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Overproduction of Nitrogenase by Nitrogen-Limited Cultures of *Rhodopseudomonas palustris*

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Rhodopseudomonas palustris cells grown on limiting nitrogen produced four- to eightfold higher nitrogenase specific activity relative to cells sparged with N_2. The high activity of N-limited cells was the result of overproduction of the nitrogenase proteins. This was shown by four independent techniques: (i) titration of the Mo-Fe protein in cell-free extracts with Fe protein from *Azotobacter vinelandii*; (ii) direct detection of the subunits of Mo-Fe protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis; (iii) monitoring of the electron paramagnetic resonance spectrum of Mo-Fe protein in whole cells; and (iv) immunological assay of the Fe protein level with an antiserum against the homologous protein of *Rhodospirillum rubrum*. The derepressed level of nitrogenase found in N_2-grown cells was not due to an increased turnover of nitrogenase. The apparent half-lives of nitrogenase in N_2-grown and N-limited cells were 58 and 98 h, respectively, but were too long to account for the difference in enzyme level. Half-lives were determined by measuring nitrogenase after repression of de novo synthesis by ammonia and subsequent release of nitrogenase switch-off by methionine sulfoximine. Observations were extended to *R. rubrum*, *Rhodopseudomonas capsulata*, and *Rhodomicrobium vannielii* and indicated that overproduction of nitrogenase under nitrogen limitation is not an exceptional property of *R. palustris*, but rather a general property of phototrophic bacteria.

Expression of the at least 17 genes (*nif*) for nitrogen fixation is linked to the control of the genes (*gln*) for glutamine synthetase (see reference 3 for review). Glutamine synthetase [L-glutamate:NH_3 ligase (ADP-forming); EC 6.3.1.2] and several other proteins are controlled by the availability of nitrogen in the growth medium, and their synthesis is increased under nitrogen-limiting conditions (2, 15). The linkage between the *nif* and *gln* genes involves interaction of a *glnR* product (8, 31) with the *nifALR* operon (10, 18). The *nifA* and *nifL* gene products, in turn, activate and repress, respectively, the synthesis of all other *nif* genes (see reference 14 for review). According to a model proposed by Buchanan-Wollaston et al. (4) and Kennedy et al. (14), the *glnR* gene product activates transcription of the *nifAL* operon. Whereas the *nifA* product activates transcription of all other *nif* genes, the repressor (*nifL* product) remains inactive through interaction with a regulatory metabolite, perhaps guanosine 3′,5′-bis(diphosphate). Upon addition of fixed nitrogen, the concentration of the regulatory metabolite is assumed to decrease and, as one mode of action, would allow the *nifL* product to become a repressor of *nif* transcription. Applying this model, one infers that under conditions of N limitation or severe N starvation, an increased level of the regulatory metabolite would lower the repressor level, thereby allowing continued transcription of the *nif* genes.

Although these views were elaborated with enteric bacteria, some of the more general phenomena allow a cautious analogy with members of the non-sulfur purple bacteria. Their ability to synthesize nitrogenase [reduced ferredoxin:dinitrogen oxidoreductase (ATP-hydrolyzing); EC 1.18.2.1] is universally repressed by ammonia (33); the glutamine synthetase inhibitor L-methionine *SR*-sulfoximine (MSX) allows derepression of nitrogenase in the presence of ammonia (30), and glutamine auxotrophic mutants synthesize active nitrogenase, albeit in the presence of ammonia (29). Some *nif* genes of the phototrophic bacteria might have the same arrangement as those found in *Klebsiella pneumoniae*; this was indicated for two structural genes of nitrogenase for which hybridization of *Rhodopseudomonas capsulata* DNA was shown with a cloned *Klebsiella* DNA fragment containing *nifDH* (23).

Previously, we have shown that N-limited

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cultures of *Rhodopseudomonas palustris* have severalfold higher levels of nitrogenase activity than cultures supplied with N_2 (1, 34). Those cultures also lost their ability to regulate nitrogenase activity in response to ammonia. In a preliminary report, a higher level of Mo-Fe protein was demonstrated in extracts from N-limited cultures relative to diazotrophically grown cells (35). In this work we show that the increased nitrogenase activity in N-limited cultures is due to overproduction of the nitrogenase proteins. Because of the ability of phototrophic bacteria to regulate nitrogenase activity by covalent modification of the Fe protein (34), specific probes had to be used to discriminate clearly between mere activation under N limitation and de novo synthesis of nitrogenase. The mechanism of nitrogenase overproduction was investigated, and observations were extended to *Rhodospirillum rubrum*, *R. capsulata*, and *Rhodomicrobium vannielii*, which also showed this phenomenon. Our findings are consistent with the current model of nitrogenase regulation in *Klebsiella* as outlined above.

