in a

photosynthetic Warburg bath. The assay was terminated by adding 20 % (v/v) perchloric acid (0.5 ml), and ethylene was assayed gas chromatographically using a POROPAK Q column (Burris, 1972).

Preparation of cell-free extracts and assay of nitrogenase. Cell-free extracts were prepared from cells harvested in late-exponential growth by sonication under N_2 in the presence of sodium dithionite (1 mg/ml), and cell debris was removed by centrifugation at 10000 g for 15 min. Assays were carried out in 8 ml bottles with 1.7 ml medium containing 50 mM-HEPES buffer pH 7.5, creatine phosphate (10 mM), creatine phosphokinase (1.5 units/ml), ATP (4 mM) and $MgCl_2$ (20 mM). After flushing the bottles with N_2 , 0.1 M-sodium dithionite (0.1 ml) was added immediately before the extract. Otherwise the assay was performed as for intact cells but without illumination.

Asparaginase (EC. 3.5.1.1) was assayed at 35 °C in intact cells by the method of Wade, Robinson & Phillips (1971). Samples of the culture (5 to 10 ml) were removed and the cells were collected by centrifuging, washed with 0.15 M-NaCl containing 10 mM- Na_2EDTA , and suspended in 0.5 ml water. Portions (0.2 ml) containing between 1.0 and 2.8 mg protein were used for assay.

Protein. Samples (1 ml) were centrifuged for 10 min, the pellet suspended in 1 M-NaOH (1 ml) and heated at 70 °C for 10 min. Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin, fraction V (Sigma), as standard.

Ammonia was determined by the indophenol method of Chaykin (1969) on 1 ml samples after removal of cells by centrifugation. Standards were prepared by adding various concentrations of NH_4^+ to 1 ml of N-free medium. Blanks gave zero absorbance relative to water.

Photophosphorylation. Chromatophores were prepared (Baltscheffsky, 1960) from cells grown with N_2 - CO_2 and photophosphorylation estimated from the rate of esterification of inorganic phosphate (Nishimura, Ito & Chance, 1962).

RESULTS

Oxygen sensitivity

Representative results for the inactivation of nitrogenase in intact cells are given in Fig. 1. If cells were shaken in the dark for 20 min with varying partial pressures of oxygen and then flushed with N_2 for 5 min before assay, a substantial part of the inactivation at low pressures could be reversed. At oxygen concentrations above 1.75 %, however, irreversible damage occurred. These observations are in reasonable agreement with those of Pratt & Frenkel (1959) who observed reversible inactivation at oxygen concentrations up to 4 %.

Regulation of nitrogenase synthesis

The rate of enzyme synthesis was followed in non-growing cultures under N_2 - CO_2 after transferring cells grown with NH_4^+ to an NH_4^+ -free medium. The maximum specific activities attained in unsupplemented medium and in medium supplemented with several amino acids are given in Table 1. Enzyme synthesis was totally repressed by L-glutamine and L-asparagine, but was little affected by the other amino acids. Maximum specific activities observed in growing cultures were significantly higher (mean of four experiments: 48 nmol C_2H_4 /min/mg protein).

Experiments were also carried out under an atmosphere of Ar- CO_2 . In unsupplemented N-free medium there was little protein synthesis and only relatively low levels of nitrogenase were synthesized. Addition of L-glutamine or L-asparagine supported good growth, but

Fig. 1

Fig. 2

Fig. 1. Inactivation of N_2 -fixation in intact cells by oxygen. $\circ$, O_2 present in the gas phase during assay; $\bullet$, cells shaken in the dark for 20 min with O_2 and then assayed anaerobically.

Fig. 2. Kinetics of nitrogenase synthesis in a culture growing under N_2 - CO_2 . $\circ$, Total activity/0.5 ml culture; $\blacktriangle$, protein.

Table 1. Maximum specific activities of nitrogenase attained under N_2 - CO_2 and Ar - CO_2 in unsupplemented N -free medium and in media supplemented with L-amino acids

Amino acid (10 mM) added	Maximum specific activity (nmol C_2H_4 /min/mg protein)						
	None	Glu	Asp	His	Leu	Gln	Asn
N_2 - CO_2	27	28	24	23	ND	0	0
Ar - CO_2	11	75	64	51	34	0	0.6

ND, Not determined.

repressed enzyme synthesis to levels below that found in unsupplemented medium. Maximum specific activities in cultures derepressed in the presence of the other amino acids which supported only moderate growth, were about three times those observed under N_2 (Table 1).

