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Evidence for an Involvement of Glutamine Synthetase in Regulation of Nitrogenase Activity in *Rhodopseudomonas capsulata*

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Abstract. In the present studies with whole cells and extracts of the photosynthetic bacterium *Rhodopseudomonas capsulata* the rapid inhibition of nitrogenase dependent activities (i.e. N_2-fixation acetylene reduction, or photoproduction of H_2) by ammonia was investigated. The results suggest, that the regulation of the nitrogenase activity by NH_4^+ in *R. capsulata* is mediated by glutamine synthetase (GS). (i) The glutamate analogue methionine sulfoximine (MSX) inhibited GS in situ and in vitro, and simultaneously prevented nitrogenase activity in vivo. (ii) When added to growing cultures ammonia caused rapid adenylylation of GS whereas MSX abolished the activity of both the adenylylated and unadenylylated form of the enzyme. (iii) Recommencement of H_2 production due to an exhaustion of ammonia coincided with the deadenylylation of GS. (iv) In extracts, the nitrogenase was found to be inactive only when NH_4^+ or MSX were added to intact cells. Subsequently the cells had to be treated with cetyltrimethylammonium bromide (CTAB). (v) In extracts the nitrogenase activity declined linearily with an increase of the ration of adenylylated *vs.* deadenylylated GS. A mechanism for inhibition of nitrogenase activity by ammonia and MSX is discussed.

Key words: *Rhodopseudomonas capsulata* – Nitrogenase activity – Glutamine synthetase regulation – Ammonia – Methionine sulfoximine.

In diazotrophic microorganisms generally the synthesis of nitrogenase is repressed when nitrogen is provided by ammonium salts (Gordon et al., 1976; Brill, 1975). The mechanism of this repression has intensively been studied in *Klebsiella* by Magasanik and coworkers (Magasanik et al., 1974; Streicher et al., 1974). The current model assumes that the deadenylylated form of GS acts directly as a positive effector for transcription of various enzymes involved in nitrogen metabolism, including nitrogenase (Shanmugam and Valentine, 1975; Tyler et al., 1974). The deadenylylated GS represents the catalytically active and the adenylylated GS the inactive form of the enzyme. This concept has been extended to the regulation of nitrogenase synthesis in other N_2 fixing bacterial systems (Weare and Shanmugam, 1976; Gauthier and Elmerich, 1977; Kondorosi et al., 1977). In *Rhodopseudomonas capsulata* the control of GS by adenylylation and deadenylylation has recently been confirmed by Johansson and Gest (1977), and its participation in regulation of nitrogenase synthesis is suggested.

Only in photosynthetic bacteria ammonia causes in addition to repression of nitrogenase synthesis a rapid, complete, and reversible inhibition of the nitrogenase catalyzed activities of N_2 fixation (Schick, 1971), acetylene reduction (Zumft and Castillo, 1978), and photoproduction of H_2 (Hillmer and Gest, 1977). This short term variation can be demonstrated in intact cells not, however, in cell extracts. Therefore, a mechanism was considered that required the structural coherence of the cell and a factor that mediated the regulation of the nitrogenase activity by ammonia.

The experiments with the non sulfur purple bacterium *R. capsulata*, described in this communication, are consistent with GS being involved as a mediating factor. The results suggest that nitrogenase activity is under negative control of the adenylylated form or GS.

Materials and Methods

Organisms and Growth Conditions. Rhodopseudomonas capsulata strain B 10 has been described by Weaver et al. (1975). Cells were

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Abbreviations. BSA = bovin serum albumine; CTAB = cetyltrimethylammonium bromide; GOGAT = L-glutamine: 2-oxoglutarate amino transferase; GS = glutamine synthetase; HEPES = N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid; MSX = L-methionine- D,L-sulfoximine

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grown photosynthetically with lactate and glutamate (Hillmer and Gest, 1977) in a mineral medium, designated as RCVB and specified by Johansson and Gest (1977). When N_2 was the source of nitrogen cultures were continuously sparged with 10% CO_2 in N_2.

Resting Cell Suspension. Preparation of resting cells was carried out as described by Hillmer and Gest (1977).

