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Hydrogenase Activity in *Rhodopseudomonas capsulata*: Relationship with Nitrogenase Activity

ANNETTE COLBEAU, BRUCE C. KELLEY,† AND PAULETTE M. VIGNAIS*

Biochimie, Département de Recherche Fondamentale, Centre National de la Recherche Scientifique (ER n235), Institut National de la Santé et de la Recherche Médicale (U. 191), Centre d'Etudes Nucléaires, 85X, 38041 Grenoble-cedex, France

Hydrogenase activity was found in cells of *Rhodopseudomonas capsulata* strain B10 cultured under a variety of growth conditions either anaerobically in the light or aerobically in the dark. The highest activities were found routinely in cells grown in the presence of H_2. The hydrogenase of *R. capsulata* was localized in the particulate fraction of the cells. High hydrogenase activities were usually observed in cells possessing an active nitrogenase. The hydrogen produced by the nitrogenase stimulated the activity of hydrogenase in growing cells. However, the synthesis of hydrogenase was not closely linked to the synthesis of nitrogenase. Hydrogenase was present in dark-grown cultures, whereas nitrogenase synthesis was not significant in the absence of light. Unlike nitrogenase, hydrogenase was present in cultures grown on NH_4^+. Conditions were established which allowed the synthesis of either nitrogenase or hydrogenase by resting cells. We concluded that hydrogenase can be synthesized independently of nitrogenase.

Photoproduction of molecular hydrogen by photosynthetic bacteria was first observed with *Rhodospirillum rubrum* growing photoheterotrophically in the presence of dicarboxylic acid and glutamate or aspartate as the nitrogen source (9, 10). Subsequently, it was demonstrated that the light-dependent production of H_2 was linked to N_2 metabolism (8, 13) and was catalyzed by nitrogenase (28).

Under appropriate nutritional conditions, the purple nonsulfur photosynthetic bacterium *Rhodopseudomonas capsulata* also evolves large amounts of H_2, either in growing cultures (11) or in resting cells (12). This H_2 production, which is light dependent and inhibited by NH_4^+ (11, 12), is mediated by nitrogenase.

These bacteria also use molecular hydrogen for photoautotrophic growth (7, 18, 22), and certain strains of *R. capsulata* are among the most efficient bacteria of this class to use H_2 as an energy source (15). This ability is associated with the activity of another enzyme, hydrogenase (3, 15, 28). During photoautotrophic growth, hydrogenase activates molecular hydrogen for the photoreduction of CO_2 and therefore functions as an H_2 uptake hydrogenase.

R. capsulata is a facultative anaerobe and phototroph and can also grow in the dark in the presence of oxygen. When grown in the dark under autotrophic conditions, this bacterium develops a highly active hydrogenase. Under such conditions H_2 uptake is linked to respiration, resulting in ATP synthesis, as occurs in other aerobic hydrogen bacteria (23); hydrogenase again acts as an H_2 uptake hydrogenase and feeds electrons to the respiratory chain.

Dixon (4-6) postulated a possible coupling between the nitrogenase and hydrogenase activities in aerobic H_2-fixing organisms; such a coupling would involve a hydrogen cycle in which the H_2 produced by a nitrogenase could serve as a substrate for a unidirectional, H_2 uptake hydrogenase. Such H_2 recycling has since been demonstrated in various N_2-fixing organisms (1, 19, 25-27), including *Rhodopseudomonas capsulata* (14). The purpose of this investigation was to determine the growth conditions and factors affecting hydrogenase activity in *R. capsulata*, the location of this enzyme within the cell, and the functional interactions occurring in vivo between hydrogenase and nitrogenase activities. *R. capsulata* strain B10, which is known to produce large amounts of H_2 through nitrogenase activity (11), was chosen to study the possibility of employing such bacteria to produce H_2 at the expense of light energy.