Inactivation of N_2 -fixing activity in intact cells

In a culture growing under N_2 - CO_2 there was a loss in total activity after maximum levels were reached (Fig. 2). A much more dramatic loss was, however, observed during experiments in which nitrogenase activity was maximally derepressed under Ar - CO_2 in media supplemented with amino acids and the gas phase then changed to N_2 - CO_2 . Only moderate growth occurred under Ar , and shortly after changing the gas phase to N_2 - CO_2 more rapid growth took place. There was always a rapid and substantial fall in total activity, even when great care was taken to eliminate the possibility of inhibition due to the introduction of trace amounts of oxygen. Typical results are shown in Fig. 3(a); comparable observations were also made in experiments using several strains of *Rhodospseudomonas palustris* (Fig. 3b).

Rapid inactivation could also be obtained by adding low concentrations of ammonium acetate, L-glutamine or L-asparagine to cultures growing under N_2 (Fig. 4). The rate of inactivation by L-asparagine was significantly lower, even though the final degree of inacti-

Fig. 3. Inactivation of N_2 -fixation in intact cells grown in medium supplemented with glutamate (10 mM) after changing the gas phase from $Ar-CO_2$ to N_2-CO_2 at the time indicated by the arrows. ○, Total activity; ●, specific activity; ▲, protein or turbidity. (a) *Rhodospirillum rubrum*; (b) *Rhodopseudomonas palustris*.

vation was about the same, and this presumably reflects the existence of a less effective transport system. Following a lag, there was a rapid increase in the total activity to 80 to 90 % of the initial value. Since the amount of protein synthesis at this time was almost negligible, the inactivation appears to be reversible. The shorter time for recovery from inactivation by the amino acids was presumably due to the lower concentrations used. The initially high intracellular concentrations then fell to levels insufficient to maintain inactivation. Experiments could not be carried out at concentrations above 0.5 mM since higher concentrations caused lysis of the cells within 20 to 30 min.

Within 5 min after adding chloramphenicol (10 μg/ml) to the cultures, 90 % of the initial activity was lost and thereafter activities remained constant for 2 h. In cell-free extracts, chloramphenicol at a concentration of 20 μg/ml brought about only 12 % loss of nitrogenase activity; we were therefore unable to investigate whether reactivation of the N_2 -fixing system occurred in the presence of concentrations of chloramphenicol sufficient to inhibit protein synthesis. A similar inactivation of $^{15}N_2$ incorporation during 2 h experiments with intact cells of *A. vinelandii* was observed by Strandberg & Wilson (1968). Although these authors attributed this to the accumulation of inhibitory concentrations of NH_4^+ as a result of the cessation of protein synthesis, this would not apply to our experiments which used the acetylene-reduction assay, since acetylene is a competitive inhibitor of N_2 fixation (Schöllhorn & Burris, 1967). Acetylene is therefore preferentially reduced and little NH_4^+ produced.

The effect of adding various concentrations of ammonium acetate, L-glutamine and L-asparagine to the assay medium is shown in Fig. 5. Inactivation does not appear to be due merely to a rapid increase in ionic strength within the cell, since in assays to which 0.5 and

Fig. 4. Kinetics of inactivation and recovery of total N_2 -fixing activity in intact cells. The numbers in parentheses, below, are the concentrations in the medium after the additions. ●, Protein concentration. (a) ○, Addition of NH_4^+ (1 mM); ◆, control to which N-free medium was added. (b) □, Addition of L-glutamine (0.25 mM). (c) △, Addition of L-asparagine (0.25 mM).

Fig. 5. Inactivation of N_2 -fixing activity in cells derepressed under N_2 - CO_2 by addition of (○) NH_4^+ , (□) L-glutamine and (△) L-asparagine.

5.0 mM-sodium acetate was added, the relative activities were insignificantly different from the control values (102 and 88 %).

In contrast, inactivation was observed in cell-free extracts only at much higher concentrations. Comparison of the effects of ammonium acetate and sodium acetate, and of ammonium sulphate and sodium sulphate (Table 2), shows that the inactivation was not specific to NH_4^+ , but must be ascribed primarily to the increasing ionic strength. Correspondingly, inactivation of nitrogenase in cell-free extracts by L-glutamine and L-asparagine was hardly significant in comparison to that observed in intact cells (Table 2 and Fig. 5).