Cell-Free Extracts. Cell-free extracts were obtained by passage through a French pressure cell following the procedure of Johansson of Gest (1977). For determination of nitrogenase activity in cell extracts, cells were washed and resuspended in 60 mM HEPES-buffer (pH 7.0) instead of imidazol-buffer containing 1 mM $MnCl_2$ and 5 mM $Na_2S_2O_4$.

Photoproduction of H_2. The activity was followed manometrically as described by Hillmer and Gest (1977).

Glutamine Synthetase Assays. The activity was determined by the γ-glutamyltransferase assay according to Bender et al. (1977) or by the biosynthetic assay as described by Shapiro and Stadtman (1970). In the transferase assay the pH was 7.25 i.e. the isoactivity point of GS in *R. capsulata* under these conditions. The isoactivity point was determined with whole cells (Bender et al., 1977). Extract or CTAB-treated cells were preincubated in the reaction mixture at 34° C for 5 min before the reaction was started. In the transferase assay the adenylylated enzyme is inhibited by Mg^{2+} and the total activity is measured with Mn^{2+}. The relative state of adenylylation is expressed by the activity ratio $+Mg^{2+}/-Mg^{2+}$. A value of 0 means complete, a value of 1 no adenylylation.

Nitrogenase Assays. Acetylene reduction activity in whole cells was determined in 21 ml flasks as described by Hillmer and Gest (1977). The reaction mixture for measuring C_2H_2 reduction activity in cell-free preparations contained in a final volume of 2 ml, 20 mM HEPES (pH 7.0), 4 mM ATP, 10 mM creatine phosphate, 0.1 mg creatine phosphokinase (EC 2.7.3.2.), 5 mM $Na_2S_2O_4$, 10 mM $MgCl_2$ and 0.2 mM $MnCl_2$. The reaction was started with cell extract and terminated with 0.2 ml of 20% trichloroacetic acid. Other conditions were identical to that of the whole cell assay.

Analytical Methods. Protein was estimated by the biuret procedure after repeated extraction with 5 ml acetone (Schmidt et al., 1963). BSA was used as a standard.

Ammonia was determined by modification of a method described by Gutmann and Bergmeyer (1974). 2.5 ml of a solution of 0.106 M phenol and 0.17 mM Na-nitroprussid and 2.5 ml of a solution of 11 mM Na-hypochlorite and 0.125 M NaOH were added to 0.5 ml of an ammonia containing sample. The solutions were thoroughly mixed and incubated for 20 min at 30° C. The extinction was determined at 540 nm and the ammonia concentration was established by comparison with appropriate standards.

Chemicals. All chemicals were reagent grade and commercially available.

Results

Inhibition of Photoproduction of H_2 and Acetylene Reduction by MSX

In resting cells of *Rhodopseudomonas capsulata* derived from lactate-glutamate medium, nitrogenase dependent photoproduction of H_2 is rapidly inhibited by low concentrations of ammonia (Hillmer and Gest, 1977). In Fig. 1 it is shown that in such cells H_2 production and acetylene reduction were also sensitive towards the glutamate analogue MSX. At 300 μM and 70 μM, respectively, the activities were completely inhibited.

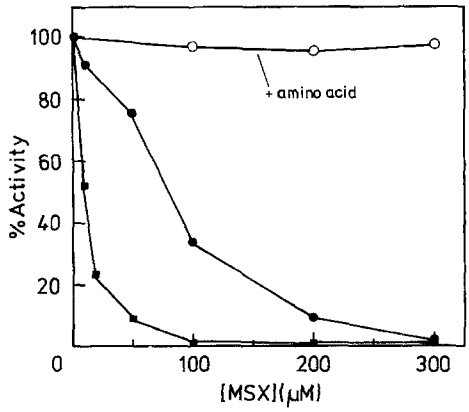

Fig. 1. Inhibition of photoproduction of H_2 and acetylene reduction by MSX in resting cells of *Rhodopseudomonas capsulata*. Cells were grown photosynthetically in lactate-glutamate medium. H_2 production assays contained 2.5 mg dry wt of cells, 100% activity = 120 μl $H_2 \cdot h^{-1} \cdot$ mg dry wt^{-1}; C_2H_2 reduction assays contained 1.5 mg dry wt of cells, 100% activity = 1.5 μmol $C_2H_4 \cdot h^{-1} \cdot$ mg dry wt^{-1}. MSX as indicated. (●), H_2 production; (■), C_2H_2 reduction; (○), both assays supplemented in addition with 7 mM L-glutamate, L-alanine, L-threonine, or L-histidine, respectively