MATERIALS AND METHODS

Cell growth. *R. palustris* (ATCC 17001), *R. capsulata* B10 (obtained from B. Marrs), *R. rubrum* (ATCC 11170), and *R. vannielii* (DSM 166, obtained from N. Pfennig) were grown photoheterotrophically on a basal medium for *Rhodospirillaceae* consisting of the following compounds (in g/liter): KH_2PO_4, 0.5; $MgSo_4 \cdot 7H_2O$, 0.4; NaCl, 0.4; $CaCl_2 \cdot 2H_2O$, 0.05; sodium malate, 4.0; and 1 ml of trace element solution SL8, which also incorporated iron and was a slightly modified version of a previously used solution, SL6 (21). Media were adjusted to pH 6.9 before autoclaving. Those for *R. palustris* and *R. vannielii* were supplemented with 0.5 g of yeast extract (E. Merck AG, Darmstadt, Germany) per liter; those for *R. capsulata* and *R. rubrum* were supplemented with 100 μg of thiamine-hydrochloride and 300 μg of biotin per liter. Glutamate or NH_4Cl was added as a nitrogen source where specified, in the concentrations indicated below.

Cells were grown in completely filled 1-liter Roux bottles which were loosely capped to allow H_2 to escape or were filled to within 100 ml when sparged with 95% N_2–5% CO_2 at 10 to 20 ml/min. Cultures were inoculated with 30 ml of an ammonia-grown culture. They were incubated up to 5 days in an illuminated water bath at 30 ± 1°C and an incident illuminance of 5,000 lx, which was supplied by a bank of 60-W tungsten bulbs.

C_2H_2 reduction assays. The nitrogenase activities of whole cells and cell-free extracts were determined by the C_2H_2 reduction technique. A sample of cell suspension was withdrawn anaerobically by syringe from the growth vessel and assayed without further manipulation by injecting 1 ml of the sample into a 13-ml stoppered vial containing a gas phase of 92% Ar and 8% C_2H_2. The vials were incubated at 30°C under continuous shaking and illumination with saturating light (10,000 lx). Activities of cell-free extracts were measured at 30°C in 9-ml vials under an atmosphere of 89% Ar and 11% C_2H_2. The reaction mixture (total volume, 0.5 ml), with a final pH of 7.0, was composed of 2.5 μmol of ATP, 5 μmol of Mg^{2+}, 15 μmol of creatine phosphate (Sigma Chemical Co., Munich, W. Germany), and 30 μg of creatine phosphokinase (Sigma), 25 μmol of TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid], and 10 μmol of $Na_2S_2O_4$. Gas samples of 50 μl were taken periodically and analyzed by gas chromatography for C_2H_4.

Protein assays. Whole cells were digested, and the protein content was estimated according to the method of Stickland (26), or a cell sample was incubated overnight at 30°C in 2 ml of 1 N NaOH and centrifuged, and the supernatant was assayed for protein, like cell-free extracts, by the Lowry procedure. Bovine serum albumin was used as a standard.

Cell-free extracts and nitrogenase components. Cells were harvested by centrifugation at 10,000 × *g* for 10 min. The sediment of a 1-liter culture was suspended in 10 ml of 50 mM TES, pH 7.5, containing 2 mM dithionite. Cells were broken by two passages through a French press (137 MPa). Cell debris and unbroken cells were removed by centrifugation (27,000 × *g*, 30 min), and the dark-red supernatant was used for experiments. All manipulations were done anaerobically at room temperature. Centrifugation of the 27,000 × *g* supernatant at 135,000 × *g* at 4°C for 90 min removed chromatophores and yielded the high-speed supernatant that was used for gel electrophoresis.

Nitrogenase of N-limited *R. palustris* was purified from high-speed supernatants by adsorbing it on DEAE-Sepharose. The enzyme was eluted by 0.5 M NaCl and separated into its two components by gel filtration. The Mo-Fe protein and Fe protein were purified separately to near homogeneity by ion-exchange chromatography and further gel filtration (W. G. Zumft, unpublished data).

Fe protein from *Azotobacter vinelandii* was purified according to a published method (25). The preparation was free of Mo-Fe protein, because, when assayed by itself, it showed no reduction of C_2H_2.

Double-diffusion immunoassay. The Ouchterlony method was used (11) with 1% agar slides (Agar Noble; Difco Laboratories, Detroit, Mich.) and 0.85% NaCl in 50 mM Tris-hydrochloride buffer, pH 8. Lyophilized rabbit antiserum against *R. rubrum* Fe protein was a gift from P. W. Ludden. It was dissolved in 10 mM phosphate buffer, pH 7.2, containing 0.85% NaCl and was partially purified by precipitating the immunoglobulins with $(NH_4)_2SO_4$ (37% saturation). The precipitate was collected by centrifugation and dissolved in a minimal volume of the above Tris buffer. Immunodiffusion was carried out for 30 h at room temperature. Afterward, the slides were washed for 24 h in 0.1% NaCl, followed by 6 h in water. They were then dried overnight at 30°C, stained with Coomassie brilliant blue, and destained as described previously (11).

