JOURNAL OF BACTERIOLOGY, July 1983, p. 107–112
0021-9193/83/070107-06$02.00/0

Vol. 155, No. 1

Nitrogen Fixation and Ammonia Switch-Off in the Photosynthetic Bacterium *Rhodopseudomonas viridis*

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Received 10 January 1983/Accepted 21 April 1983

Rhodopseudomonas viridis ATCC 19567 grows by means of nitrogen fixation in yeast extract-N_2 or nitrogen-free medium when sparged with 5% CO_2 and 95% N_2 in the light at 30°C. Acetylene reduction assays for nitrogenase activity revealed an initially high level of activity during early-logarithmic growth phase, a lower plateau during mid- to late-logarithmic phase, and a dramatic reduction of activity at the beginning of the stationary phase. When viewed by electron microscopy, nitrogen-fixing *R. viridis* cells appeared to be morphologically and ultrastructurally similar to cells grown on nitrogen-rich media. Whole cells prepared under reducing conditions in the dark for electron spin resonance spectroscopy yielded *g*4.26 and *g*3.66 signals characteristic of the molybdenum-iron protein of nitrogenase. During growth on N_2 in the absence of fixed-nitrogen sources, the nitrogenase activity of *R. viridis* measured by acetylene reduction stopped rapidly in response to the addition of NH_4Cl as has been observed in other *Rhodospirillaceae*. However, unlike the nitrogenase of *Rhodopseudomonas palustris* or *Rhodospirillum rubrum*, which recover from this treatment within 40 min, the nitrogenase activity of *R. viridis* was not detectable for nearly 4 h.

All members of the family *Rhodospirillaceae* that have been tested have been shown to fix nitrogen (36). In these organisms, nitrogen fixation usually occurs photosynthetically under anaerobic conditions although low levels of nitrogen fixation have been demonstrated in the dark during microaerophilic or fermentative growth (22, 32). Nitrogen reduction occurs as a result of the concerted interaction of an iron protein (Fe protein) and a molybdenum-iron protein (MoFe protein) whose properties are similar to those of the nitrogenase components of non-photosynthetic, nitrogen-fixing bacteria (20). These proteins have been isolated in purified form (5, 20, 25) from *Rhodospirillum rubrum*. Unlike the Fe protein from non-photosynthetic bacteria, the Fe protein of *R. rubrum* can be isolated in either an active or inactive form (5, 19, 24), which is due to the absence or presence, respectively, of a covalently bound modifying group (15, 20, 21). Inactive Fe protein can be activated in vitro by a presumably intracytoplasmic membrane-bound enzyme in conjunction with divalent metal ion and ATP (19).

In vivo nitrogenase activity in several *Rhodospirillaceae* is subject to short-term regulation by certain fixed-nitrogen-containing compounds (1, 12, 31), a phenomenon known as ammonia switch-off (39). Although the mechanism of ammonia switch-off is unknown, a direct relationship has been suggested between this event in vivo and the observed modification of the Fe protein (5, 15).

Nitrogen fixation in *Rhodopseudomonas viridis*, also a member of *Rhodospirillaceae*, has been little characterized. Some nitrogen-fixing properties of a *Rhodopseudomonas* isolate identified as *R. viridis* have been reported (D. C. Pratt, P. L. Bergad, and G. E.Ham, Bacteriol. Proc. 71:139, 1971), and in a review article in 1981, Postgate listed it as a nitrogen-fixer (27). We further characterized the nitrogen-fixation and ammonia switch-off properties of this species because of several features which make *R. viridis* unusual among the purple, non-sulfur bacteria. First, it is one of two members which contain bacteriochlorophyll *b* (BChl *b*) in its photosynthetic reaction centers instead of BChl *a*. Since BChl *b* in reaction centers absorbs light of longer wavelengths than does BChl *a*, the amount of energy in the first excited singlet state of *R. viridis* is less than that in other *Rhodospirillaceae* (29). Thus, the energy coupling between photosynthesis and nitrogen fixation in this organism may differ from that of the BChl *a*-containing species. Second, *R. viridis* bears a similarity in size, shape, and thylakoid structure

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to *Rhodopseudomonas palustris*, another member of the *Rhodospirillaceae* (35). However, electron microscopic examination of the intracytoplasmic membrane supramolecular structure of *R. viridis* reveals a nearly crystalline periodicity unlike the photosynthetic membranes of other purple, non-sulfur bacteria (14, 26).

