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Regulation of Nitrogenase A and R Concentrations in *Rhodopseudomonas capsulata* by Glutamine Synthetase

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Nitrogen-starved purple non-sulphur bacteria have an active unregulated form of nitrogenase (nitrogenase A); however, the nitrogenase of a glutamine synthetase-negative mutant of *Rhodopseudomonas capsulata*, when nitrogen-starved, was predominantly inactive and required activation by Mn^{2+} and activating-factor protein. This regulatory form of nitrogenase has been called nitrogenase R. Treatment of wild-type cells (containing nitrogenase A) with methionine sulphoximine, an inhibitor of glutamine synthetase, converted the enzyme into nitrogenase R. Glutamine synthetase thus appears to control the intracellular concentrations of nitrogenase A and R and in this way regulates nitrogenase activity in the photosynthetic bacterium.

Nitrogenase in the phototrophs *Rhodospirillum rubrum* and *Rhodopseudomonas capsulata* exists in two enzymic forms (Carithers *et al.*, 1979; Yoch, 1979*a*). Nitrogenase A, found only in N-starved cells, is identical with that of the other N_2-fixers and can be converted into a second low-molecular-weight form, nitrogenase R, by the addition of either NH_4^+ or glutamate to an N-starved culture. Nitrogenase R (whose Fe protein is inactive) can be distinguished from nitrogenase A *in vitro* by its need for activation by an Mn^{2+}-(and ATP)-dependent activating-factor protein (Ludden & Burris, 1976, 1978; Nordlund *et al.*, 1977). The rapid conversion of nitrogenase A into nitrogenase R in response to N_2, amino acids and NH_4^+ represents a new dimension in the regulation of nitrogenase activity (Carithers *et al.*, 1979; Yoch, 1979*a*). Isolation of an *R. capsulata* glutamine auxotroph deficient in glutamine synthetase activity (Wall & Gest, 1979) provided an opportunity to determine if this enzyme had a role in nitrogenase A $\leftrightharpoons$ nitrogenase R conversions. Evidence from work with this mutant, together with effects of methionine sulphoximine on wild-type cells, strongly suggests that glutamine synthetase is involved in converting nitrogenase A into nitrogenase R and therefore involved in regulating nitrogenase activity in these photosynthetic bacteria.

Materials and Methods

Organism and growth conditions

Rhodopseudomonas capsulata strain G29 (a glutamine auxotroph) and the wild-type strain B10 were kindly provided by Dr. Judy Wall, Department of Biochemistry, University of Missouri, Columbia, MO, U.S.A. Both *R. capsulata* and *R. rubrum* were grown photosynthetically in a medium described by Ormerod *et al.* (1961), which was modified by deleting the glutamate and replacing biotin with thiamin (300 μg/litre) for growth of *R. capsulata*. Growth of the auxotroph (strain G29) further required that the NH_4Cl be replaced with glutamine. Wild-type cells (which contain nitrogenase A) were obtained by growing them on low NH_4^+ (2.5 mM) to ensure eventual N-starvation, which is identified by the vigorous photoevolution of H_2 from the culture. N-starvation of the auxotroph (strain G29) was achieved by growing it on 1.5 mM-glutamine. To obtain wild-type cells that contain nitrogenase R, glutamate (0.75 mM) was added to N-starved cultures, which could then be harvested at any time from 6 to 24 h later, and the nitrogenase activity was predominantly Mn^{2+}-dependent (indicative of nitrogenase R). Similar results are obtained by adding NH_4^+ to the culture.

The glutamine auxotroph (strain G29) was induced to revert to prototrophy by replacing glutamine in the medium with NH_4^+, which allowed the naturally occurring revertants to grow. Although initial growth was slow, it increased on subsequent transfers; after three such transfers the prototroph-enriched culture was plated on NH_4^+-containing medium and several isolates were selected. One G29 revertant isolate (G29-R1), which grew well on NH_4^+, was selected and used for the nitrogenase experiments.

