JOURNAL OF BACTERIOLOGY, Aug. 1984, p. 693–699
0021-9193/84/080693-07$02.00/0

Vol. 159, No. 2

# Carbon Monoxide Dehydrogenase from *Rhodospirillum rubrum*

DUANE BONAM, SCOTT A. MURRELL, AND PAUL W. LUDDEN*

*Department of Biochemistry, The College of Agriculture and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706*

Received 26 March 1984/Accepted 15 May 1984

**The carbon monoxide dehydrogenase from the photosynthetic bacterium *Rhodospirillum rubrum* was purified over 600-fold by DEAE-cellulose chromatography, heat treatment, hydroxylapatite chromatography, and preparative scale gel electrophoresis. In vitro, this enzyme catalyzed a two-electron oxidation of CO to form $CO_2$ as the product. The reaction was dependent on the addition of an electron acceptor. The enzyme was oxygen labile, heat stable, and resistant to tryptic and chymotryptic digestion. Optimum in vitro activity occurred at pH 10.0. A sensitive, hemoglobin-based assay for measuring dissolved CO levels is presented. The in vitro $K_m$ for CO was determined to be 110 μM. CO, through an unknown mechanism, stimulated hydrogen evolution in whole cells, suggesting the presence of a reversible hydrogenase in *R. rubrum* which is CO insensitive in vivo.**

The ability of bacteria to tolerate and utilize carbon monoxide has been studied since the beginning of this century. The first demonstration of in vitro CO oxidation to yield $CO_2$ was by Yagi in 1958, using extracts of *Desulfovibrio desulfuricans* (37). The enzyme which carries out this reaction has been named carbon monoxide dehydrogenase (CODH) and has since been found in a diverse set of species, including both aerobic and anaerobic bacteria (4–7, 12, 17, 18, 21, 23, 29, 30; for reviews, see references 13, 24 and 31).

The CODHs from the aerobic bacteria *Pseudomonas carboxydovorans* (22, 23) and *Pseudomonas carboxydohydrogena* (17) have been purified and characterized. These enzymes are induced by CO, stable to oxygen, and contain FAD as a prosthetic group. In addition, the CODH from *P. carboxydovorans* contains molybdenum, iron, and acid-labile sulfur. Nickel is not present in these enzymes.

In contrast, the CODHs from anaerobic organisms are oxygen-labile enzymes and do not require CO for induction. Several are reported to be heat stable (10, 29, 38) and inhibited by cyanide, and all are able to reduce low potential electron acceptors such as the viologen dyes.

The metabolic role of CODH has been the subject of considerable discussion, and there may be different roles for the enzyme in different organisms. In the best-understood anaerobic system, that of *Clostridium thermoaceticum*, the CODH plays a key role in the synthesis of acetyl coenzyme A from CO (11, 16). The enzyme from *C. thermoaceticum* contains nickel (10, 26), and experiments on the electron paramagnetic resonance signal of the enzyme indicate that this nickel interacts with the substrate CO (27). The CODHs from other anaerobes have also been suspected to contain nickel (6, 8, 9).

The ability of nonsulfur photosynthetic bacteria to tolerate CO was first noted by Hirsch (14). Uffen has documented the ability of *Rhodospirillum rubrum* grown in the dark under 100% CO to oxidize CO with concomitant production of $H_2$ (31). Uffen has also characterized the CO-oxidizing system of *Rhodopseudomonas gelatinosa* strain 1 and has shown that stoichiometric amounts of $CO_2$ and $H_2$ are produced during CO oxidation by these cells (30, 32). The organism will grow readily with CO as the sole carbon and energy source, and evidence suggests that $CO_2$ is refixed via the Calvin cycle, with the $H_2$ produced during CO oxidation as a reductant. How the cell is able to generate ATP anaerobically in the dark with only carbon monoxide as a substrate remains a question.

In this investigation the CODH from *R. rubrum* was studied. The enzyme was purified 600-fold and some of its properties were determined. The enzyme appears to be similar in some respects to the CODHs from other anaerobic bacteria. *R. rubrum* does not readily take up nickel, and definitive evidence for or against the presence of nickel in this enzyme has not yet been obtained. A method is presented for determining dissolved carbon monoxide levels in anaerobic solutions.

## MATERIALS AND METHODS

**Growth of *R. rubrum*.** *R. rubrum* ATCC 11170 was grown on the glutamate-malate medium of Ormerod et al. (25) under anaerobic, phototrophic conditions at 30°C. Cells were concentrated by a Millipore pellicon cassette system with an HVLP filter (pore size, 0.5 μm; Millipore Corp., Bedford, Mass.) before being collected by centrifugation at 6,500 × *g* for 10 min. The cell paste was frozen and stored in liquid nitrogen until needed. Cell cultures used for experiments in which the gas exchange by whole cells was measured were grown on an ammonium chloride-malate medium to repress synthesis of nitrogenase.

