

# Regulation of Carbon Monoxide Dehydrogenase and Hydrogenase in *Rhodospirillum rubrum*: Effects of CO and Oxygen on Synthesis and Activity†

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Exposure of the photosynthetic bacterium *Rhodospirillum rubrum* to carbon monoxide led to increased carbon monoxide dehydrogenase and hydrogenase activities due to de novo protein synthesis of both enzymes. Two-dimensional gels of [<sup>35</sup>S]methionine-pulse-labeled cells showed that induction of CO dehydrogenase synthesis was rapidly initiated (<5 min upon exposure to CO) and was inhibited by oxygen. Both CO dehydrogenase and the CO-induced hydrogenase were inactivated by oxygen in vivo and in vitro. In contrast to CO dehydrogenase, the CO-induced hydrogenase was 95% inactivated by heating at 70°C for 5 min. Unlike other hydrogenases, this CO-induced hydrogenase was inhibited only 60% by a 100% CO gas phase.

A diverse set of bacteria possess the ability to oxidize CO to CO<sub>2</sub> (for reviews, see references 10 through 12, 14, 15, 18, 26, and 30). In acetogenic and methanogenic bacteria, CO is oxidized by multisubunit nickel-containing CO dehydrogenase complexes (8, 9, 21). The expression of these enzymes is not affected by the presence or absence of CO, and these enzymes appear to be involved in acetate metabolism, with CO oxidation being a secondary reaction (15, 29, 30).

Aerobic carboxydophilic bacteria oxidize CO by using carbon monoxide oxidase, an oxygen-stable iron-sulfur enzyme containing flavin and molybdopterin (16-18). In these bacteria CO oxidase is induced by the presence of CO, and hydrogenase is induced in cells grown with CO or with CO<sub>2</sub> and H<sub>2</sub> (18).

Some photosynthetic bacteria tolerate CO (13), and *Rhodocyclus* (formerly *Rhodospseudomonas*) *gelatinosus* has been shown by Uffen and co-workers to utilize CO as its sole carbon and energy source during anaerobic growth in the dark (25). The membrane-bound CO-oxidizing system of *R. gelatinosus* is inducible by CO and produces CO<sub>2</sub> and H<sub>2</sub> as products of the oxidation of CO (27, 28). CO-dependent evolution of H<sub>2</sub> from extracts of *Rhodospirillum rubrum* S1 has been noted (26). We have previously reported CO-dependent formation of H<sub>2</sub> and CO<sub>2</sub> by *R. rubrum* cells grown in the light on ammonium-malate medium (6) and that exposure of light-grown *R. rubrum* cultures to CO led to significantly increased levels of CO dehydrogenase (4).

In this paper these initial observations are extended to demonstrate that CO induces two enzymatic activities, CO dehydrogenase and a CO-insensitive hydrogenase, which appear to function together to accomplish the oxidation of CO to CO<sub>2</sub> and H<sub>2</sub>. These activities are inactivated by O<sub>2</sub> both in vivo and in vitro, and the synthesis of CO dehydrogenase is shown to be inhibited by oxygen.

## MATERIALS AND METHODS

**Cell growth.** *R. rubrum* ATCC 11170 was grown in the medium of Ormerod et al. (20), except that ammonium chloride (1 g/liter) was used as a nitrogen source in place of glutamate to repress nitrogenase. Cells were grown phototrophically at 30°C under a nitrogen gas phase before exposure to CO.

**Chromatophore suspensions.** Membrane preparations (chromatophores) were derived from cells which had been treated with CO for 24 h before harvest. Cells were broken and the chromatophores were collected by centrifugation as previously described (4) and stored in liquid nitrogen.

**Enzyme assays.** CO dehydrogenase activity was assayed by using a CO-dependent methyl viologen reduction assay as previously described (6). Cells were lysed by grinding before the assay for CO dehydrogenase (5).