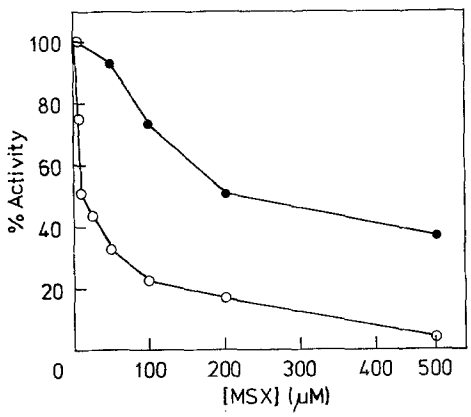

Fig. 2. Inhibition of GS by MSX in extracts of *Rhodopseudomonas capsulata*. Biosynthetic activity was assayed in crude extracts of cells grown in lactate-glutamate medium. (○), 20 μl of extract and MSX were incubated in the assay mixture of which glutamate was omitted; after 10 min the reaction was started with glutamate. (●), 20 μl of extract and glutamate were incubated for 10 min in the reaction mixture of which NH_4Cl was omitted. After 10 min MSX was added and incubation continued for 5 min before the reaction was started with NH_4Cl; controls without NH_4Cl were included. 100% activity = 0.13 μmol P_i released $\cdot$ 10 min^{-1}. Further details are given under „Materials and Methods"

When, however, amino acids, MSX, and cells were contained together in the assays, the activities were increased hyperbolically with increasing concentrations of amino acids. At 5 mM inhibition was completely prevented (Fig. 1). This indicated an unspecific antagonistic relationship between amino acids and the analogue.

Inhibition of GS by MSX in Vitro

Meister and coworkers demonstrated that MSX is tightly bound to GS of sheep brain (Ronzio et al., 1969) and of *Escherichia coli* (Weisbrod and Meister, 1973) and causes irreversible inhibition of enzyme activity. In cell extracts of *R. capsulata* the biosynthetic and the transferase activity of GS were inactivated by MSX with 50% activity remaining at 20 μM in either assays (Fig. 2, results with the transferase activity are not shown). Inhibition was substantially reduced when the extract was preincubated with glutamate as has been reported for the enzymes from sheep brain and *E. coli*. In vitro the nitrogenase activity was, in contrast to GS, not affected by even 5 mM MSX; 14 mM NH_4^+, 7 mM glutamate, or glutamine did not alter the activity either. The regulatory response of nitrogenase was lost simultaneously with the structural integrity in extracts. Therefore, a direct regulation of the enzyme could be excluded and an indirect mechanism with GS as a mediating factor was envisaged.

Effect of NH_4^+ and MSX on Activity and Adenylylation State of GS and on Nitrogenase Activity

In order to elucidate a regulatory connection between GS and nitrogenase activity, experiments were designed to follow changes in activity and adenylylation state of GS. Cultures growing with glutamate or N_2 as the nitrogen source were pulsed with ammonia or MSX and at appropriate intervals the cells were exposed to CTAB (Figs. 3 and 4). Bender et al. (1977) introduced CTAB as a reagent that prevents alteration of the adenylylation state of GS during harvesting of cells of *Klebsiella aerogenes*. Johansson and Gest (1977) confirmed the stabilizing effect in their experiments with GS of *R. capsulata*. Addition of 7 mM NH_4^+ led to a drastic decrease of the Mg^{2+} dependent activity while the Mn^{2+} dependent activity, i.e. the total activity of GS, was inhibited only slightly (Fig. 3). The transferase activity of the adenylylated GS in *E. coli* (Stadtman et al., 1970) is inhibited by Mg^{2+} and, consequently, these findings indicate rapid adenylylation of GS by NH_4^+. The same effect of NH_4^+ on GS activity has been shown in *E. coli* by Schutt and Holzer (1972). The slow decrease of the total activity may indicate repression of GS by NH_4^+.