MATERIALS AND METHODS

Bacterial strains and culture. *R. capsulata* strains B10 and W15 were generous gifts from the Photosynthetic Bacteria Group, Department of Microbiology, Indiana University, Bloomington. Cultures were grown at 30°C in a mineral salts medium (11, 29) supplemented with organic acids as carbon sources and either glutamate or ammonia as the nitrogen source. For photoheterotrophic growth, carbon was

† Present address: Department of Agricultural Biochemistry, Waite Agricultural Research Institute, The University of Adelaide, Glen Osmond, South Australia 5064, Australia.

provided as either lactate (30 mM), malate (30 mM), or succinate (30 mM), and nitrogen was provided as glutamate (7 mM) or ammonium sulfate (7 mM). For autotrophic growth, the medium was supplemented with ammonium sulfate (7 mM) and sodium bicarbonate (1 g/liter) and was bubbled with a mixture of H_2 and CO_2 (80:20, vol/vol) in the light or H_2, CO_2, and O_2 (80:10:10, vol/vol) in the dark. Cultures were illuminated by 100-W incandescent lamps (ca. 10,000 lux).

Preparation of broken cells and membranes. Bacterial cell suspensions (5 mg of protein per ml) were passed through a French pressure cell at 20,000 lb/in^2 and 10°C. The homogenate was centrifuged at 20,000 × *g* for 15 min in a Sorvall centrifuge. The pellet was suspended in 20 mM Tris-hydrochloride (pH 8) in about one-half the volume of the homogenate, and this suspension was submitted to the French press treatment and centrifuged as described above. The two supernatant fluids were mixed and centrifuged at 105,000 × *g* for 1 h in a Spinco 40 rotor at 12°C. The membrane pellets were suspended in 20 mM Tris-hydrochloride buffer (pH 8) and gassed with H_2.

Hydrogenase assays. Hydrogenase activity was assayed either by H_2 production or by H_2 uptake. H_2 production was determined by gas chromatography, using 2.5 mM methyl viologen and 10 mM dithionite as the electron donor (2). The reaction was carried out in 2-ml glass tubes fitted with gas-tight rubber stoppers and containing between 0.5 and 1 mg of whole cells or cell-free extract in 0.5 ml of 20 mM Tris-hydrochloride buffer (pH 8). With whole cells, 1 mM $(NH_4)_2SO_4$ was added to inhibit H_2 production by nitrogenase. The reaction vessels were gassed with argon and incubated in a water bath at 30°C. The reaction was started by adding 10 mM dithionite; samples (50 μl) of the gas phase were withdrawn at intervals and injected into a model IGC 120 gas chromatograph (Intersmat Instrument, Pavillon-sous-Bois, France) equipped with a thermal conductivity detector and provided with a Porapak Q column (80 to 100 mesh; 2 m by 3.2 mm).

H_2 uptake was measured by following the reduction of methylene blue or benzyl viologen with a Zeiss PMQII spectrophotometer. Diluted fractions (0.2 ml) in 2-ml glass tubes fitted with rubber septa were gassed with H_2, and then 1.5 ml of H_2-saturated dye solution (0.2 mM methylene blue or 5 mM benzyl viologen in 20 mM Tris-hydrochloride, pH 8) equilibrated at 30°C was added. Control tubes gassed with N_2 were included to evaluate nonspecific reduction of the dyes. The reduction of methylene blue was read at 570 nm (ϵ_{mM}, 16.5 $mM^{-1} \times cm^{-1}$ [Colbeau, unpublished data]), and 50 to 300 μg of protein was used. The reduction of benzyl viologen was read at 555 nm (ϵ_{mM}, 7.55 $mM^{-1} \times cm^{-1}$ [24]), using 100 to 500 μg of protein. With whole cells and membrane fractions, 1% Triton X-100 was included to unmask all hydrogenase activity in the benzyl viologen assay.

Nitrogenase assays. Assays were performed in 4-ml vials containing 1 ml of cell suspension. Reaction mixtures were supplemented with lactate (final concentration, 20 mM) and chloramphenicol (final concentration, 20 μg/ml). The assay technique has been described elsewhere (20).

Other assays. Proteins were determined by the method of Lowry et al. (17). For whole cells, samples of the cell suspension (0.2 ml) were digested with 0.2 ml of 4% (wt/vol) cholate and 1 ml of 10% (wt/vol) NaOH for 15 min in a boiling water bath. A fraction of the mixture was assayed by using the Folin reagent.