(A preliminary report of the present work has been presented previously [K. S. Howard, B. J. Hales, and M. D. Socolofsky, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, p. 153, K95].)

MATERIALS AND METHODS

Organisms and growth conditions. Unless otherwise specified, *R. viridis* ATCC 19567 and *R. palustris* ATCC 17001 used in these experiments were grown anaerobically at 30°C under incandescent light of ca. 6 mW/cm^2 of intensity. Stock cultures of *R. viridis* (10) and *R. palustris* (13) were grown as previously described. To derepress nitrogenase, we grew batch cultures of both organisms in 200-ml volumes of the yeast extract-N_2 medium of Pfennig (23), which contains sodium succinate and 0.1% yeast extract, and continually sparged them with 95% N_2 and 5% CO_2.

Cell growth analysis. The turbidity of the batch cultures was monitored at 660 nm with a Klett-Summerson colorimeter. The colorimeter was calibrated to correct for deviations from Beer's Law at high concentrations of cells (17). The reported growth curves were corrected for this deviation, and the data were converted to absorbancy units.

Protein determination. After acetone-methanol extraction of pigments (6), total cell protein was determined by the method of Lowry et al. (18), with bovine serum albumin as the standard. The micrograms of protein per milliliter of whole cells per absorbancy unit remained essentially constant throughout the period of growth and equaled 890 for *R. palustris* and 442 for *R. viridis*.

Acetylene reduction assay. Acetylene reductions were performed by a modification of the Burris method (3). A 15-min preincubation of 10-ml cell samples in 50-ml serum bottles was conducted under an Ar atmosphere in the light (6 mW/cm^2) at 30°C to allow temperature and gas equilibration. The concentration of acetylene used was 10 kPa. The length of incubation varied with experimental conditions.

ESR spectroscopy. Electron spin resonance (ESR) samples of mid-logarithmic growth stage cells were prepared similarly to previously published methods (11). Throughout the procedure, all solutions were Ar flushed for 3 to 5 min before use. The cultures were harvested at 8,000 × *g* for 10 min in Ar-flushed centrifuge bottles, suspended in 0.025 M Tris-hydrochloride buffer (pH 7.4), and recentrifuged at 8,000 × *g* for 10 min. From this point until frozen, the samples were kept in darkness or dim light. After centrifugation, the cells were suspended under Ar in an equal volume of 0.10 M glycylglycine buffer (initial pH, 10.1 with KOH) containing 0.10 M KCl, 0.10 mM methyl viologen, plus freshly added 40 mM $Na_2S_2O_4$ (final pH, 8.0). This suspension was further diluted by one half with an equal volume of glycerol and thoroughly mixed with a stream of Ar delivered from a Pasteur pipette. The cells were then transferred to Ar-flushed quartz ESR tubes, capped, frozen within 1 min with liquid-nitrogen-cooled N_2, and stored in liquid nitrogen. ESR spectra were observed at X-band wavelengths with either a Varian E12 spectrometer with an Oxford liquid He cryostat (Princeton University) or a Varian E-109 ES spectrometer with a Heli-Tran liquid He cryostat (model LTD-3-110, Air Products, Inc.) at temperatures between 4 and 9°K.

Chemicals. All growth media were prepared with reagent grade salts, yeast extract (Difco Laboratories), and sodium succinate (Nutritional Biochemicals Corp.) or ammonium succinate (ICN Pharmaceuticals, Inc.). Buffer components included reagent grade TRIZMA, Tris-hydrochloride (pH 7.4; Sigma Chemical Co.), or glycylglycine (Sigma), reagent grade salts, methyl viologen (Mann Research Laboratories, Inc.), and sodium dithionite (Sigma).