**Purification of CODH.** All procedures were carried out anaerobically, and dithionite was present in all enzyme-containing solutions. Frozen cells were thawed in 200 mM morpholinepropanesulfonic acid (MOPS) buffer (pH 7.5) containing 5 mM dithionite and 1 mM dithiothreitol. The cells were broken by mechanical shearing with a bead-beater (Biospec Products, Bartlesville, Okla.) in an anaerobic glove box under an atmosphere of $N_2$-$H_2$ (96:4, vol/vol). A total of 400 g of cells were broken in a total volume of 1,200 ml, and the extract was centrifuged at 53,000 × *g* for 16 h to remove unbroken cells and membrane fragments (chromatophores). The supernatant was applied to an anaerobic DEAE-cellulose column (16 by 4 cm), and the column was washed with 100 mM NaCl in 50 mM Tris buffer (pH 7.7). The enzyme was eluted from the column during a 100 to 400 mM NaCl gradient in 50 mM Tris buffer (pH 7.7). Fractions containing CODH activity were pooled and heat treated. The following protocol was followed for heat treatment. Samples of 20 ml

* Corresponding author.

at room temperature were placed in serum-stoppered Oakridge tubes under an atmosphere of $N_2$ and heated at 60°C for 5 min. The tubes were then heated at 80°C for an additional 5 min before being cooled on ice. Precipitated proteins were removed by centrifugation at 35,000 × *g* for 30 min. The supernatant containing CODH was removed and applied to a column (12 by 2.5 cm) of Calbiochem fast-flow hydroxylapatite. This column was washed with 60 ml of 5 mM potassium phosphate in 50 mM Tris-hydrochloride buffer (pH 7.7), followed by elution of the enzyme with a total volume of 260 ml of potassium phosphate gradient (5 to 50 mM) in 50 mM Tris-chloride buffer (pH 7.7). Fractions containing activity were concentrated with an Amicon model 52 ultrafiltration cell with a YM5 membrane. The concentrated fraction was applied to a preparative polyacrylamide gel electrophoresis unit as previously described (20). The following alterations in the preparative gel electrophoresis were made: a 7%, instead of an 8.3%, separating gel was used, and 1% Triton X-100 was included in the separating gel, stacking gel, and sample. The presence of Triton X-100 improved resolution but also prevented the stacking and separating gels from adhering to the glass electrophoresis apparatus; thus, it was necessary to include a 0.5-cm support spacer made from glass beads and plastic mesh between the lower supporting gel and the upper Triton X-100-containing gels to prevent the slipping of the upper gel. The CODH was washed from the gel with 50 mM Tris-hydrochloride (pH 7.7) and then applied to a small hydroxylapatite column for concentration. Activity was eluted from the hydroxylapatite column with 50 mM phosphate in 50 mM MOPS buffer (pH 7.5).

**CODH assays.** CODH activity was measured by the change in absorbance at 578 nm due to the reduction of methyl viologen (MV). A 1-ml assay mixture containing 10 mM MV and 50 mM lysine buffer (pH 10.0) in a serum-stoppered quartz cuvette was made anaerobic by being bubbled with 100% CO for 5 min and then prereduced with a trace of dithionite before injection of the enzyme.

Metabolism of CO, $CO_2$, and $H_2$ by whole cells was followed by measuring these gases with a Gow-Mac series 550 thermal conductivity gas chromatograph. A molecular sieve 5A (Supelco) column (5 feet [ca. 1.5 m] by 1/4 inch [ca. 0.6 cm]) was used when measuring CO, and a Porapak R column (5 feet [ca. 1.5 m] by 1/4 inch [ca. 0.6 cm] was used for $CO_2$ measurements. Helium was used as the carrier gas in both cases at a flow rate of 60 ml/min. The column chamber was heated to 150°C. $H_2$ was measured by use of either of these columns with $N_2$ as the carrier gas.

For radioactive assays of CODH activity, $^{14}CO$ was generated in individual serum-stoppered reaction vials containing two 1.5-ml microfuge tubes. An appropriate volume of formate (5 μCi/ml in 79% formic acid) enriched with $^{14}C$ label was injected into 175 μl of concentrated $H_2SO_4$ contained in one of the microfuge tubes. The formation of $^{14}CO$ was complete after 2 h at room temperature. CODH assays were initiated by the addition of enzyme to the reaction mixture in the bottom of the vial and terminated by the addition of $H_2SO_4$ to the reaction mixture. The product $^{14}CO_2$ was trapped from the gas phase by injecting 250 μl of Carbo-Sorb (United Technologies Packard, Downers Grove, Ill.) into the second microfuge tube. The Carbo-Sorb was removed after 30 min, and $^{14}CO_2$ was measured by scintillation counting.

**Materials.** All materials required for preparation of growth medium, including malate, glutamate, EDTA, and MOPS buffer were purchased from Sigma Chemical Co., St. Louis, Mo. Dimethyl glyoxime, α,α'-dipyridyl, and crystallized bovine hemoglobin were also obtained from Sigma. Formic acid was obtained from Fisher Scientific Co., Pittsburgh, Pa.; $^{63}NiCl_2$ and [$^{14}C$]formic acid were obtained from Amersham Corp., Arlington Heights, Ill.; dithionite was obtained from J. T. Baker Co., Phillipsburg, N.J.; cylinder gases were obtained from Matheson Gas Company, Joliet, Ill.; and Triton X-100 was obtained from Research Products International Corp., Elk Grove Village, Ill.