Materials. Benzyl viologen, methyl viologen, and methylene blue were from Serva, Heidelberg, Germany; dithionite was from Merck, Darmstadt, Germany; and carbonyl cyanide-*p*-trifluoromethoxyphenyl hydrazone was from Pierce Chemical Co., Rockford, Ill.

RESULTS

Intracellular location of hydrogenase. The hydrogenase from *R. capsulata* is a cold-labile enzyme which can be stabilized under an atmosphere of H_2 (3). Most efficient cell breakage (little loss of hydrogenase activity) was achieved by two successive passages through a French pressure cell. After the first passage, unbroken cells were sedimented by centrifugation at 20,000 × *g* for 15 min at 20°C. Only the unbroken cells were pressed a second time. Under these conditions about 90% cell breakage was achieved. In all operations care was taken to ensure that the temperature did not go below 10°C. Subsequent centrifugation steps were carried out at 12°C. A total of 80% of the hydrogenase activity was recovered in the particulate fraction after centrifugation at 105,000 × *g* for 120 min. Some activity (about 8%) remained in the supernatant. However, a prolonged centrifugation (105,000 × *g* for 16 h) sedimented all material absorbing in visible light together with the hydrogenase activity. The small amounts of hydrogenase activity which remained in the supernatant fluid were thus attributed to light membrane fragments.

There was no evidence for a soluble NAD-linked hydrogenase activity in the cytoplasmic fraction. We could not detect hydrogen production with NADH (and traces of dithionite) or H_2 consumption when NAD was added as an electron acceptor.

The membrane-bound hydrogenase of *R. capsulata* could function reversibly, but the rate of H_2 production was approximately 200 times lower than the rate of H_2 uptake (3). Because of this low activity, the study of the hydrogenase was limited to H_2 uptake. Nevertheless, we observed that during cell fractionation the uptake and H_2 evolution patterns were similar (data not shown).

Hydrogenase activity in growing cells of *R. capsulata*: relationship with nitrogenase activity. Hydrogenase activity was observed in cells of *R. capsulata* under a variety of growth

conditions, even in the absence of H_2. The lowest activity was found during aerobic dark growth (Table 1). Hydrogenase activity was strongly stimulated when H_2 was present during growth, either from the addition of H_2 to the medium (for example, autotrophic growth with H_2 and CO_2) or due to nitrogenase-mediated H_2 evolution during growth (for example, heterotrophic growth in a lactate-glutamate medium) (Table 1 and Fig. 1A). The stimulatory effect of H_2 was seen clearly by examining the hydrogenase level in cells grown aerobically in the dark. If 5% H_2 was added at the beginning of growth, the hydrogenase activity was three times higher, although the growth was slightly slower (Table 1).

TABLE 1. *Hydrogenase and nitrogenase activities in R. capsulata cells grown under various conditions*[a]

Strain	Conditions	Light	Hydrogenase activity[b]	Nitrogenase activity[c]
B10	$H_2 + CO_2 + NH_4$	+	38	0
	$H_2 + CO_2 + NH_4 + O_2$	−	40	0
	$H_2 + CO_2$ + glutamate	+	39	ND[d]
	Lactate + glutamate	+	20	7
	Malate + glutamate	+	26	6
	Succinate + glutamate	+	12	8
	Lactate + NH_4	+	4	0
	Malate + NH_4	+	4	0
	Lactate + glutamate, semi-aerobiosis	−	2-6	0
	Lactate + glutamate, aerobiosis	−	2	0
	Lactate + glutamate, aerobiosis + 5% H_2	−	10-14	ND
W15	Lactate + glutamate	+	8-10	0
	$H_2 + CO_2 + NH_4^+$	+	10-12	0

[a] Cells were grown in the light as described in the text. For aerobic dark growth, 500 ml of a culture was shaken in a 2-liter flask fitted with a cotton plug and covered with aluminum foil. For semiaerobic dark growth, the culture flask was fitted with a sterile rubber septum and covered with aluminum foil. Such cultures were grown under an atmosphere containing 10% pure O_2 and 90% argon. All dark growth was carried out in a thermostatically controlled room at 30°C. Cells were harvested at different times during growth, washed, and suspended in 20 mM Tris-hydrochloride (pH 8). The cell mass was determined by measuring the absorbance at 660 nm and by protein determination. Hydrogenase activity was measured by the reduction of methylene blue, and nitrogenase activity was measured by the C_2H_2 reduction assay. The values reported correspond to the maximum specific activities of the enzymes during the growth curve.