In contrast to NH_4^+, MSX caused an immediate, parallel loss of both the Mg^{2+} and the Mn^{2+} stimulated activities (Fig. 4), thereby leaving the adenylylation state unaltered. Incubation of the cells with 1 μM MSX for 1 min reduced the activity by 80–90%. In vitro the same decrease of activity was achieved only with much higher concentratations of MSX (Fig. 2). Inhibition by MSX occurred regardless, if CTAB was added or not, possibly because MSX is tightly bound to GS.

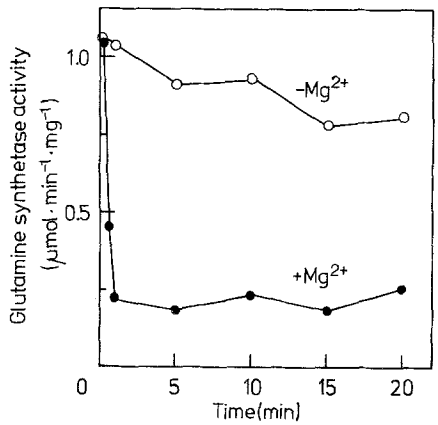

Fig. 3. Effect of ammonia on GS activity in growing cells of *Rhodopseudomonas capsulata*. Cells were grown photosynthetically with lactate-glutamate or lactate-N_2. At culture densities of $E_{660\ nm}$ = 0.3–0.5 cells were exposed to 15 mM NH_4^+ for the time periods indicated, supplemented with 0.1 mg CTAB/ml for 1 min and collected by centrifugation. Transferase activity of GS was determined in whole cells in the presence and absence of Mg^{2+}

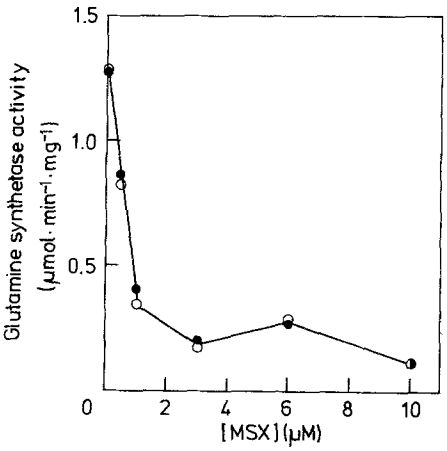

Fig. 4. Effect of MSX on GS activity in growing cultures of *Rhodopseudomonas capsulata*. Cells were grown as described in Fig. 3. At culture densities of $E_{660\ nm}$ = 0.3–0.5 cells were exposed for 1 min to concentrations of MSX as indicated. Extracts were prepared and transferase activity was determined in the presence (●) and absence (○) of Mg^{2+}. Treatment with CTAB did not alter the results

In extracts nitrogenase activity was not affected when NH_4^+ or MSX were included in the assay mixture. When, alternatively, the effectors were added to growing cells which then were supplemented with CTAB, in extracts only a small fraction of nitrogenase activity was detected (Table 1). Accordingly, an intact structure is required to trigger inhibition of the nitrogenase. CTAB seems to conserve protein conformations, so inhibition can be seen in extracts.