RESULTS

Nitrogen fixation by whole cells. *R. viridis* ATCC 19567 was capable of continued photosynthetic growth at 30°C after transfer from nitrogen-rich stock medium to yeast extract-N_2 medium containing 0.1% yeast extract when supplied with 95% N_2 and 5% CO_2. The growth of a 200-ml batch culture (Fig. 1) proceeded for 82 to 85 h before entering the stationary phase at a turbidity of ca. 0.5 absorbancy units. The stationary-phase turbidity and the 65- to 70-h logarithmic growth phase were consistent characteristics of the nitrogen-fixing batch cultures

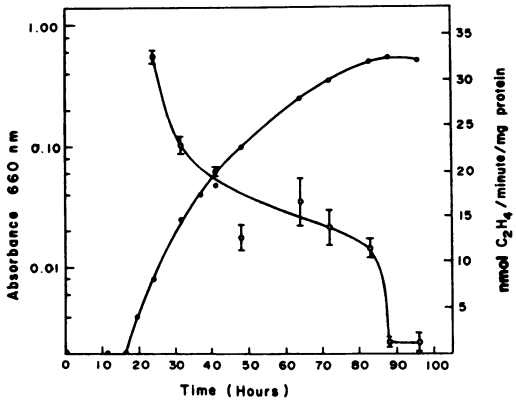

FIG. 1. *R. viridis* growth and acetylene reduction rates in batch culture. A 1% inoculum of *R. viridis* stock culture grown in nitrogen-rich medium was transferred to 200 ml of yeast extract-N_2 medium, grown, and monitored turbidimetrically as described in the text. Acetylene reductions were performed with duplicate 10-ml samples taken from duplicate 200-ml batch cultures such that each data point is the average of four samples. The standard deviation among the samples is denoted by bars. After a 15-min incubation for acetylene reduction, each sample was frozen and used to determine the milligrams of whole cell protein per milliliter of culture per absorbancy unit. Symbols: ●, turbidity; ○, rate of ethylene production.

of *R. viridis*, which had a generation time of 4.5 h as compared with 1.75 h for *R. palustris* grown under identical conditions. *R. viridis* transferred during mid-logarithmic growth from the original Pfennig medium to Pfennig medium free of yeast extract showed no alteration in growth rate as compared with cells held in the original medium (data not shown). These data suggest that the cells in the original Pfennig medium had utilized the nitrogen available in the yeast extract by mid-logarithmic growth phase and did not require it for continued growth while fixing nitrogen in nitrogen-free medium.

Figure 1 shows the rate of nitrogen-fixing activity during *R. viridis* growth in batch culture. Ethylene was not detectable in samples taken during the lag or the initial logarithmic growth phase of cultures inoculated with either nitrogen-rich or yeast extract-N_2 stocks. This may have been due either to the lack of sufficient cell numbers to produce detectable amounts of ethylene during the 15-min incubation time or to repression of nitrogenase by the initial fixed-nitrogen sources present in the medium. The rate of ethylene production was greatest during early-logarithmic phase, decreased at mid- to late-logarithmic phase, and was marked by an immediate reduction at the beginning of the stationary phase.

Acetylene reduction appeared to be reversibly inhibited by exposure to small amounts of O_2 as reported in other photosynthetic bacteria (16, 28). Occasionally, one of a duplicate or triplicate set of samples would be inactivated after transfer from the batch culture to the reaction vessel, presumably due to exposure to air. However, the inactive sample usually regained its acetylene-reducing activity after about 1 h of incubation, suggesting that *R. viridis* nitrogenase was protected upon exposure to very low concentrations of O_2 even though the organism is reportedly a strict anaerobe (8).

Electron microscopy of nitrogen-fixing cells. We were interested in comparing the morphology and ultrastructure of *R. viridis* growth in both nitrogen-enriched and nitrogen-limited media. Negative stains and thin sections of exponentially growing cells from 0.5% yeast extract–ammonium succinate-enriched medium and yeast extract-N_2 medium were indistinguishable. Typically, cells observed from mid-logarithmic-phase cultures were in the process of budding. In negative stains, round, dense bodies appeared in all cells, generally located at one or both poles. Longer cells often contained three bodies nearly equally spaced, whereas shorter cells sometimes contained only one. The size of the dense bodies varied and could be reduced to some extent by beam damage in the electron microscope.