**Hydrogen production by whole cells.** Whole-cell production of hydrogen (Fig. 1 and 2) was measured in 25.0-ml serum-stoppered vials incubated in a shaking water bath at 30°C. Vials were filled with the appropriate gas mixtures, 15 ml of cells was injected, and the vials were vented to leave a 10.0-ml gas phase. The cells used in this experiment were grown in the light under a nitrogen gas phase to an A<sub>600</sub> of 1.5 on ammonium-malate medium.

For the experiments in Fig. 3, 4, and 6, 20 U (A<sub>600</sub> × ml) of cells were collected onto Whatman GF/C microfiber filters at the indicated times and stored in liquid nitrogen. These filters were added to 13.2-ml assay vials that contained 3.0 ml of 100 mM MOPS (morpholinepropanesulfonic acid) buffer (pH 7.5) plus 1 mM methyl viologen. Hydrogenase assays were initiated by injecting 170 μl of 1 M sodium dithionite to give a final dithionite concentration of 50 mM. Vials were incubated in a shaking water bath at 30°C. H<sub>2</sub> was measured by gas chromatography as previously described (6).

**Hydrogen assays in extracts.** Hydrogenase assays of chromatophore suspensions were performed as described above for microfiber filters, except that the buffer was 200 mM MOPS (pH 7.0) and the reactions were initiated by injection of the chromatophore suspension.

**Preparation of [<sup>35</sup>S]methionine-labeled extracts, two-dimensional gel electrophoresis, and autoradiography.** Cell samples

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† This paper is dedicated to Professor R. H. Burris with admiration for his contributions as a scientist and mentor on the occasion of his 75th birthday.

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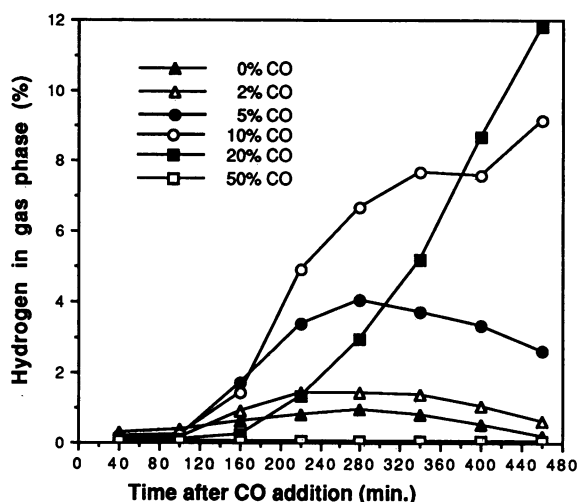


FIG. 1. CO effect on  $H_2$  production by *R. rubrum* cells in the light. Assay vials were incubated in a shaking water bath at  $30^\circ\text{C}$  in the light. Each vial contained 15 ml of cell culture ( $A_{600}$ , 5) under a 10-ml gas phase composed of CO and  $N_2$ , with CO present at the levels indicated.  $H_2$  was measured by thermal conductivity gas chromatography.

(1.0 ml) were transferred by syringe to sterile anaerobic test tubes containing  $15 \mu\text{Ci}$  of [ $^{35}\text{S}$ ]methionine and incubated in the light at  $30^\circ\text{C}$  for 5 min in the absence of CO. Samples were then frozen in dry ice and stored at  $-80^\circ\text{C}$ . For analysis, these samples were thawed, and the cells were collected by centrifugation in Eppendorf microfuge tubes. Cell pellets were then prepared for two-dimensional gel analysis and electrophoresis as previously described (22) with the following changes. Phenylmethylsulfonyl fluoride was added to the sonication solution to a final concentration of 3 mM to inhibit proteolysis, and 50- $\mu\text{l}$  samples were loaded onto the first-dimension tube gels. Two-dimensional polyacrylamide gel electrophoresis and autoradiography were performed as previously described (19, 22), except that the two-dimensional gels were fixed for 30 min with formaldehyde (23) rather than trichloroacetic acid before autoradiography.

**Protein and bacteriochlorophyll assays.** Protein was assayed by the method of Peterson as previously described (4). Bacteriochlorophyll was determined by the method of Clayton (7).