Correlation of Reactivation of Nitrogenase Activity and Deadenylylation of GS in R. capsulata

In the experiment depicted in Fig. 5 the process of reactivation of H_2 production was investigated in intact

Table 1. Demonstration of inhibition of nitrogenase activity by NH_4^+ and MSX in cell extracts when the effectors were added to growing cells

Additions	% Activity
None	100
+ CTAB	105
+ NH_4^+	90
+ NH_4^+ + CTAB	13
+ MSX	114
+ MSX + CTAB	15

Cells growing in lactate-N_2 medium were exposed to 7 mM NH_4^+, 0.5 mM MSX or 0.1 mg CTAB · ml^{-1}, respectively, for 1 min before they were harvested. Where indicated, 1 min after addition of NH_4^+ or MSX, cultures were supplemented with CTAB for 1 min. Assays contained 2–6 mg protein. 100% activity = 8–15 nmol C_2H_4 · min^{-1} · mg $protein^{-1}$. Nitrogenase activity was determined as detailed in "Materials and Methods"

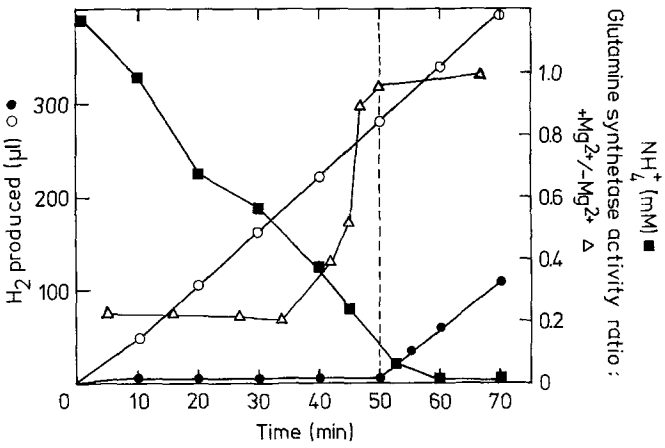

Fig. 5. Kinetics of H_2 production, NH_4^+ consumption, and rel. adenylylation of GS in intact cells of *Rhodopseudomonas capsulata*. A series of identical Warburg vessels contained each 30 mM lactate, 1.2 mM NH_4Cl and 2.5 mg dry wt of cells grown in lactate-glutamate medium (●). In a control vessel NH_4Cl was omitted (○). H_2 production was followed and at intervals 0.25 mg CTAB was added and two vessels each were sacrificed for determination of NH_4^+ concentration and of transferase activity with and without Mg^{2+}. The vertical dashed line relates the beginning of H_2 production to the other parameters

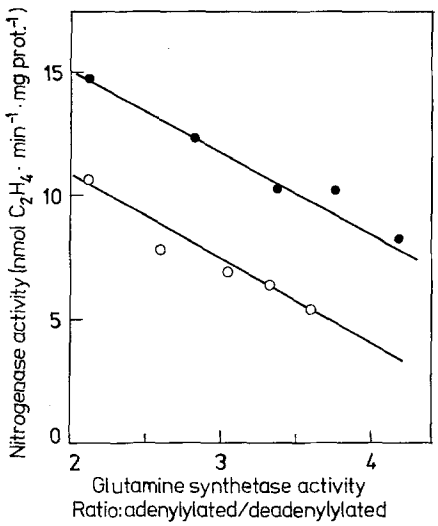

Fig. 6. Dependence of nitrogenase activity on the ratio of adenylylated *vs.* deadenylylated GS in extracts of *Rhodopseudomonas capsulata*. Extracts were prepared from lactate-glutamate and lactate-ammonia grown cells (Glu extract and NH_4^+ extract, respectively). Nitrogenase assays contained 3.3 mg (●) and 4.9 mg (○) protein, respectively, of Glu extract, and increasing amounts of NH_4^+ extract (1.4–5.8 mg protein). Relative adenylylation values for GS in the respective extracts were 0.32 and 0.12. The amount of adenylylated and deadenylylated GS was calculated. Nitrogenase and GS activity were determined as described under "Materials and Methods"

cells. In the lactate-glutamate grown cells which have the capacity for H_2 production (note the activity in the control where NH_4^+ was omitted) initially nitrogenase was inhibited. As the ammonia concentration decreased with a rate of 50 nmol · min^{-1}, at *ca.* 0.4 mM the relative adenylylation value started to increase and a few minutes later after deadenylylation was complete (at *ca.* 0.1 mM NH_4^+) H_2 production resumed with the same rate as in the control vessel. 0.1 mM NH_4^+ can be considered the lowest concentration that inhibits nitrogenase activity in *R. capsulata*. It is evident from these and the data shown in Fig. 3, that the state of adenylylation of GS represents a signal that turns nitrogenase activity on and off in reverse relation to the availability of ammonia.