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Preparation of nitrogenase extracts and activating factor

Cells were harvested and disrupted by sonic oscillation by using the method previously described for *R. rubrum* (Yoch, 1979*a*). The nitrogenase-containing extracts were the supernatant fluid resulting from centrifugation for 10 min at 30000 *g* (Table 1 and Fig. 2 below). An additional centrifugation for 60 min at 250000 *g* removed the chromatophores (and activating factor); this extract was used to determine the activating-factor requirement of *R. capsulata* nitrogenase (Fig. 1 below). Activating factor from *R. rubrum* was prepared as described by Ludden & Burris (1976). Chromatophores (30 ml) were washed once with 0.5 M-NaCl and the washings treated with poly(ethylene glycol) 4000. The protein precipitating from these washings between 10 and 30% (w/v) poly(ethylene glycol) was used as the activating-factor preparation.

Nitrogenase assays

Crude extracts containing either nitrogenase A or nitrogenase R were assayed for nitrogenase activity (with the acetylene technique) by methods previously described for *R. rubrum* (Yoch, 1979*a*). The basic nitrogenase reaction mixture contained, in a total volume of 1.5 ml: 50 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer, pH 7.4, 25 mM-phosphocreatine, 20 μg of creatine kinase, 15 mM-$MgCl_2$, 2.9 mM-ATP, 8 mM-sodium dithionite ($Na_2S_2O_4$) (33 mM-$Na_2S_2O_4$ was used in *R. rubrum* nitrogenase assays). This reaction mixture (non-activating) is complete for nitrogenase A; to assay for nitrogenase R (i.e., activating conditions) 0.5 mM-$MnCl_2$ and activating factor are also required. Previous work with *R. rubrum* (Carithers *et al.*, 1979; Yoch, 1979*a*) showed that nitrogenase R can be distinguished from nitrogenase A in crude extracts (which contain chromatophores and therefore bound activating factor) by the absolute requirement of nitrogenase R for Mn^{2+}. The percentage of nitrogenase A and nitrogenase R in any extract can be determined by assaying for nitrogenase in the absence (nitrogenase A) and presence (nitrogenase A plus nitrogenase R) of added Mn^{2+}.

In the absence of added Mn^{2+}, the endogenous Mn^{2+} in the crude extract is probably insufficient to allow expression of any nitrogenase R. This assumption is based on the fact that the K_m for Mn^{2+} in the nitrogenase-R-activation process is 0.2 mM (Yoch, 1979*a*), and crude extracts used here contain less than one-tenth this concentration of Mn^{2+}.

Results

Previous kinetic data suggested that the nitrogenase of *R. capsulata* (strain B10) was similar to that of *R. rubrum*, because Mn^{2+} greatly stimulated the nitrogenase obtained from cells grown on glutamate (Yoch, 1979*a*). The presence of nitrogenase R in *R. capsulata* is confirmed here by showing its requirement for activating factor and Mn^{2+}. The nitrogenase activity with saturating amounts of activating factor prepared from *R. rubrum* is shown in Fig. 1. The nitrogenase activity is approximately twice that attained with endogenous (chromatophore-bound) activating factor. The Mn^{2+}-dependence of this system is shown in Fig. 1(*b*).

To determine if active glutamine synthetase was essential for the control of intracellular amounts of nitrogenase A and nitrogenase R, as suggested from previous observations of *R. rubrum* (Yoch, 1979*b*), a glutamine synthetase-negative mutant of *R. capsulata* (strain G29) was cultured photosynthetically on low concentrations of glutamine and allowed to reach a state of N-starvation. Under these conditions the wild-type *R. capsulata* and *R. rubrum* would produce almost 100% nitrogenase A. As Table 1 shows, the nitrogenase of the *R. capsulata* glutamine auxotroph (G29), was predominantly (65%) in the R-form. When wild-type cells (strain B10 were grown on either glutamine or NH_4^+, N-starvation resulted in the production of a nitrogenase whose activity could not be enhanced by Mn^{2+} (i.e. nitrogenase A). This result indicates that glutamine itself played no part in the auxotroph's production of nitrogenase R under these conditions. A revertant of strain G29 (strain G29-R1) having