Thin sections of *R. viridis* ATCC 19567 revealed similar amounts of intracytoplasmic membrane development regardless of the nitrogen source. However, the extent of thylakoid development in this strain was significantly less than in another strain of *R. viridis* (gift of H. Frank) grown under similar conditions (11) and observed by electron microscopy (G. C. Dismukes, personal communication). Although both strains grew equally well under nitrogen-fixing conditions, they differed in two other respects. Our ATCC 19567 strain retained its yellow-green color regardless of the age of the culture and did not adhere to glassware, whereas the second *R. viridis* strain gradually developed a brownish color in aging cultures, as previously reported (8), and did adhere to the sides of growth vessels even in cultures constantly mixed by sparging gas.

ESR spectroscopy of whole cells. Nitrogen-fixing *R. viridis* and *R. palustris* were prepared for ESR spectroscopy in the presence of sodium dithionite and methyl viologen in the dark. We have shown that these conditions enhance the *g*4.3 and *g*3.6 peaks associated with the active MoFe protein of nitrogenase by reducing background signals in this region of the whole cell ESR spectra (manuscript in preparation). Under these reducing conditions, *R. viridis* showed peaks at *g*4.26 and *g*3.66 comparable to the signals observed in the nitrogenase MoFe protein in whole cells of other nitrogen-fixing bacteria, e.g., *R. palustris* (Fig. 2; 40) and *Azotobacter vinelandii* (7). In whole cells of all three organisms, the *g*2.01 peak observed in purified MoFe protein is obscured by other strong signals in the *g*2 region.

Ammonia switch-off in *R. viridis*. Members of the *Rhodospirillaceae* family demonstrate a reversible loss of in vivo nitrogenase activity when exposed to NH_4^+ under specific nutritional and environmental conditions (5, 31, 33, 37, 38). This phenomenon has been characterized most extensively in *R. rubrum*, although *R. palustris* shows similar characteristics in its response to NH_4^+ when growing by means of nitrogen fixation in the light on sparged N_2 (1, 40). Under these conditions, both organisms reversibly stop nitrogenase production of ethylene or hydrogen (12, 31, 33) within minutes after the addition of NH_4Cl. Furthermore, the length of the inactive period has been shown to depend on the amount of NH_4Cl added (31, 40). Once the ammonia concentration in the medium dropped below 0.10 mM, nitrogenase activity rapidly resumed. Since *R. palustris* and *R. viridis* are closely related species (35), we chose to compare ammonia switch-off of nitrogenase activity in these two organisms.

Figure 3 shows the effect of 0.20 mM NH_4Cl

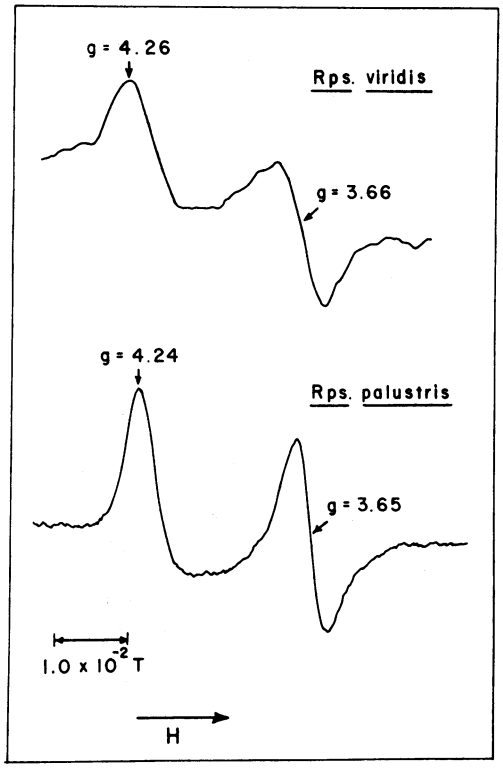

FIG. 2. First-derivative ESR spectra of *R. viridis* and *R. palustris* whole cells grown under nitrogen-fixing conditions. The organisms were dark adapted for approximately 15 min before reduction by $Na_2S_2O_4$. The samples were held in the dark until they were frozen to 77°K for storage. The spectra were obtained under the following instrumental conditions: *R. viridis*—power, 5.0 mW; frequency, 9.0 GHz; modulation amplitude, 12.5 G; time constant, 1 s; temperature, 9°K. *R. palustris*—power, 5.0 mW; frequency, 9.233 GHz; modulation amplitude, 20 G; time constant, 3 s; temperature, 4°K. T, Tesla; H, magnetic field strength.