[b] Micromoles of methylene blue reduced per hour per milligram of protein.

[c] Micromoles of ethylene formed per hour per milligram of protein.

[d] ND, Not determined.

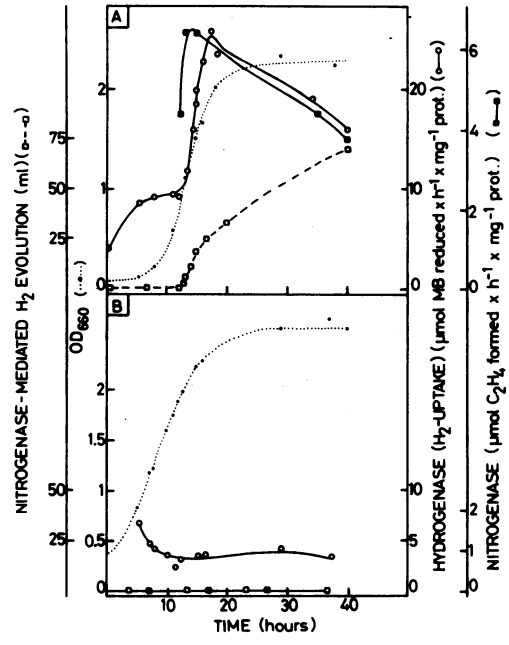

FIG. 1. *Typical hydrogenase and nitrogenase activities during photoheterotrophic growth of R. capsulata. Cells were harvested at varying times, washed, and suspended in 20 mM Tris-hydrochloride (pH 8). (A) Growth with lactate and glutamate as carbon and nitrogen sources, respectively. (B) Growth with lactate and ammonium sulfate as carbon and nitrogen sources, respectively. H_2 uptake was estimated from the reduction of methylene blue (MB) by using 0.050 to 0.2 mg of cell protein (prot.) per assay; nitrogenase activity was estimated from acetylene reduction by using 0.2 to 0.5 mg of cell protein per assay. Symbols: □, H_2 evolution; ●, optical density at 660 nm (OD_{660}); ○, hydrogenase activity; ■, nitrogenase activity.*

The highest hydrogenase activity was found under autotrophic growth conditions. However, although hydrogenase synthesis was independent of light, maximum growth rates were dependent on illumination. Under dark autotrophic conditions, a slow growth rate was observed (doubling time, 20 to 25 h compared with 3 to 5 h in the light). This is in agreement with recent findings in our laboratory which showed that phosphorylation associated with respiration on H_2 is much lower than photophosphorylation (21).

Under photoheterotrophic growth conditions (lactate-glutamate medium [Fig. 1A]) there was a sharp increase in hydrogenase activity at about the same time that visible nitrogenase-mediated H_2 production was observed. It should be noted

that intracellular H_2 production probably occurs some time before gas accumulation is observed. NH_4^+ inhibits neither the activity nor the synthesis of hydrogenase. However, when NH_4^+ replaced glutamate as the source of nitrogen, thus repressing nitrogenase biosynthesis and hence nitrogenase-mediated H_2 production, a low but constant hydrogenase activity was observed during growth (Fig. 1B).

Strain W15 of *R. capsulata* (*Nif*$^-$), which cannot evolve hydrogen during photoheterotrophic growth, had a hydrogenase activity lower than that of the wild-type strain when grown on a medium containing lactate and glutamate (Table 1). Photoautotrophic growth resulted in only a very slight increase in hydrogenase activity (Table 1).