## RESULTS

**Induction of carbon monoxide dehydrogenase and hydrogenase by CO.** The time course of  $H_2$  accumulation by *R. rubrum* cells placed under various CO concentrations in the light led to the production and accumulation of  $H_2$  after a lag phase of about 100 min. Increased initial concentrations of CO led to higher levels of  $H_2$  accumulation in these cells (Fig. 1), consistent with the model that CO functioned not only as an inducer but also as the source of reductant for  $H_2$  production. The rate of  $H_2$  evolution increased as the concentration of CO was increased up to 20% CO. Above that concentration there was a longer lag phase before  $H_2$  accumulated; under 50% CO,  $H_2$  did not accumulate in the gas phase.

Figure 2 shows the time course of  $H_2$  accumulation from cells kept in the dark under various levels of CO. In the absence of CO,  $H_2$  was produced, indicating hydrogenase

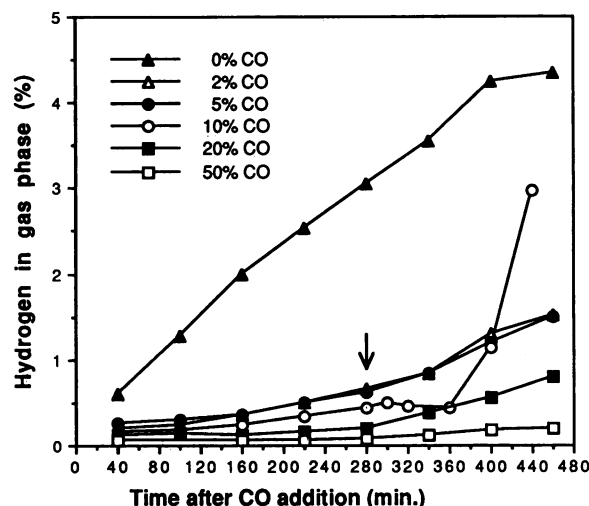


FIG. 2. CO effect on  $H_2$  production by *R. rubrum* cells in the dark. Conditions were as described for Fig. 1, except that assay vials were kept in the dark. The vial under 10% CO was shifted into the light at 280 min (arrow).

activity in these cells before their exposure to CO. CO inhibited this  $H_2$  production, and the extent of inhibition increased with increasing CO concentration. There was no CO-dependent evolution of  $H_2$  from cells in the dark, but such cells evolved  $H_2$  after a lag when placed in the light (Fig. 2, 10% CO curve). The lag phase was observed after the addition of an energy source (light), suggesting that the  $H_2$ -evolving enzyme system is induced, rather than activated, in the presence of CO.

Both CO dehydrogenase and hydrogenase enzymatic activities were low in *R. rubrum* cells grown under  $N_2$  (Fig. 3) and remained low throughout growth. When 100% CO was added to a duplicate culture (Fig. 4), both CO dehydrogenase and hydrogenase activities were induced, and these enzyme activities continued to increase even after the cells ceased to grow.

**Regulation by CO occurs at the level of gene expression.** To analyze the rate and extent of CO induction of CO dehydrogenase, the two-dimensional gel method of O'Farrell (19) was employed in conjunction with [ $^{35}\text{S}$ ]methionine pulse-labeling. Figure 5 shows the autoradiograms of two-dimensional gels of extracts from cells that had been pulse-labeled with [ $^{35}\text{S}$ ]methionine for 5 min. Figure 5A shows the proteins that were being synthesized by cells under  $N_2$ . No CO dehydrogenase protein (arrow) was produced. Figure 5B and C show the proteins that were synthesized in cells after exposure to 100% CO for 10 min and 6 h, respectively. Cells exposed to CO for only 10 min synthesized CO dehydrogenase at a rate near the maximum seen after 6 h, indicating that *R. rubrum* responded rapidly to the presence of CO. CO dehydrogenase also accumulated (as shown by staining gels for protein) and constituted one of the major proteins produced by these cells (data not shown).