on ethylene production in exponentially growing, nitrogen-fixing cultures of *R. palustris* and *R. viridis*. Duplicate 10-ml samples of each culture were transferred anaerobically to 50-ml serum bottles under an Ar atmosphere. After a 15-min preincubation, acetylene (10 kPa) was added at time zero. An Ar-flushed NH_4Cl solution (0.4 ml, 5.0 mM) was added at the times indicated by the arrows (Fig. 3) to produce a final concentration of 0.20 mM. *R. palustris* nitrogenase responded in the manner previously reported by Zumft and Castillo (40) by rapidly halting the production of ethylene and then resuming its activity after approximately 35 min. Under the same conditions, *R. viridis* nitrogenase was rapidly inactivated within 5 min, but did not resume for nearly 4 h. Approximately 4 h after the addition of ammonia, the amount of ethylene in the sample had increased from 120 to 220 nmol/mg of protein. During the same time span, ethylene in the control sample increased at a nearly linear rate to 780 nmol/mg of protein. Finally, increasing concentrations of NH_4Cl up to 0.10 M did not lengthen the period of inactivation.

The inadvertent introduction of O_2 with the NH_4Cl can occur with any given sample, resulting in O_2 switch-off as opposed to ammonia switch-off. The possibility of introducing contaminating O_2 was controlled by repeating the experiment identically on eight samples of *R. viridis* and four samples of *R. palustris* on various dates. The consistency of results among these samples suggests that O_2 contamination was not a contributing factor.

DISCUSSION

R. viridis grew vigorously by nitrogen fixation under anaerobic, photosynthetic conditions.

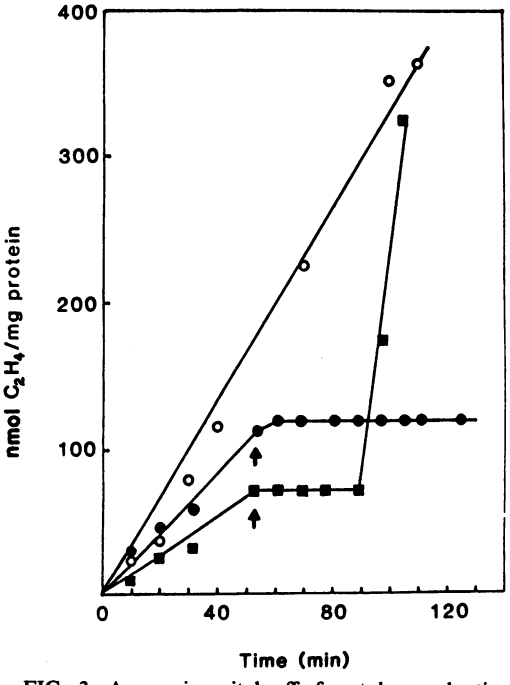

FIG. 3. Ammonia switch-off of acetylene reduction in *R. viridis* and *R. palustris*. These data from a single experiment are representative of four *R. viridis* and two *R. palustris* experiments, each run in duplicate. The absorbancies of the batch cultures at the beginning of this experiment were 0.235 for *R. viridis* and 0.185 for *R. palustris*. Ar-flushed NH_4Cl was added by syringe to a final concentration of 0.20 mM at the times indicated by the arrows. Symbols: ●, *R. viridis* treated with NH_4Cl; ■, *R. palustris* treated with NH_4Cl; ○, *R. viridis* control (the line was determined by a least-squares fit of the data points).

The highest rate of nitrogenase activity was observed in early-log phase, consistent with other data which suggest that nitrogenase derepression is greatest at this time because of the lack of high concentrations of nitrogenase-produced ammonia in the cells (7). It is also possible, however, that the unidentified mechanism responsible for ammonia switch-off in several *Rhodospirillaceae* represents a finer control than repression for nitrogenase activity.

Upon entering stationary growth phase, *R. viridis* nitrogenase activity was rapidly reduced to a very low level, although not completely inactivated. The loss of activity and the initiation of the stationary phase occurred within 5 h. This trait is unlike *R. palustris*, in which both nitrogenase activity and ammonia switch-off can be demonstrated in stationary phase (1).