EPR spectroscopy. Electron paramagnetic resonance (EPR) spectra were recorded at 15 K with an X-band Bruker B-ER 420 instrument. Approximately 0.8 ml of cell suspensions of N_2-grown and N-limited cells were injected into Ar-filled quartz tubes 4 mm in diameter. The tubes were centrifuged for 10 min at 900 × *g* to pack cells densely to approximately 3 cm in height at

the bottom of the tube. The supernatant was removed by syringe, and the samples were immediately frozen in liquid nitrogen.

RESULTS

Cell growth and development of nitrogenase activity in diazotrophic and N-limited cultures. Optimal growth conditions for *R. palustris* required yeast extract in the medium. When no additional N source was provided, growth was limited by the N content of yeast extract (1). Cultures grown in the presence of 0.1, 0.3, 0.5, and 1.0 g of yeast extract per liter and assayed in the stationary phase showed a proportional increase in optical density and total protein content from 0.1 to 0.5 g of yeast extract per liter. If those cultures were subsequently sparged with N_2 or supplemented with NH_4Cl, the cultures grew to higher optical densities. This indicated that the nitrogen of yeast extract was the primary growth-limiting factor. In all further experiments, cultures were supplemented with 0.5 g of yeast extract per liter to obtain cell densities comparable to N_2-sparged cultures and to obtain sufficient cell mass for the preparation of crude extracts.

Cultures that were grown on limiting nitrogen reached a several times higher specific nitrogenase activity than cultures sparged with a mixture of N_2 and CO_2, irrespective of the reference basis that was applied to the activity measurement (Table 1, Fig. 1). The difference in whole cell nitrogenase specific activity on a protein basis between sparged and nonsparged cultures was at least fourfold, and differences up to eightfold were frequent. When cultures were sparged with pure N_2 or with Ar, the pH increased rapidly. However, the differences in the levels of nitrogenase activity were still apparent. In the cultures that were sparged with N_2-CO_2 or in those that were not sparged, the pH increased only slightly, from 6.9 upon inoculation to 7.2 at day 3 of growth.

TABLE 1. Comparison of nitrogenase activity in N-limited and N_2-grown cultures of *R. palustris*[a]

Sp act unit[b]	Sp act in:		Sp act ratio (N limited/N_2 grown)
	N-limited cells	N_2-grown cells	
U/mg of protein	48.5	9.5	5.1
U/optical density at 660 nm	7.1	1.7	4.1
U/mg fresh weight	4.5	1.1	4.2
U/mg dry weight	20	5	4

[a] Cells were assayed after 3 days of growth.

[b] Nitrogenase activity is expressed as 1 U = 1 nmol of C_2H_4 formed per min.

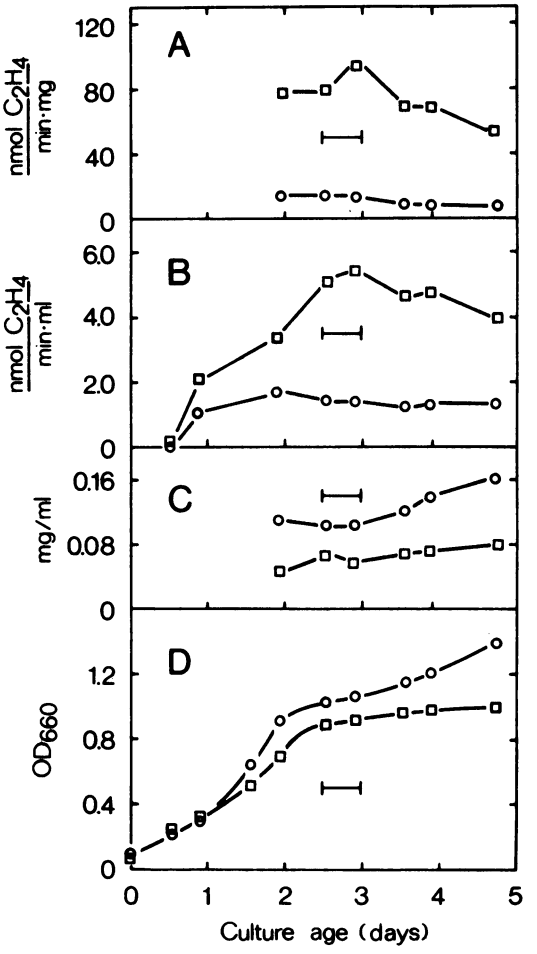

FIG. 1. Growth curves of N_2-grown and N-limited cultures of *R. palustris*. Cultures were grown as described in the text. Sampling from the N-limited culture generated a head space which was filled with Ar. Panel A, specific activity of C_2H_2 reduction (nmol of C_2H_4 formed per min per mg of protein); panel B, total activity of C_2H_2 reduction (nmol of C_2H_4 formed per min per ml of cell suspension); panel C, protein content of the growing cultures (mg of protein per ml of cell suspension); panel D, optical density at 660 nm (OD_{660}). Symbols: ○, N_2-grown cells; □, N-limited cells. The difference in the specific activity of nitrogenase between the two cell types at day 3 was sevenfold.