To investigate further a possible relationship between hydrogenase and nitrogenase activities, strain B10 was grown photoheterotrophically in modified 60-ml syringes (11) in the presence of glutamate and varying concentrations of NH_4^+. Both hydrogenase activity and the amount of H_2 produced by the culture (reflecting nitrogenase activity) were followed. When ammonium sulfate (final concentrations, 0.5 to 3 mM) was added at the beginning of growth, there was a delay in the appearance of H_2 gas in the culture medium. A parallel delay in the appearance of hydrogenase activity was observed. Figure 2 shows the results obtained with 2.25 mM ammonium sulfate (Fig. 2B) and compares them with the data obtained in absence of ammonium salt (Fig. 2A). Apparently no hydrogenase was synthesized until NH_4^+ had been metabolized by the cells.

If higher NH_4^+ concentrations were used, so that no nitrogenase was synthesized during growth, hydrogenase activity remained low and constant, as shown in Fig. 1B. The shapes of the hydrogenase activity curves in cells growing with and without NH_4^+ are strikingly different, and the maximum activity in the cells grown with NH_4^+ was only about one-half the activity obtained in cells grown without NH_4^+. The maximum activity did not depend on the NH_4^+ concentration used.

Hydrogenase activity curves similar to that shown in Fig. 2B were obtained when cells were grown in aerobic medium in the dark and transferred at varying times to photosynthetic autor heterotrophic conditions (data not shown). A rapid increase in hydrogenase activity occurred only if the transfer was made before the onset of the stationary phase.

Effect of chloramphenicol on hydrogenase activity in resting cells: relationship with nitrogenase activity. We next investigated the possibility of a relationship between hydrogenase and nitrogenase activities in resting cells. Resting cell preparations of cultures grown in the dark in the presence of 10% oxygen lacked nitrogenase but had low yet significant hydrogenase activity (about 2 to 6 μmol of methylene blue reduced per h per mg of protein) (Table 1). It has already been shown in this laboratory that such cell preparations, when incubated in the light, rapidly synthesize nitrogenase (20). Nitrogenase and hydrogenase activities were compared in resting cells derived from cultures grown in the dark and subsequently incubated in the light. On illumination an immediate decrease in hydrogenase activity was observed, followed by a subsequent increase (data not shown). As was the case with nitrogenase, the maximum activity was reached after 15 to 20 h of incubation and was of the same order of magnitude as the activity found in cells grown photoheterotrophically (e.g., in medium containing lactate and glutamate) (Table 1).

Increases in both nitrogenase and hydrogenase activities were blocked by the addition of chloramphenicol either at the beginning of the incubation or after several hours of incubation

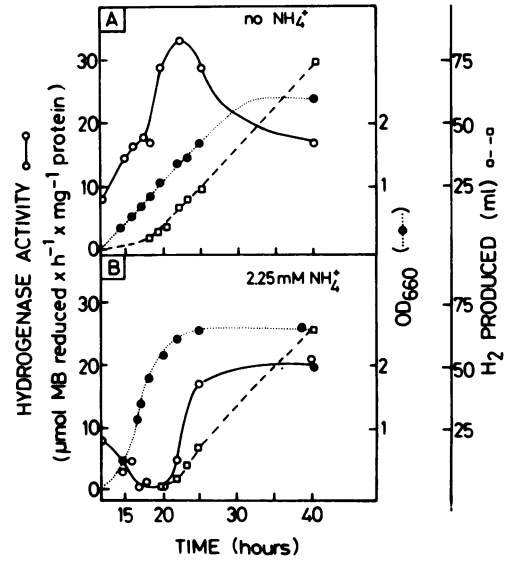

FIG. 2. *Effect of NH_4^+ ions on H_2 production and hydrogenase activity in growing cells of R. capsulata. Cells were grown in 60-ml syringes as described by Hillmer and Gest (11) in lactate-glutamate medium with or without ammonium sulfate (2.25 mM). Fractions were collected at different times, and hydrogenase activity was determined with the methylene blue (MB) assay. The amount of H_2 gas produced by nitrogenase activity and accumulated over the culture was read directly in the calibrated syringes. OD_{660}, Optical density at 660 nm.*

(unpublished data). It has already been shown (20) that the increase in nitrogenase activity upon illumination represents synthesis of this enzyme, and it is tempting to speculate a similar synthesis in hydrogenase activity. Indeed, the inhibitory effect of chloramphenicol supports this hypothesis.