The N_2 -fixing system was increasingly sensitive towards inactivation by NH_4^+ , L-glutamine and L-asparagine during growth (Figs. 6 and 7). Presumably a less effective uptake system for L-asparagine accounts for the markedly lower degree of inactivation in the early stages of the experiment.

During growth under N_2 - CO_2 , measurements of turbidity, total nitrogenase activity, relative inactivation by NH_4^+ , and levels of NH_4^+ in the culture medium were made (Fig. 7).

Table 2. Inactivation of nitrogenase activity in cell-free extracts by addition of ammonium and sodium salts, and L-amino acids

Concn (mm) ...	Percentage activity relative to control			
	5	10	20	50
Ammonium acetate	94	83	85	71
Sodium acetate	92	84	86	70
Ammonium sulphate	97	63	35	5
Sodium sulphate	92	70	37	6
L-Asparagine	105	104	90	61
L-Glutamine	110	105	120	101
L-Aspartate	116	90	78	23
L-Glutamate	84	84	76	60

Fig. 6

Fig. 7

Fig. 6. Dependence of the degree of inactivation by L-glutamine (0.5 mM; $\square$) and L-asparagine (0.5 mM; $\triangle$) on the age of a culture growing with N_2 - CO_2 .

Fig. 7. Total nitrogenase activity ($\circ$), turbidity ($\bullet$), degree of inactivation of N_2 -fixation by 0.5 mM- NH_4^+ ($\square$) and extracellular NH_4^+ concentration ($\triangle$) during growth in unsupplemented medium under N_2 - CO_2 .

Asparaginase activity

Assays for periplasmic asparaginase activity were carried out with intact cells at several stages of growth. Enzyme activity was undetectable even when 2.8 mg cell protein were used for assay.

Photophosphorylation

Chloramphenicol (at 20 μ g/ml) and sodium acetate, ammonium acetate, L-asparagine, L-glutamine, L-aspartate and L-glutamate (each at 50 mM) were without effect on the rates of photophosphorylation by chromatophore preparations.

DISCUSSION

Regulation of enzyme synthesis. That nitrogenase can be synthesized by *R. rubrum* under an atmosphere putatively lacking N_2 is in agreement with the results of Munson & Burris (1969), and is consistent with the view that nitrogenase is not positively induced by N_2 . Repression by L-glutamine and L-asparagine has been noted in *K. pneumoniae* (Parejko &

Wilson, 1970) and in *A. vinelandii* (StJohn & Brill, 1972), though in the latter only 60 % repression by L-asparagine was reported. It seems, in general, that other amino acids do not repress nitrogenase synthesis (Munson & Burris, 1969; Tubb & Postgate, 1973). From experiments of this type, however, it is clearly impossible to determine whether glutamine and asparagine act directly or only via NH_4^+ produced by hydrolysis.

Among the bacteria examined by Wade *et al.* (1971), activities of glutaminase were uniformly much lower than those of asparaginase, and in the study of Imada *et al.* (1973), glutaminase activity was not found in any strain which did not also display asparaginase activity. Since we failed to demonstrate the presence of periplasmic asparaginase, which is considered to be the high-affinity form of the enzyme (Cedar & Schwartz, 1967), we tentatively conclude that asparaginase, and possibly also glutaminase, activities are absent, and suggest that asparagine and glutamine can act as repressors without intermediate formation of NH_4^+ . Asparaginase activity has been reported in *Rhodopseudomonas capsulatus* (Tchan, Asano & Kobayashi, 1971) though only insignificant levels were found in *Rhodopseudomonas spheroides* by Wade *et al.* (1971).

Inactivation of nitrogen fixation. Our data have conclusively shown that, in intact cells, N_2 -fixation is rapidly inactivated by concentrations of NH_4^+ , L-asparagine and L-glutamine which are completely without effect on cell-free extracts of the enzyme prepared from cells which are maximally sensitive towards inactivation (Figs. 6 and 7). This suggests that loss of N_2 -fixing activity may be due not to inhibition of nitrogenase activity but to inactivation of some part of the electron transport system or the coupling between ATP and nitrogenase. Our data showing lack of inhibition of photophosphorylation in chromatophores suggest that inhibition of the ATP-generating system itself is unlikely. The inactivation at much higher concentrations which occurs in cell-free extracts is primarily an effect of increased ionic strength (Hardy *et al.* 1968).