Nitrogenase activity in batch cultures of N_2-grown *R. rubrum* increased rapidly to a maximum and then declined (20). If cultures of *R. palustris* behaved similarly and if the activity maxima of nitrogenase were off-phase for N_2-grown and N-limited cultures, one would observe an apparent difference among specific activities in cultures of the same age. Figure 1 shows no drastic changes in specific or total activity from days 2 to 5 of growth in either cell

type. Usually, cells were assayed and harvested after 3 days of growth. Figure 1 also shows that cultures sparged with N_2-CO_2 grew to a higher optical density and a higher protein content than N-limited cultures, despite their lower nitrogenase activity.

Determinations of the nitrogenase level in both cell types. To determine whether the observed difference in the activity levels of N_2-grown and N-limited cells was correlated with a comparable difference in the intracellular concentration of Mo-Fe protein, C_2H_2 reduction of crude extracts was titrated to maximal activity by the addition of purified Fe protein from *A. vinelandii*. This method was chosen to circumvent interference by the regulatory mechanism that operates in this organism and inactivates the Fe protein in response to ammonia (34). *R. palustris* Mo-Fe protein and *A. vinelandii* Fe protein form a catalytically active heterologous cross (36), and with saturating amounts of Fe protein the activity would be proportional to the concentration of active Mo-Fe protein in a cell-free extract. Titration of the two types of cell-free extracts indicated a higher specific activity for the Mo-Fe protein in N-limited cells (Fig. 2), which correlated well with the difference observed with whole cells (Table 2). The reciprocal experiment with *R. palustris* Fe protein and *A. vinelandii* Mo-Fe protein would not give a conclusive result because of the abovementioned inactivation of *R. palustris* Fe protein (34, 36).

The difference between the intracellular levels of Mo-Fe protein in N-limited and N_2-grown cells was also apparent after gradient polyacrylamide gel electrophoresis of high-speed supernatants of cell-free extracts (Fig. 3). Purified *R. palustris* Mo-Fe protein was resolved into two subunits, and each was identified in gels of extracts from N_2-grown and N-limited cells. Although the total amount of protein loaded in each lane was identical, the bands corresponding to the Mo-Fe subunits were clearly more dense in the supernatant derived from N-limited cells. Unequivocal identification of the Fe protein of *R. palustris* by this technique was not possible because a prominent protein band, also present in ammonia-grown cells, comigrated with the Fe protein.

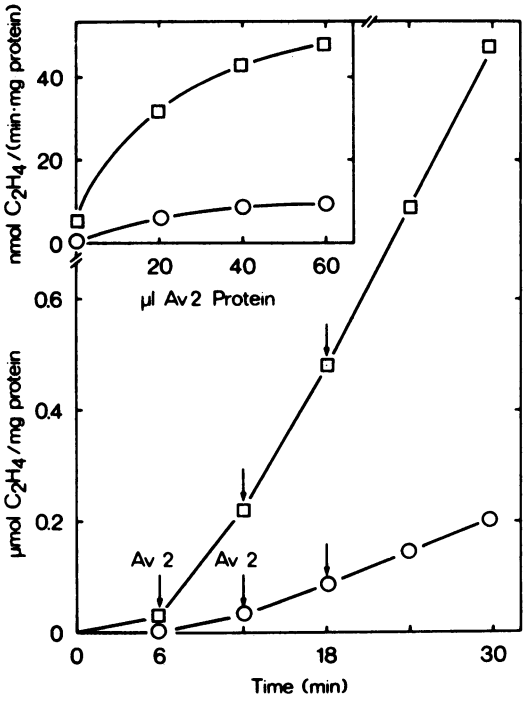

FIG. 2. Titration of cell-free extracts from N_2-grown and N-limited cell types to maximal nitrogenase activity with *A. vinelandii* Fe protein. At the times indicated by arrows, 20 µl (92 µg) of Fe protein (Av 2) was added to a reaction mixture with a cell-free extract (ca. 1 mg). Three additions usually were sufficient to reach maximal activity. The inset shows the rate of C_2H_4 formation as a function of the amount of *A. vinelandii* Fe protein added. Symbols: ○, N_2-grown cells; □, N-limited cells.

TABLE 2. Correlation of whole-cell and cell-free nitrogenase activities of N-limited and N_2-grown cells of *R. palustris*[a]

Expt	Cell type	Whole-cell activity (U/mg protein)	Ratio (N-limited/N_2-grown)	Cell-free activity (U/mg protein)[b]	Ratio (N limited/N_2 grown)
1	N limited	29.4	4.6	31.9	4.5
	N_2 grown	6.4		7.1	
2	N limited	61.7	5.6	47.9	5.2
	N_2 grown	10.9		9.2	
3	N limited	56.9	4.8	63.6	4.5
	N_2 grown	11.8		14.0	

[a] Cells were assayed after 3 days of growth. For each experiment, both types of cultures were grown in parallel from identical subcultures. Activity (U) is defined in Table 1, footnote *b*.