Selective synthesis of hydrogenase or nitrogenase in resting cells. Although hydrogenase and nitrogenase activities increase in similar fashions when resting cells are incubated in the light, the syntheses of these two enzymes are not linked since conditions can be established where the activity of one enzyme increases but activity of the other does not. In the experiment shown in Fig. 3A, cells were incubated with 5% C_2H_2 in the gas phase. The synthesis of nitrogenase was not modified, but hydrogenase activity decreased to a low value, comparable to the activity found in cells grown photosynthetically in lactate and NH_4^+, for example. It should be noted that hydrogenase activity was determined with 0.1-ml fractions of the cell suspension, as described above; thus, no acetylene was present during the hydrogenase assay. Jouanneau et al. (12a) observed that the hydrogenase activity of *R. capsulata* strain B10 was not inhibited by 5% acetylene when assessed by either H_2 uptake or hydrogen-deuterium exchange. We have confirmed this observation. The decrease in hydrogenase activity observed (Fig. 3A) did not seem to result merely from the binding of C_2H_2 to the active site of hydrogenase, as is the case for nitrogenase.

When resting cells were incubated in the light in the presence of 1 mM ammonium sulfate (Fig. 3B), no nitrogenase activity was detected, whereas hydrogenase activity increased. This increase was abolished in the presence of chloramphenicol. Thus, Fig. 3 shows two sets of conditions, which produced opposing results. In the experiment shown in Fig. 3A, only nitrogenase activity increased; this increase has been shown to be linked to nitrogenase synthesis (20). In the experiment shown in Fig. 3B, there was synthesis of hydrogenase only.

Factors affecting hydrogenase synthesis. The effects of light and oxygen on the synthesis of hydrogenase by resting cells are shown in Fig. 4. The best synthesis of hydrogenase was obtained when resting cells were incubated in the light. The effect of O_2 was dependent on the presence of light. In the light and in the presence of 5% O_2, the final level of hydrogenase was 40% of the level in the anaerobic control (Fig. 4B). In the dark with 5% O_2, there was no significant increase in hydrogenase activity. Controls showed that O_2 gas (up to 10%) did not inhibit cellular hydrogenase activity.

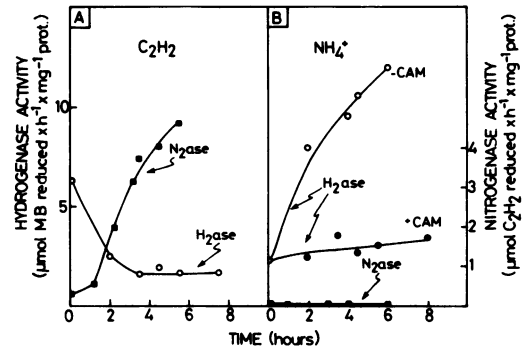

FIG. 3. *Selective synthesis of nitrogenase (N_2ase) and hydrogenase (H_2ase) upon incubation of resting cell suspensions in the light. Resting cells were prepared from a culture grown semi-aerobically (10% O_2 in argon) in the dark on 300 ml of lactate-glutamate medium. At harvest the absorbance at 660 nm was 0.8. The cells were washed, suspended in 120 ml of mineral salts medium, and distributed into four conical flasks saturated with argon (25 ml of resting cells per flask; 0.4 mg of protein per ml). The flasks were again sparged with argon and incubated at 30°C in an illuminated water bath (ca. 5,000 lx). (A) C_2H_2 (5%) was injected at the beginning of the incubation. (B) Incubation medium contained 1 mM ammonium sulfate (pH 7) and, as indicated, chloramphenicol (CAM) (20 μg/ml). At the times indicated 1 and 0.1 ml of the liquid suspension were sampled to determine nitrogenase and hydrogenase activities, respectively. MB, Methylene blue; prot., protein.*