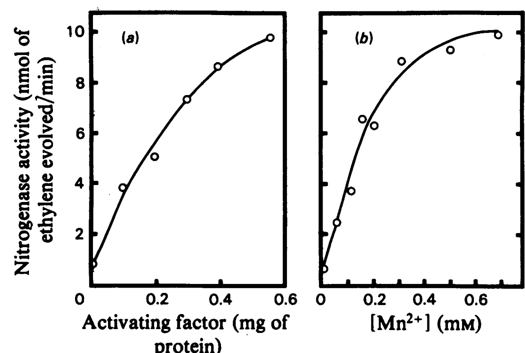

Fig. 1. *Requirement of Rhodopseudomonas capsulata (B10) nitrogenase R for activating factor and* Mn^{2+}
(*a*) The basic reaction mixture (see the Materials and Methods section) was supplemented with 0.5 mM-Mn^{2+} and the concentration of *R. rubrum* activating factor indicated on the Figure. *R. capsulata* nitrogenase (1.02 mg of protein) was supplied as a chromatophore-free extract. (*b*) The basic reaction mixture was supplemented with activating factor (0.4 mg of protein) and Mn^{2+} at the concentration indicated.

Table 1. *Characterization of nitrogenase from the R. capsulata glutamine synthetase-negative mutant (strain G29)*
The nitrogenase activity is expressed as nmol of ethylene/min per mg of protein; the initial velocity was determined by averaging the activity over a 20-min period. Assay methods are described in the Materials and Methods section.

Strain	Nitrogen source before N starvation	Nitrogenase activity: Activating conditions	Nitrogenase activity: Non-activating conditions	Nitrogenase R (% of total nitrogenase)
R. capsulata G29 (glutamine auxotroph)	Glutamine	13.1	4.5	65.5
R. capsulata B10 (wild-type)	Glutamine	13.9	13.7	1.6
	NH_4^+	13.5	15.7	0
R. capsulata G29-R1 (revertant)	Glutamine	9.0	9.3	0

normal glutamine synthetase activity was again identical with the wild-type in that it had no nitrogenase R activity under these conditions. Thus glutamine synthetase is implicated in controlling the concentrations of nitrogenase A and R in the purple non-sulphur bacteria.

Additional evidence that glutamine synthetase is involved in the control of nitrogenase A and nitrogenase R comes from experiments in which illuminated N-starved wild-type *R. capsulata* cultures ($A_{660} \simeq 0.3$) were treated for 18h with methionine sulphoximine, an inhibitor of glutamine synthetase. The addition of 15mM-methionine sulphoximine directly to the N-starved culture resulted in cells whose nitrogenase activity in extracts could be stimulated by the addition of Mn^{2+} and *R. rubrum* activating factor (Fig. 2). Thus inhibition of glutamine synthetase activity by methionine sulphoximine results in the cells converting nitrogenase A into nitrogenase R. Control extracts derived from untreated N-starved cells showed nitrogenase kinetics (in the absence of Mn^{2+}) indicative of nitrogenase A (Fig. 2). Similar results were obtained with *R. rubrum* (Yoch, 1979*b*).

Although nitrogenase A in these photosynthetic bacteria was converted into nitrogenase R by adding methionine sulphoximine to N-starved cultures, considerable variation was noted in the results. This variation ranged from observing no effect (i.e., Mn^{2+} added to crude extracts had no stimulating effect on nitrogenase) to cases where activity in the presence of Mn^{2+} was three times that in its absence. Neither the concentration of methionine sulphoximine used (5–15mM) nor the time of exposure (0.5–24h) added to the reproducibility of this process. The variability in the amount of nitrogenase R produced by methionine might be related to previous observations that inhibition of whole-cell nitrogenase activity by this inhibitor required an actively growing culture (Hillmer & Fahlbush, 1979). In the experiments reported here, N-starved and therefore non-growing cultures had, by necessity, to be used in order to have cells that contained nitrogenase A. The fact that N-starved cells are not growing may, therefore, explain the variation observed in the concentration of nitrogenase R produced in response to this inhibitor. In conclusion, it appears that