[b] Nitrogenase activity was titrated to its maximal value with Fe protein from *A. vinelandii* (cf. Fig. 2).

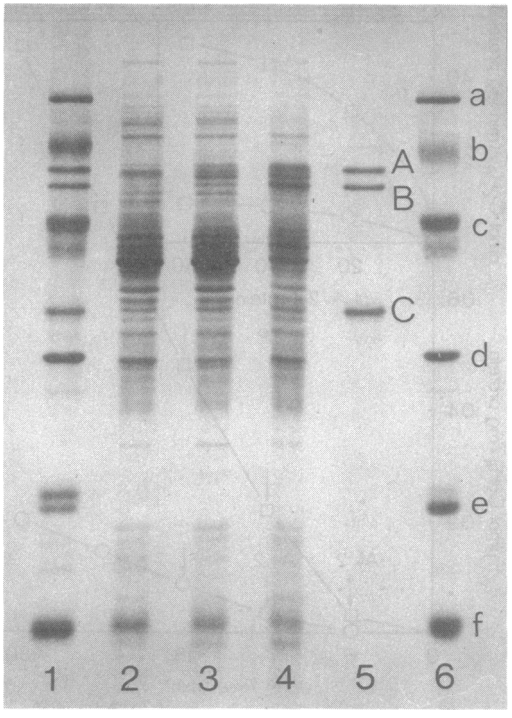

FIG. 3. Comparison of protein profiles of N_2-grown and N-limited cells of *R. palustris* by gradient polyacrylamide gel electrophoresis of cell-free extracts. Proteins were separated electrophoretically in a polyacrylamide gradient (14 to 20%) with a Tris-glycine buffer system and in the presence of sodium dodecyl sulfate (17). Before electrophoresis, proteins were heated for 5 min at 100°C in Tris buffer containing 3% sodium dodecyl sulfate and 5% β-mercaptoethanol. Lanes 2 to 4 were each loaded with 50 μg of protein of high-speed supernatants from ammonia-grown cells (lane 2), N_2-grown cells (lane 3), and N-limited cells (lane 4). Lane 5 shows the purified nitrogenase proteins of *R. palustris*: A, large subunit of the Mo-Fe protein; B, small subunit of the Mo-Fe protein; C, subunit of the Fe protein. Lanes 1 and 6 show the following molecular weight standards: a, phosphorylase *b* (94,000); b, bovine serum albumin (67,000); c, ovalbumin (43,000); d, carbonic anhydrase (30,000); e, soybean trypsin inhibitor (20,100); and f, lactalbumin (14,400). Lane 1 also included the purified nitrogenase components.

Since the two previous techniques required cell breakage, the use of a method probing the intracellular concentration of nitrogenase in intact cells was indicated. The Mo-Fe protein has a distinctive EPR signal in the resting state, which is detectable in whole cells around $g = 4.3$ and 3.6 without interference from other paramagnetic centers (7, 36). The EPR spectra of equally densely packed cells from N_2-grown and N-limited cultures showed a dramatically higher signal intensity in the N-limited cell type (Fig. 4). The ratio of the signal amplitudes between cell types was in agreement with other measurements of the differing nitrogenase levels.

An indication was obtained from immunological evidence that the Fe protein concentration, which could not be monitored by any previous method, was also higher in N-limited cells than in N_2-grown cells. An Ouchterlony double diffusion immunoassay with *R. palustris* Fe protein and an antiserum against the Fe protein from *R. rubrum* was positive, indicating that some antigenic determinants were closely related in the two species. Similarly intense precipitin lines were observed when equal concentrations of *R. palustris* Fe protein, purified from N_2-grown and N-limited cells, were assayed. However, cell-free extracts from the two cell types showed a much higher titer for the Fe protein in the N-limited cell type, an observation which was also supported by the precipitin line intensity and position (Fig. 5).

Half-life of nitrogenase. A higher rate of degradation of nitrogenase in N_2-grown cells relative to N-limited cells or, alternatively, an increased rate or prolonged period of nitrogenase synthesis could account for the difference in the level of enzyme in the two cell types. Studies of nitrogenase turnover in diazotrophic cells are scarce. The common technique is to add ammonia to N_2-fixing cells to repress de novo nitrogenase synthesis and to determine the fate of the preformed enzyme (19). With phototrophic bacteria this type of approach is only applicable if one differentiates experimentally between irre-