The effect of oxygen upon the synthesis of CO dehydrogenase was tested. Cells that had been exposed to 100% CO for 6 h and were synthesizing CO dehydrogenase (Fig. 5C) were shifted into an atmosphere of 80% CO–20%  $O_2$ , resulting in the loss of over 80% of the existing CO dehydrogenase activity (data not shown). Additionally, the rate of synthesis of CO dehydrogenase was drastically decreased after a 5-min exposure to oxygen (Fig. 5D) and was undetectable after 10

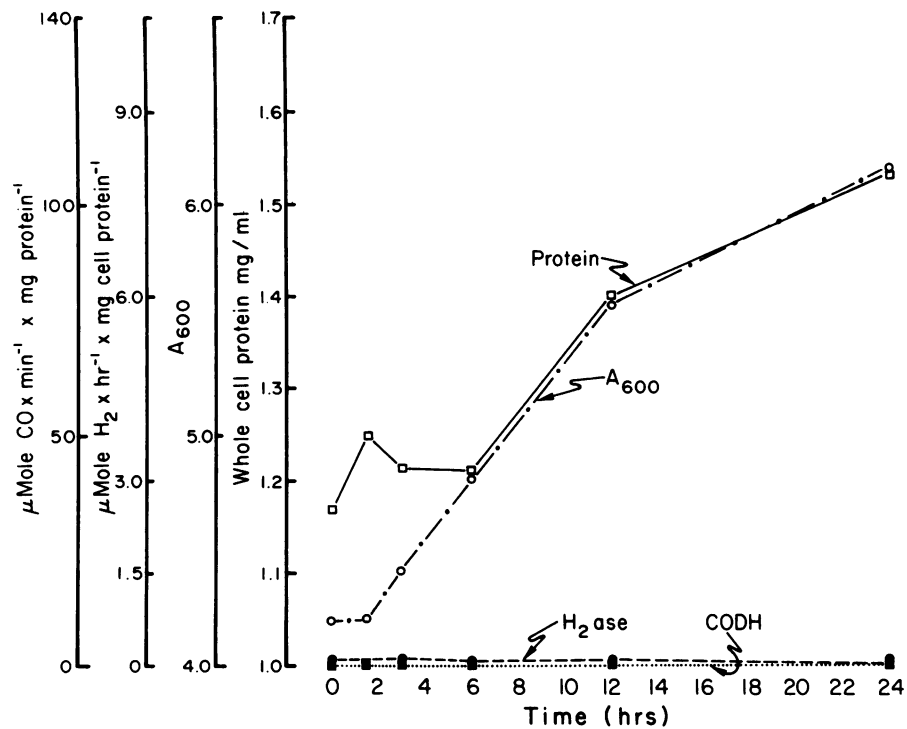


FIG. 3. Levels of CO dehydrogenase and hydrogenase activities in *R. rubrum* cells under  $N_2$ . Cells were grown at  $30^\circ C$  on ammonium-malate medium in a 5-liter illuminated fermentor under  $N_2$ . At the time points indicated, cell samples were collected by filtration and assayed for enzyme levels as described in Materials and Methods.

min (data not shown). Thus, oxygen functioned not only to inactivate the existing CO dehydrogenase in these cells but also to inhibit further synthesis of the protein. The two-dimensional gels shown in Fig. 5 demonstrate that CO induced the synthesis of the CO dehydrogenase protein.

The hydrogenase has not been purified, and therefore it is not known which, if any, of the protein spots in Fig. 5 correspond to this enzyme. Thus, even though exposure of

cells to CO led to increased hydrogenase activity (Fig. 4), it could not be concluded that this was due to de novo protein synthesis because it is possible that a preexisting hydrogenase was activated by CO treatment of these cells.