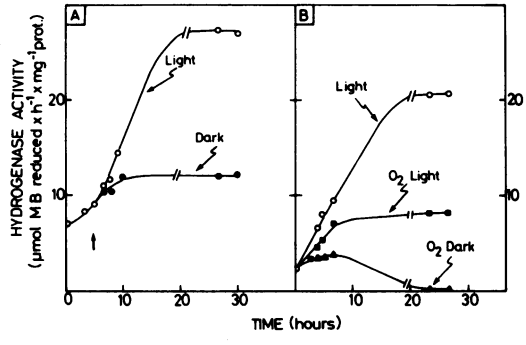

FIG. 4. *Effect of light and O_2 on hydrogenase synthesis in resting cell suspensions. Resting cells were prepared from cultures grown semi-aerobically (10% O_2 in argon) in the dark as described in the legend to Fig. 3. Samples (25 ml) of the cell suspension were distributed into 100-ml flasks, which were sparged with argon and incubated at 30°C in a water bath. Incubation conditions were as follows. (A) Two flasks were incubated in the light. At the time marked by the vertical arrow, light was shut off for one flask by wrapping it in aluminum foil. (B) Dark or light conditions were provided from the beginning of the incubation period. The O_2 concentration in the gas phase was 5%. The gas was added at zero time. MB, Methylene blue; prot., protein.*

Table 2 provides further data on hydrogenase synthesis in resting cells obtained from cultures grown under anaerobic, photosynthetic, or dark semiaerobic conditions. The maximum increase in hydrogenase activity was observed when resting cell suspensions were incubated in the light under an atmosphere containing O_2, H_2, and Ar (5:5:90, vol/vol). Replacement of this gas phase with pure 100% H_2 significantly reduced hydrogenase synthesis. The uncoupler carbonyl cyanide-*p*-trifluoromethoxyphenyl hydrazone nearly abolished hydrogenase synthesis (Table 2). Taken together, these facts indicate that the increase in activity observed in the light or in presence of oxygen (and with H_2 as electron donor) is probably linked to a sufficient availability of ATP for the synthesis of hydrogenase.

TABLE 2. *Effects of light, O_2, and H_2 on hydrogenase activity during incubation of resting cells from cultures grown semi-aerobically in the dark or from cultures grown photosynthetically in lactate-NH_4^+ medium*[a]

Addition(s)	Light	Hydrogenase activity in:	
		Dark-grown cells (semi-aerobiosis)	Light-grown cells (lactate-NH_4^+ medium)
None	+	100	100
	−	23	14
5% O_2	+	58	ND[b]
	−	22	ND
5% O_2 + 5% H_2	+	128	152
	−	57	77
H_2	+	62	60
FCCP (2 μM)[c]	+	30	22

[a] Resting cells were prepared either from cells grown semi-aerobically in the dark in lactate-glutamate medium as described in the legend to Fig. 3 or from cells grown photosynthetically on lactate-NH_4^+ medium. The optical densities at 660 nm of the resting cell suspensions were adjusted to 2.0. Portions (5 ml) of the suspensions in 15-ml flasks were gassed with argon and incubated at 30°C in an illuminated water bath. All gasses were added at zero time. For dark incubation, flasks were wrapped in aluminum foil. The incubation time was 10 h. The specific activity of hydrogenase during incubation increased from 0.6 to 3.8 μmol of methylene blue reduced per h per mg of protein in cells grown in lactate-NH_4^+ medium and from 1.7 to 16.5 μmol of methylene blue reduced per h per mg of protein in cells grown semi-aerobically. Results are given as percentages of activity increase during the 10-h incubation. The 100% control was incubated in the light without any addition.

[b] ND, Not determined.

[c] FCCP, Carbonyl cyanide-*p*-trifluoromethoxyphenyl hydrazone.

DISCUSSION

Nonsulfur purple bacteria can metabolize molecular hydrogen (7), and certain strains of *R. capsulata* are among the most efficient bacteria of this class to use H_2 as an energy source. This ability is associated with high hydrogenase activity (15).

This paper provides additional evidence that two different systems catalyze the production and utilization of H_2 in *R. capsulata* and confirms earlier reports (11, 12) that production of H_2 is mediated by the ATP-consuming light-dependent nitrogenase, whereas utilization of H_2 can occur in the dark and is catalyzed by the enzyme hydrogenase (19).