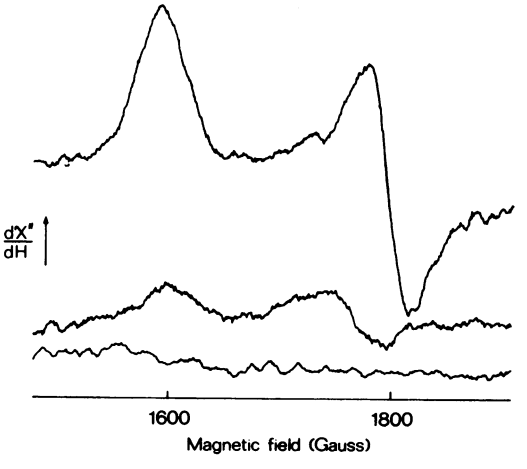

FIG. 4. EPR spectra of N_2-grown and N-limited cell types. Top trace, N-limited cells (123 mg of protein/ml); middle trace, N_2-grown cells (115 mg of protein/ml; recorded with identical spectrometer settings as top trace); bottom trace, ammonia-grown cells. Temperature, 15 K; microwave power, 20 mW; modulation amplitude, 10 Gauss; frequency, 9.44 GHz.

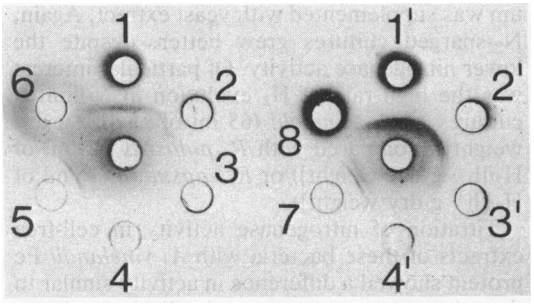

FIG. 5. Immunoassay of *R. palustris* Fe protein in extracts of N-limited and N_2-grown cells. Each center well contained 10 μl of rabbit antiserum against *R. rubrum* Fe protein. Wells 1 to 4 contained 110, 37, 12, and 4 μg of protein, respectively, from a cell-free extract of N_2-grown cells. Wells 1' to 4' contained the same amounts of protein from a cell-free extract of N-limited cells. Well 5 contained 6 μg of purified Fe protein from N_2-grown cells, and well 7 contained 6 μg of Fe protein from N-limited cells. Wells 6 and 8 were controls, with purified *R. palustris* Mo-Fe protein and an extract of ammonia-grown cells, respectively.

versible inactivation or degradation and reversible inactivation by ammonia switch-off. One way to do this was to use L-methionine *SR*-sulfoximine (MSX), which overcame or released ammonia switch-off in *R. palustris* (Fig. 6) (27). Ten to 20 min after the addition of MSX to ammonia-inactived cells, nitrogenase activity was fully restored. The rate established after the addition of MSX was usually 10 to 20% higher than the initial rate of the enzyme, indicating that not all of the nitrogenase was fully active before. The partial switch-off observed in N-limited cells was also released by MSX, and the glutamine synthetase inhibitor still counteracted nitrogenase switch-off 50 h after addition of ammonia (Fig. 6).

Upon addition of ammonia to an N-limited culture, nitrogenase activity decreased with a half-life of about 10 h (data not shown). However, addition of MSX to a sample of the same culture restored almost initial activity levels, which indicated that the activity decline was due to nitrogenase switch-off and not due to enzyme degradation. The rate of C_2H_2 reduction in N_2-grown and N-limited cells that was restored on addition of MSX was followed over a 50-h period after addition of ammonia to repress de novo nitrogenase synthesis (Fig. 7). The results obtained by adding NH_4Cl to a 3-day-old culture indicated a high stability for nitrogenase in both cell types. Half-lives of 58 and 98 h for nitrogenase activity in N_2-grown and N-limited cultures were determined, respectively. These figures indicate a rather slow apparent turnover rate of nitrogenase or of the most labile enzyme component. A similar high stability for nitrogenase was observed when the same experiment was started with a 2-day-old culture.

Overproduction of nitrogenase in other *Rhodospirillaceae*. We have extended the observations on *R. palustris* to *R. rubrum*, *R. capsulata*, and *R. vannielii*. In each case, cells grown on limit-