Since NH_4^+ , L-glutamine and L-asparagine can serve as excellent N-sources under Ar-CO_2 , *R. rubrum* must possess effective transport systems for all three compounds; the intracellular concentrations were therefore probably much higher than those occurring in the culture medium. Since, however, there was no significant inactivation upon addition of sodium acetate to intact cells at concentrations ten times greater than those giving 80 % inhibition by NH_4^+ , inactivation in intact cells is probably a specific phenomenon and not due simply to an increase in ionic strength.

Inactivation of N_2 -fixing activity by addition of NH_4^+ (40 mM) to the assay medium has been observed in intact cells of *A. vinelandii* (Hardy *et al.* 1968), and it has frequently been noted in *Azotobacter* that addition of NH_4^+ to N_2 -fixing cultures causes a loss of activity at a rate greater than would be expected if the enzyme had merely been diluted out by continued protein synthesis (Strandberg & Wilson, 1968; Drozd *et al.* 1972; Kleiner, 1974). Since, moreover, this has been noted even when the enzyme was assayed in cell-free extracts (Strandberg & Wilson, 1968), inactivation would appear to have been irreversible.

Daesch & Mortenson (1972) observed that in continuous cultures of *C. pasteurianum* derepressed under Ar, change in the gas phase to N_2 brought about a rapid loss in nitrogenase activity. However, they found no inactivation of activity by addition of NH_4^+ to the assay medium at a concentration of 50 μM .

The results (Fig. 7) show that cultures of *R. rubrum* excreted NH_4^+ towards the end of exponential growth. Maximum concentrations appeared in the medium after the levels of nitrogenase had begun to decline. This was presumably a reflection of the increasing accumulation within the cell of physiologically-deleterious levels of NH_4^+ . This interpretation is supported by the corresponding increase in the sensitivity of the N_2 -fixing system towards

inactivation by NH_4^+ throughout growth. By the beginning of the stationary phase, the activity had been sufficiently inactivated for excessively high concentrations of NH_4^+ no longer to be produced, and part of the NH_4^+ excreted earlier had been utilized for growth.

This rapid and apparently reversible inactivation of nitrogen fixation provides the cell with a sensitive and efficient method for controlling the levels of NH_4^+ produced. In cultures with high levels of nitrogenase, such as those approaching the end of exponential growth or those in which enzyme synthesis has been strongly derepressed under Ar, this inactivation prevents the synthesis of excessively high levels of NH_4^+ with the consequent depletion of the pools of glutamate and 2-ketoglutarate.

It has been well established in the Enterobacteriaceae that glutamine synthetase is reversibly inactivated by NH_4^+ (Gancedo & Holzer, 1968) and in *Klebsiella* that glutamine synthetase is the regulator both of nitrogenase synthesis (Streicher *et al.* 1974; Tubb, 1974) and of the enzymes involved in the synthesis of glutamate (Magasanik *et al.* 1974). The ammonium ion therefore occupies a central position in regulating not only the synthesis and functioning of nitrogenase but also the overall nitrogen metabolism of the cell.

Inactivation by NH_4^+ of other enzymes of nitrogen metabolism has also been observed. The rapid and reversible inactivation of glutamine synthetase by NH_4^+ in enteric bacteria differs from inactivation of nitrogenase in *R. rubrum*, in occurring both in intact cells and in cell-free extracts (see Holzer & Duntze, 1971). Although inactivation of nitrate reductase in eukaryotic cells seems to occur only in intact cells, in *Neurospora crassa*, at least, it is accompanied by destruction of the enzyme (Sorger, Debanne & Davies, 1974).

We have no data on the mechanism of inactivation of nitrogenase in *R. rubrum*, and the extreme sensitivity of the enzyme towards inactivation by oxygen makes technically inaccessible the kind of experiments which have been used in elucidating the mechanism of inactivation in other enzyme systems. Nevertheless our results clearly show that in intact cells nitrogen fixation is rapidly and apparently reversibly inactivated by low concentrations of NH_4^+ , L-glutamine and L-asparagine.

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