The hydrogenase of *R. capsulata* strain B10 grown under phototrophic conditions is membrane bound. This observation allowed total cellular hydrogenase activity to be determined in whole cells. However, methylene blue does not react directly with membrane-bound hydrogenase, but rather with other redox components of the membrane. This is shown by the apparent shift in the pH optimum for the reduction of methylene blue by solubilized hydrogenase (3). The problem is to determine whether changes in the compositions of membrane components may affect the value of hydrogenase activity estimated from methylene blue reduction. Indeed, all controls with the benzyl viologen method showed that qualitatively comparable results are obtained in growing cells and in resting cells, irrespective of the electron acceptor used. Both methods gave the same results when applied to the study of the distribution of hydrogenase in *R. capsulata* cells.

The *R. capsulata* strain B10 used in this study exhibited rather similar hydrogenase activities when grown either autotrophically or heterotrophically. Under autotrophic conditions, only twofold increases in hydrogenase activity were observed compared with the values obtained under photoheterotrophic conditions (Table 1). This pattern differs from the patterns in other strains of *R. capsulata*, in which hydrogenase is approximately 10 times more active in cells grown autotrophically (15). It is noteworthy that a good hydrogenase activity was also found in cells grown photoheterotrophically on lactate-glutamate or malate-glutamate medium. This differs from the situation which occurs in some knallgas bacteria (e.g., *Paracoccus denitrificans*), in which the synthesis of hydrogenase requires not only the presence of H_2 but also the absence of organic electron donors (16). This difference probably results from the presence in *R. capsulata* of a nitrogenase which actively evolves H_2 during photoheterotrophic growth (11). In fact, when nitrogenase synthesis was

repressed by NH_4^+ (photoheterotrophic growth in lactate-NH_4^+ medium [Fig. 1]) or by O_2 (dark growth in aerobiosis), the hydrogenase activity remained at the minimum level (Table 1).

Although these results indicate that the H_2 produced via the nitrogenase may influence hydrogenase synthesis and/or activity, the data presented in this paper further indicate that the syntheses of the two proteins are not linked. Indeed, conditions could be readily established whereby synthesis of one protein occurred quite independently of synthesis of the other (Fig. 3). H_2 produced by nitrogenase can activate hydrogenase synthesis, but it is not the only factor. We observed consistently that resting cell suspensions of semiaerobically grown cultures developed a high hydrogenase activity when incubated for several hours in the light. It is difficult to determine whether no H_2 is produced under these conditions, for when there is an H_2 uptake hydrogenase sufficiently active, there is no release of H_2 gas into the medium. Our data show that a combination of H_2 and O_2 resulted in maximum hydrogenase biosynthesis, whereas O_2 alone was inhibitory (Table 2). On the other hand, maximum nitrogenase synthesis required light and anaerobic conditions (20). For both enzymes, synthesis occurred when enough ATP was available. In the presence of 2 μM carbonyl cyanide-*p*-trifluoromethoxyphenyl hydrazone, which completely abolishes photophosphorylation (21), hydrogenase synthesis was strongly inhibited (Table 2); however, hydrogenase synthesis was maintained in the dark in the presence of H_2 and O_2, where oxidative phosphorylation could occur (21). The fact that chloramphenicol inhibited the increase in hydrogenase activity (Fig. 3) suggests that a true synthesis and not a mere activation of the hydrogenase took place during incubation of the resting cells in the light. The data of Fig. 3B eliminate the possibility of an indirect effect of chloramphenicol on nitrogenase. Moreover, conclusive evidence indicating a true synthesis of hydrogenase has been obtained recently by directly comparing the membrane proteins of cells rich in hydrogenase activity and the membrane proteins of cells showing poor hydrogenase activity. Only the former possess the protein hydrogenase (Colbeau et al., manuscript in preparation).

We conclude that although nitrogenase may influence hydrogenase synthesis by supplying some of the necessary factors for hydrogenase synthesis (e.g., H_2), there is no strict correlation between hydrogenase synthesis and nitrogenase synthesis.

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