ESR spectra of *R. viridis* whole cells prepared in dim light with sodium dithionite were comparable to the ESR spectra of whole, nitrogen-fixing *R. palustris*, *A. vinelandii*, and *R. rubrum* observed in our laboratory and others (4, 7, 9). Under these conditions, *R. viridis* and *R. palustris* spectra contained peaks at *g*4.26 and 3.66 and *g*4.24 and 3.65, respectively, indicative of the presence of the MoFe protein of nitrogenase (7). Although they did not report *g* factors, Zumft and Castillo (40) showed similar spectra for nitrogen-fixing and ammonia switched-off *R. palustris* whole cells. Their preparations were made under Ar without sodium dithionite and contain additional peaks in the *g*4.5 to 3.5 region which we eliminated by reducing the sample with sodium dithionite in the dark. We typically observed two peaks of equal amplitude in our reduced samples of *R. palustris* and *R. viridis*. However, the line widths of both peaks in *R. viridis* samples were broader than those of other whole cell organisms which we observed and approach those previously reported for iron-molybdenum cofactor (FeMoco) isolated from the MoFe protein of *A. vinelandii* (2, 30).

Ammonia switch-off of nitrogenase activity is a feature common to all *Rhodospirillaceae* so far examined (39). Growth conditions, such as light intensity (38), carbon source (37), and especially, nitrogen source (1, 33), regulate the rate and occurrence of ammonia switch-off, which has been suggested to be due to the covalent modification of the nitrogenase Fe protein (20) by an unidentified enzyme (34).

The nitrogenase activity of *R. palustris* and *R. rubrum* resumes in vivo upon decreased concentration of the exogenous ammonia source (31, 37, 40). The activating mechanism in vivo is unknown, but is presumably the same as that observed in vitro where the reactivation of isolated, inactive Fe protein is induced by an activating enzyme (19). The activating enzyme has been isolated from chromatophores by salt elution (20, 24), and is highly O_2 labile (19). Inactive Fe protein differs from active Fe protein by the presence of a covalently bound group which contains pentose, phosphate, and an adenine-like moiety on one of its two subunits (15; P. W. Ludden and G. G. Preston, Proc. 4th Int. Symp. on Nitrogen Fixation, Canberra, Australia, 1980, p. 71). These properties set apart the nitrogenase Fe protein of the *Rhodospirillaceae* from the analogous protein in the *Azotobacter*, *Klebsiella*, and *Clostridium* systems.

Upon the anaerobic addition of 0.20 mM NH_4Cl to cells growing on N_2 in the light, *R. viridis* completely stopped reducing acetylene within 5 min. However, unlike other *Rhodospirillaceae*, *R. viridis* nitrogenase activity did not reappear for nearly 4 h. This pattern was the same for NH_4Cl concentrations up to 0.10 M. As expected, cultures treated with 0.20 or 0.50 M NH_4Cl were still inactive after 4 h. The extended period of inactivity, regardless of NH_4Cl concentration, implies that nitrogenase reactivation may require protein synthesis of possibly the activating enzyme (if it exists in *R. viridis*) or the nitrogenase components themselves. It is not unreasonable to suspect that the activating mechanism, which seems to be associated with the intracytoplasmic membranes of those organisms studied thus far, may differ in this organism considering the significant differences in the photosynthetic apparatuses of *R. viridis* and other *Rhodospirillaceae* (14). It is also possible that, unlike *R. palustris*, the threshold level of NH_4^+ or some intermediate metabolite which controls ammonia switch-off is not reached until 4 h after the addition of 0.2 mM NH_4Cl.

ACKNOWLEDGMENTS

We thank M. M. Lajaunie, L. D. Smith, and K. A. Howard for their expert technical assistance and G. C. Dismukes and D. Abramowicz (Department of Chemistry, Princeton University) for their assistance in obtaining the *R. palustris* ESR data and for enlightening discussion. We also thank H. A. Frank (Department of Chemistry, University of Connecticut) for kindly providing us with the second *R. viridis* strain.

K. S. Howard gratefully acknowledges receipt of an American Society for Microbiology President's Fellowship. This investigation was supported in part by research grant no. GM-25049 (B.J.H.) from the National Institutes of Health and by the Louisiana State University Agricultural Experiment Station.

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