Effect of Extract from NH_4^+ Grown Cells on the Nitrogenase Activity in Vitro

Extracts from cells of *R. capsulata* grown with glutamate as the nitrogen source (Glu extract) contain active nitrogenase and partly deadenylylated GS. In contrast, extracts from cells grown with NH_4^+ (NH_4^+ extract) are devoid of nitrogenase and GS is highly adenylylated (Johansson and Gest, 1977; Hillmer and Gest, 1977). In Fig. 6 it is shown that the nitrogenase activity in Glu extracts decreased linearily with an increase of the ratio of the amount of adenylylated *vs.* deadenylylated GS. No alteration of the activity occurred, however, when equivalent amounts of BSA or NH_4^+ extract inactivated by heat was added. In NH_4^+ extract no nitrogenase activity was found. These results provide further evidence that the nitrogenase activity is under negative control of adenylylated GS.

Discussion

In purple bacteria the nitrogenase is rapidly inhibited by low concentrations of ammonia and immediately reactivated once ammonia is utilized. The data presented in the present paper contribute to the under-

standing of the hitherto unknown mechanism of this unique regulatory phenomenon.

Attempts to demonstrate a direct inhibition of the nitrogenase activity by ammonia in cell extracts were unsuccessful in *Rhodospirillum rubrum* (Bulen et al., 1965; Schneider et al., 1960) and also in our investigations with *Rhodopseudomonas capsulata.*

The first indication for an involvement of GS as a mediator of this regulation came from the finding that in growing cells not only GS was inhibited by MSX but also the nitrogenase activity (i.e. acetylene reduction, photoproduction of H_2). The glutamate analogue MSX is, according to Ronzio et al. (1969) and Weisbrod and Meister (1973), as a potent inhibitor of GS irreversibly bound to the enzyme.

From experiments with NH_4^+, the accessory natural inhibitor of the nitrogenase, a regulatory connection between GS and nitrogenase is indicated by three lines of evidence. (i) Inhibition of the nitrogenase activity by ammonia was accompanied by an immediate adenylylation of GS. (ii) Reactivation coincided with a rapid deadenylylation of GS. (iii) Nitrogenase activity in crude extracts was lowered when the amount of adenylylated GS was increased. Preliminary data by Wall and Gest (1978) with glutamine auxotrophs of *R. capsulata* are in agreement with our conclusion since the glutamine requiring mutants had only negligible GS activity and H_2 production was no longer inhibited by NH_4^+. Accordingly, GS seems to be the target enzyme which binds NH_4^+ and MSX. As a consequence the enzyme becomes adenylylated or inactivated, respectively, and these variations are likely to coincide with a conformational alteration of GS (Weisbrod and Meister, 1973). If the nitrogenase in vivo is neighbouring the GS or is even loosely attached to it, the conformational change could be transferred to the nitrogenase, and bring about its inactivation.

We empirically found a method to demonstrate inhibition of the nitrogenase by NH_4^+ and MSX in extracts, i.e., the inhibitors have to be added to growing cells, followed by exposure to CTAB. In extracts of cells treated in that way only little nitrogenase activity is found. The function of CTAB in this connection is obscure, but it may consist in stabilization of protein structures.

Recently, Ludden and Burris (1976) and Nordlund et al. (1977) reported about a membrane component in *R. rubrum* that stimulates the activity of the Fe-protein of the nitrogenase. Under physiological conditions its function is assumed to connect the nitrogenase with membranes. Evidence for such a factor has also been found in *Rhodopseudomonas palustris* by Zumft and Castillo (1978). In *R. capsulata* its presence has not been reported. If it is existing, a role in the regulation of nitrogenase activity has to be considered.

It will be necessary to elaborate a more defined in vitro system to get a closer insight into this rather complex regulation, particularly, when one assumes a connection between the mechanisms of inhibition and repression by NH_4^+.

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