To show that the increase in hydrogenase activity required de novo protein synthesis, phototrophically grown cells were added to bottles containing a 100% CO gas phase (Fig. 6). Rifampin or chloramphenicol was added at time zero or

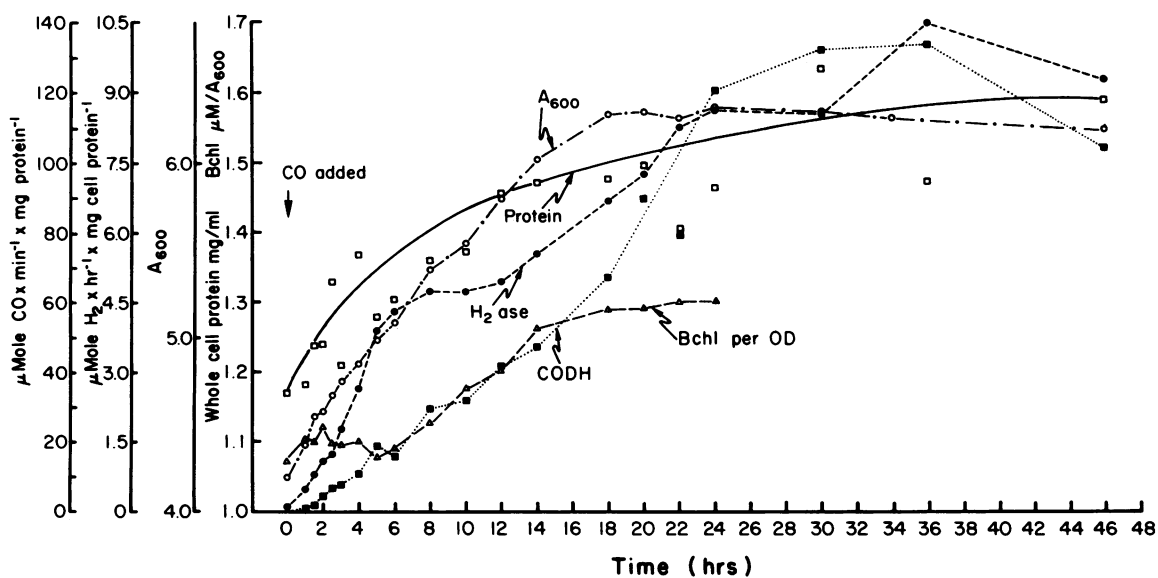


FIG. 4. Levels of CO dehydrogenase and hydrogenase activities in *R. rubrum* cells exposed to CO. Conditions were as described in Fig. 3, except that at time zero, cells were sparged with 100% CO for 30 min.