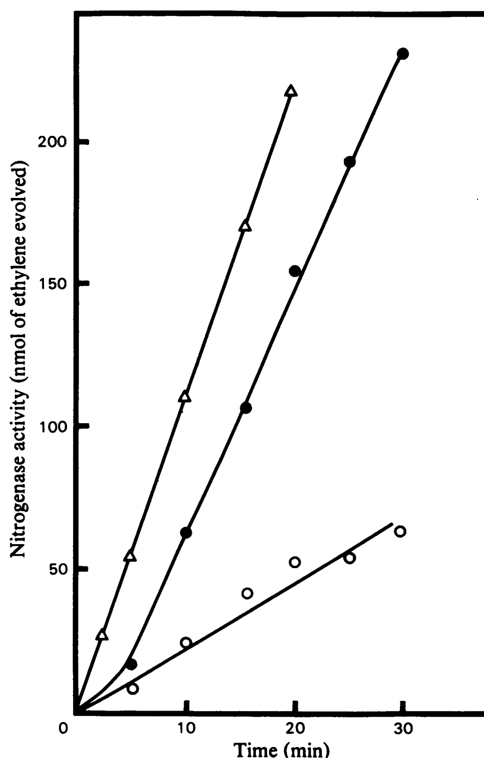

Fig. 2. *Requirement of R. capsulata (B10) nitrogenase for Mn^{2+} after treatment of the cells with methionine sulphoximine*
The basic nitrogenase assay mixture is described in the Materials and Methods section. The crude nitrogenase extract from the methionine sulphoximine-treated cells was supplemented with activating factor (0.3mg of protein) and assayed with (●) and without (○) Mn^{2+}. The nitrogenase extract from the cells not treated with methionine sulphoximine (control) was assayed without Mn^{2+} (△).

inhibition of glutamine synthetase *in vivo* causes (or permits) the conversion of a substantial amount of nitrogenase A into the R-form.

Discussion

The inhibition in the purple non-sulphur bacteria of both glutamine synthetase (Weare & Shanmugam, 1976; Johnasson & Gest, 1977) and nitrogenase activity (Gest *et al.*, 1950; Schick, 1971; Neilson & Nordlund, 1975; Hillmer & Gest, 1977) by NH_4^+ has suggested that a regulatory relationship might exist between these two enzymes. The strong correlation between NH_4^+-induced glutamine synthetase adenylylation (inactivation) and nitrogenase inhibition in *R. capsulata* and the reversal of this pattern when NH_4^+ was depleted from the culture medium, prompted Hillmer & Fahlbush (1979) to postulate that the adenylylation state of glutamine synthetase acts as a signal in turning on and off the nitrogenase activity in response to NH_4^+. However, these workers also found that methionine sulphoximine inhibits nitrogenase activity, but unlike NH_4^+, it has no effect on the adenylylation state of glutamine synthetase. The latter observation, although strongly suggesting that glutamine synthetase plays a role in inhibiting nitrogenase activity, indicates that it must occur by a mechanism not related to the adenylylation state of glutamine synthetase.

In the present paper, evidence is provided that shows that nitrogenase R (the form of nitrogenase that is normally produced in response to an NH_4^+ shock) is present 'constitutively' in *R. capsulata* mutants that have little or no glutamine synthetase activity. Furthermore, nitrogenase R arises from nitrogenase A in cells treated with methionine sulphoximine. These two lines of evidence argue strongly that glutamine synthetase is involved in the control of the nitrogenase A $\rightleftharpoons$ nitrogenase R equilibrium.

The observation that cultural conditions such as the simultaneous starvation of cells for both N and C also resulted in the formation of nitrogenase R from nitrogenase A (D. C. Yoch, unpublished work) and this conversion, unlike that induced by NH_4^+, was not accomplished by a change in the adenylylation state of glutamine synthetase, further suggests that the correlation between nitrogenase activity and glutamine synthetase adenylylation state (Hillmer & Fahlbush, 1979) is coincidental. In conclusion, an active glutamine synthetase (the adenylylation state seems to be irrelevant) appears to control nitrogenase activity (nitrogenase A $\rightleftharpoons$ nitrogenase R) in the purple non-sulphur bacteria. This enzyme may regulate nitrogenase activity directly or it may act by controlling the concentration of some essential metabolite involved in this process.

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