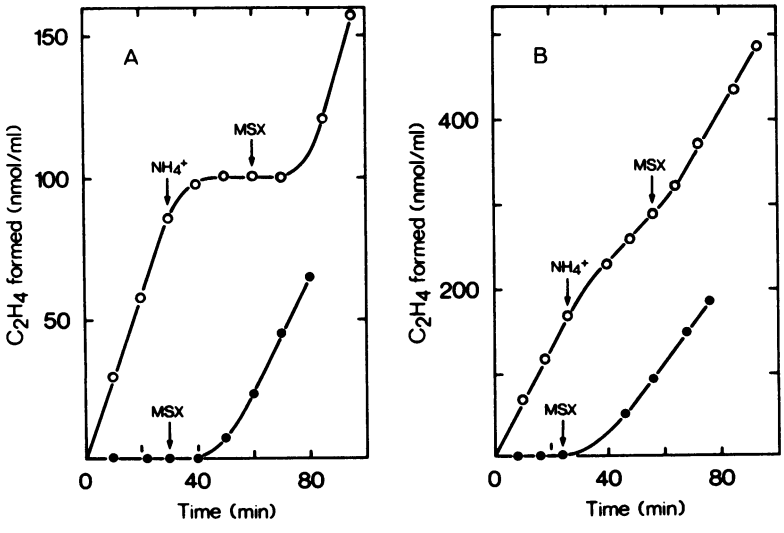

FIG. 6. Release of ammonia switch-off of nitrogenase by MSX. C_2H_2 reduction (○) was followed in a sample of a 3-day-old culture. The reaction mixture was made 1.9 mM in NH_4Cl (first arrow) and later 2 mM in MSX (second arrow). C_2H_2 reduction activity of a sample of the same culture was measured 50 h after addition of NH_4Cl (9.3 mM) (●). The sample was made 2 mM in MSX at the times indicated by the arrows. Panel A, N_2-grown culture; panel B, N-limited culture.

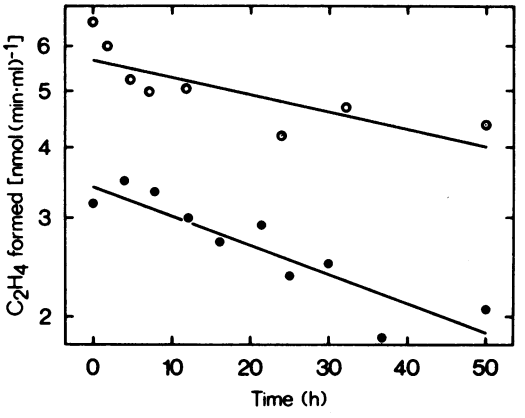

FIG. 7. Determination of the apparent turnover of nitrogenase after repression of enzyme synthesis by ammonia. NH_4Cl (final concentration, 9.3 mM) was added to a 3-day-old N_2-grown (●) or N-limited (○) culture of *R. palustris*. At the indicated times after the initial addition of NH_4Cl, samples of the two cultures were measured for C_2H_2 reduction activity after the release of ammonia switch-off of nitrogenase by MSX (2 mM).

ing nitrogen developed a higher nitrogenase activity than cells sparged with N_2 (Table 3). Cultures grown on limiting ammonia had lower total and specific activities of nitrogenase when sparged with N_2, although they grew to a higher protein content and optical density. Results similar to those obtained under N-limiting conditions were observed when *R. capsulata* and *R. rubrum* were grown on 7 mM glutamate. *R. vannielii* did not grow well on a defined medium, but rapid growth was observed when the medium was supplemented with yeast extract. Again, N_2-sparged cultures grew better, despite the lower nitrogenase activity. Of particular interest was the high rate of H_2 evolution in N-limited cultures of *R. vannielii* (65 ml of H_2/[h · g dry weight]), compared with *R. palustris* (12 ml of H_2/[h · g dry weight]) or *R. capsulata* (16 ml of H_2/[h · g dry weight]).

Titration of nitrogenase activity in cell-free extracts of these bacteria with *A. vinelandii* Fe protein showed a difference in activity similar to that observed with intact cells (Table 3). Thus, in these bacteria the higher level of activity was also correlated with an increased concentration of the Mo-Fe protein. By inference from the immunoassay with *R. palustris*, it is likely that the Fe protein concentration was again higher in nonsparged cells than in N_2-supplied cells.

DISCUSSION

Cultures of *R. palustris* grown on a limiting amount of yeast extract as nitrogen source reached and maintained four- to eightfold higher nitrogenase activity than cells grown in the presence of N_2. Cultures supplied with N_2 grew better, as expressed by their higher optical density and protein content, despite lower nitrogenase activity. Thus, the latter is not likely caused by an inhibitory effect of N_2 sparging. Diazotrophically grown cultures of *R. palustris* regulate nitrogenase at the activity level in response to ammonia (34, 36). To determine whether the difference in activity was a manifestation of this regulatory mechanism or whether higher amounts of nitrogenase were produced in N-limited versus N_2-grown cells, the intracellu-

TABLE 3. Overproduction of nitrogenase in N-limited or glutamate-grown cells of various non-sulfur purple bacteria[a]

Organism	N source	Whole-cell activity (U/mg protein)	Ratio ($-N_2/+N_2$)	Cell-free activity (U/mg protein)[b]	Ratio ($-N_2/+N_2$)
R. capsulata	NH_4Cl	96	3.6	16.9	2.8
	NH_4Cl + N_2	27		6.0	
	Glutamate	120	4.4	16.6	2.0
	Glutamate + N_2	27		8.2	
R. rubrum	NH_4Cl	92	5.1	ND[c]	
	NH_4Cl + N_2	18			
	Glutamate	94	4.7	59.8	3.7
	Glutamate + N_2	20		16.3	
R. vannielii	Yeast extract	87	3.6	14.0	2.5
	Yeast extract + N_2	24		5.6	

[a] The initial growth-limiting concentrations were : NH_4Cl, 3 mM; glutamate, 7 mM; yeast extract, 0.5 g/liter. The cultures were assayed after 3 days of growth. Activity (U) is defined in Table 1, footnote *b*.