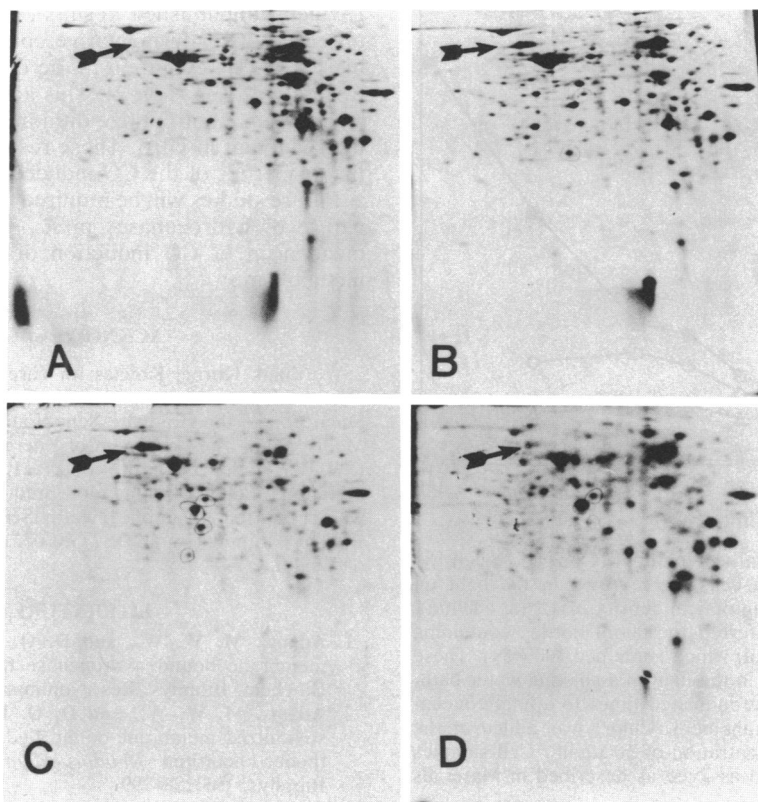


FIG. 5. Two-dimensional gel analysis of CO and O<sub>2</sub> effects on CO dehydrogenase synthesis. Each panel shows the autoradiogram of a two-dimensional gel electrophoretic analysis of an extract of cells labeled for 5 min with 15  $\mu$ Ci of [<sup>35</sup>S]methionine. Arrows indicate the positions to which CO dehydrogenase migrated in this gel system. Shown are the patterns of protein synthesis of cells (A) under N<sub>2</sub>, (B) after 10 min of exposure to CO, (C) after 6 h of exposure of CO, and (D) after 6 h under CO followed by 5 min in 20% O<sub>2</sub>-80% CO.

after 60 min, as indicated. At the times shown, cell samples were removed, collected by filtration, and assayed for hydrogenase activity.

Rifampin or chloramphenicol, added at time zero, prevented the CO-dependent increase in hydrogenase activity. However, these compounds were not inhibitors of hydrogenase activity, because the addition of either rifampin or chloramphenicol to cells 60 min after CO addition did not result in the inhibition of existing hydrogenase (Fig. 6).

**Stability of hydrogenase activities to heat and oxygen.** We have previously shown that the CO dehydrogenase from *R. rubrum* is stable to heat and is released from chromatophores by heating (4, 6). Heating the chromatophores to temperatures of 70°C or higher led to substantial loss of hydrogenase activity (data not shown), whereas CO dehydrogenase was stable under these conditions but was released from the membrane; no hydrogenase activity was present in any of the supernatant fractions.

The oxygen lability of hydrogenase was investigated in cell cultures that were grown and treated with 100% CO as described for Fig. 4. After 12 and 24 h of CO induction, samples were removed and placed in vials in the light under 80% CO-20% O<sub>2</sub> with shaking. Samples were then assayed for remaining hydrogenase activity. A 30-min exposure to O<sub>2</sub> resulted in a loss of over 15% of the original hydrogenase activity, and over 65% was lost after 4h; similar results were obtained in vitro with chromatophore suspensions, where exposure to air resulted in a 21% loss of hydrogenase activity after 30 min and a 52% loss after 3 h.

## DISCUSSION

*R. rubrum* is one of a few bacteria able to metabolize carbon monoxide; it responds to CO by inducing at least two enzymes, CO dehydrogenase and a hydrogenase, that function to carry out the oxidation of CO with concomitant production of CO<sub>2</sub> and H<sub>2</sub>. Purified CO dehydrogenase from *R. rubrum* has a high specific activity, with an activity of over 5,000  $\mu$ mol of CO per min per mg and can constitute up to several percent of the cell protein in cells induced with CO (5). Cells exposed to CO produce reducing equivalents from its oxidation. The reductant is disposed of by the reduction of protons to H<sub>2</sub>.

In this manuscript we describe the induction of CO dehydrogenase and a hydrogenase by CO and the inhibition of synthesis of at least the CO dehydrogenase by oxygen. That regulation occurs at the level of gene expression is supported by several lines of evidence: (i) pulse-labeling of cells with [<sup>35</sup>S]methionine demonstrated CODH synthesis within 5 min of CO addition and loss of synthesis within 5 min of O<sub>2</sub> addition; (ii) the addition of RNA and protein synthesis inhibitors immediately before induction prevented the expression of activity in the presence of CO but did not inhibit the activities of already synthesized CO dehydrogenase and hydrogenase; (iii) there was a considerable lag before measurable quantities of CO dehydrogenase and hydrogenase activities appeared after treatment with CO or upon transfer of cells from dark to light conditions.

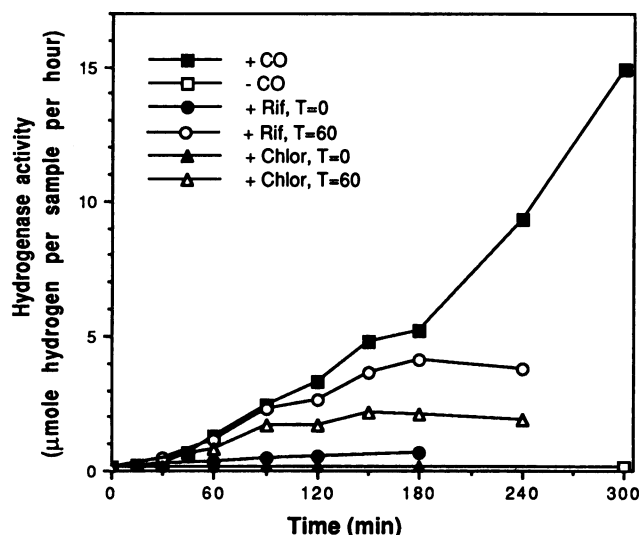


FIG. 6. Effect of protein synthesis inhibitors on whole-cell hydrogenase activity. *R. rubrum* cells were grown in the light on ammonium-malate medium to an optical density of 4.2 (at 600 nm). Samples of 200 ml were transferred to 500-ml bottles containing 100% CO, except for the control, which contained 100% N<sub>2</sub>. These bottles were incubated at 30°C in the light in a shaking water bath. Rifampin (Rif) was added at the indicated times to a final concentration of 100 µg/ml. Chloramphenicol (Chlor) was added at the indicated times to a final concentration of 10 µg/ml. Cell samples were collected by filtration and assayed as described in Materials and Methods.

These studies are complicated by the inhibition and destruction of both activities by oxygen, in vivo and in vitro. Because the protein spot responsible for hydrogenase activity has not been identified on two-dimensional gels, it is not possible to definitively distinguish between induction and activation of hydrogenase by CO and between enzyme denaturation and inhibition of hydrogenase synthesis by oxygen, although point (ii) above supports the argument that regulation of hydrogenase synthesis occurs at the level of gene expression.

The CO-induced hydrogenase activity described here is thought to represent a third class of hydrogenase in *R. rubrum*. Depending on growth conditions, *R. rubrum* may contain several enzymes capable of evolving hydrogen. These include the uptake hydrogenase purified by Adams et al. (1–3), the hydrogenase activity observed under dark, anaerobic conditions with pyruvate as the fermentable carbon source (24), and, under conditions of nitrogen starvation, nitrogenase (20). We think that the hydrogenase activity reported here is distinct from each of these. The cells used in these studies were grown on nitrogen-rich medium under conditions known to repress nitrogenase activity; thus the activity is not due to nitrogenase. The properties of this enzyme are distinct from that purified by Adams et al. in that the CO-induced activity is oxygen and heat labile, whereas the uptake hydrogenase exhibited a  $t_{1/2}$  of 6 days in air at 20°C and no loss of activity at 80°C for 20 min. Furthermore, the uptake hydrogenase was completely inhibited by 40% CO, a property not shared with the CO-induced enzyme. The data are insufficient to definitively distinguish between the hydrogenase produced by *R. rubrum* when growing fermentatively and the CO-induced hydrogenase, but CO dehydrogenase activity is not observed under conditions of dark, anaerobic, pyruvate-dependent growth (Bonam and

Ludden, unpublished results). The hydrogenase activity observed under fermentative conditions might be responsible for the H<sub>2</sub> evolution in the dark by *R. rubrum* cells not treated with CO (Fig. 2). This activity was inhibited by CO and was unable to utilize dithionite plus methyl viologen as the reductant in vitro. These results are not consistent with the properties of the CO-induced hydrogenase.

Future studies will be required to establish the number and nature of hydrogenases in *R. rubrum* and the molecular mechanism of CO induction of enzymes required for its metabolism.

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