[b] Cell-free nitrogenase activity was titrated to its maximal value with *A. vinelandii* Fe protein.

[c] ND, Not determined.

lar level of nitrogenase was measured by independent methods. The level of Mo-Fe protein in cell-free extracts was determined by titration with *A. vinelandii* Fe protein, monitoring maximal C_2H_2 reduction. The difference observed with intact cells corresponded to a large difference in the Mo-Fe protein content of crude extracts. These observations were further supported by probing the Mo-Fe concentrations of both cell types by EPR applied to intact cells and by protein profiles of cell-free extracts obtained from polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Ouchterlony double-diffusion immunoassay of extracts indicated that the higher Mo-Fe protein concentration in N-limited cells was paralleled by an increased Fe protein concentration.

Our data show that the difference in C_2H_2-reducing activity between N_2-grown and N-limited cells is due to a difference in the level of the intracellular concentration of nitrogenase. Addition of MSX to N_2-fixing cells resulted in only a 10 to 20% increase in nitrogenase activity and not by a four- to eightfold increase, which would have been expected if the activity of a high intracellular enzyme concentration were lessened by the ammonia switch-off mechanism.

An increased intracellular concentration of an enzyme may be the result of increased synthesis or a decreased rate of degradation. We, therefore, estimated the rate of nitrogenase turnover in N_2-grown and N-limited cultures after the addition of ammonia to repress de novo synthesis. Although the data varied somewhat, we did observe a longer half-life for nitrogenase in N-limited versus N_2-grown cells. However, the half-lives were too long to account for the observed levels of nitrogenase activity. Twenty-four hours after nitrogenase was derepressed, a four- to fivefold difference in nitrogenase between the two kinds of cells was observed. Thus, either an increased rate or extended time of enzyme synthesis most probably is responsible for the high nitrogenase concentration found in N-limited cells.

Increased nitrogenase activity in N-limited cultures of *R. rubrum* (27), *R. capsulata*, and *R. vannielii* suggested that the potential for overproduction of nitrogenase is not an exclusive property of *R. palustris*. Nitrogenase activity in cultures of *R. rubrum* and *R. capsulata* that were derepressed in the presence of glutamate depended upon the presence or absence of N_2. Thus, 3 to 5 times lower nitrogenase activity was found in glutamate cultures sparged with N_2. High levels of nitrogenase in glutamate-grown cultures that were not sparged with N_2 resulted in high rates of H_2 evolution. Hillmer and Gest (12, 13) proposed from their study of glutamate-grown *R. capsulata* that H_2 evolution provided an energy-idling mechanism, i.e., it would allow the cell to dispose of excess ATP and reducing equivalents under conditions of energy sufficiency but N-limitation. Our results, however, indicate that H_2 evolution was increased because of the overproduction of nitrogenase under N-limitation. Apparently the cellular concentration of this enzyme can be adjusted gradually within wide limits to respond to increasing N-deficiency. High rates of H_2 evolution, although valuable in a putative system for biological H_2 production (35), appear then to be more a consequence of nitrogenase regulation rather than a provision for cellular energy poising.

Beyond the *Rhodospirillaceae* and in otherwise diverse diazotrophic organisms, overproduction of nitrogenase under N-limitation may be a general response of the *nif* regulatory system. *Azotobacter chroococcum* (6), *Clostridium pasteurianum* (5, 28), and *K. pneumoniae* (28) had two- to ninefold higher nitrogenase activity when derepression occurred under Ar rather than N_2. This effect was described as hyperinduction and, interestingly, was shown even for *Salmonella typhimurium* carrying the F' *his nif* plasmid FN68 (22). Nitrogen starvation increased nitrogenase activity in the cyanobacteria (9, 16, 24, 32), which may be in part related to the phenomenon described here for photosynthetic bacteria. Although reports occasionally have referred to an increased concentration of nitrogenase (5, 28), evidence remained scant to support this conclusion. Our findings appear to be the first evidence linking unequivocally an increased level of nitrogenase activity in N-limited cultures to an increased intracellular concentration of the nitrogenase proteins. Moreover, we have preliminary evidence for *R. palustris* that the regulatory metabolite guanosine 3',5'-bis(diphosphate) is involved in *nif* regulation in accordance with the model outlined above. We have observed an increase in the level of this metabolite before derepression and a transient increase at the onset of overproduction of nitrogenase. After addition of ammonia to derepressed cells, there was a fast decrease in the cellular concentration of guanosine 3',5'-bis(diphosphate) followed by a slow recovery (W. G. Zumft and S. Neumann, unpublished data). When nitrogenase is derepressed in the absence of N_2 and the cell becomes exhausted of other usable N sources, the observed increase in the regulatory metabolite is consistent with the hypothesis of inactivation of the repressor molecule (*nifL* product), thereby inducing overproduction of nitrogenase.

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