## RESULTS

**Carbon monoxide determination by hemoglobin binding.** Levels of CO in anaerobic solutions were measured by utilizing the change in the absorbance spectrum of hemoglobin (Hb) upon binding CO. The differences between the spectra of Hb and carboxy Hb are particularly pronounced in the soret region of the spectrum. For example, the extinction coefficient for Hb of 420 nm increases from 109.5 to 192.0 $mM^{-1}$ $cm^{-1}$ upon binding CO. Similarly, the extinction coefficient at 430 nm decreases from about 140 to 60.0 $mM^{-1}$ $cm^{-1}$ when CO binds (33). These differences provide the basis for a sensitive assay for dissolved CO. An anaerobic stock solution of 0.2 mg of bovine Hb per ml in 0.9% NaCl–10 mM MOPS buffer (pH 7.5)–2 mM dithionite was used. Dithionite was present to convert any methemoglobin (ferric) to the reduced (ferrous) species. By removing oxygen, it also converts any oxy Hb to Hb, thus eliminating the interfering spectral contributions of both the oxidized and the oxygenated forms. One milliliter of Hb stock was added to both a reference and a sample quartz cuvette. From 1 to 5 μl of a sample solution was injected into the sample cuvette and gently mixed. A difference spectrum was recorded at room temperature of the reference versus the sample cuvette from 460 to 400 nm with a Cary 14 or a Cary 210 spectrophotometer.

Figure 1 shows typical difference spectra obtained from the Hb assay when several different volumes of CO-saturated buffer were tested. Each microliter of CO-saturated buffer contained 0.88 nmol of CO at room temperature. The total absorbance change from the peak at 419 nm to the trough at 431 nm correlated with the CO content of the added sample. The average of the second and third scans of each sample was used. The initial scan, for unknown reasons, often gave a falsely elevated reading. Figure 2 shows the standard curve generated when this procedure was used. The CO content of an unknown sample can be readily determined by comparison to this standard curve.

**Purification of CODH.** Table 1 is a summary of the purification of CODH. The first steps of this purification scheme were done in common with the purification of the Fe protein of nitrogenase. Gradient elution of the DEAE column allowed separation of the nitrogenase Fe protein and CODH. The CODH from *R. rubrum* was quite stable to heat, as are the CODHs from several other bacteria. The heating step was found to be rapid and reproducible, as evidenced by the fact that 6- to 8-fold purification with a yield of ≥85% was routinely obtained. Heating at 60 or 70°C for 5 min had no effect on the measured activity. Heating for 5 min at either 80 or 90°C led to only a 5 or 31% loss of activity, respectively. Presence of the substrate CO did not further stabilize the enzyme during heating and, in fact, led to more rapid inactivation during extended heating at temperatures between 60 and 80°C. The heat treatment removes the majority of the protein in the sample and thus prevents overloading of the hydroxylapatite column used in the next step. The yield from the hydroxylapatite column is typically above 50% with a three- to fourfold purification. A 6.6-fold

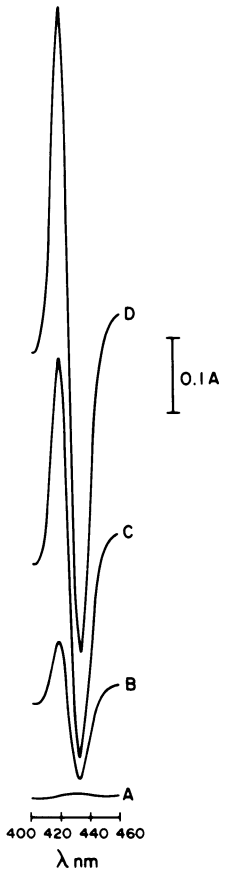


FIG. 1. Response of the Hb assay to various volumes of CO-saturated buffer. Responses with no additions (A) and with 1 (B), 3 (C), and 5 (D) μl of CO-saturated buffer are shown. Each microliter of CO-saturated buffer contained 0.88 nmol of CO.

purification with a yield of 58% was achieved in the final step, in which preparative gel electrophoresis was used. The enzyme was purified over 600-fold when this protocol was used (Table 1). A maximum specific activity of 95 μmol of MV reduced per min/mg of protein was achieved. Sodium dodecyl sulfate gel patterns of purified fractions of CODH exhibit a number of protein bands, and it is not known at this time which band(s) corresponds to the CODH protein (gel not shown).

**Properties of CODH.** The highest rate of MV reduction occurred between pH 9.5 and 10.5. Activity decreased rapidly above or below this range. At pH 7.0 the observed activity was less than 25% of that seen at pH 10.0 (Fig. 3).

The CODH from *R. rubrum* is extremely oxygen sensitive. No measurable activity could be recovered after exposure of the purified enzyme to saturating $0_2$ levels for as little as 10 s at room temperature (data not shown). In experiments run to demonstrate the proteinaceous nature of CODH activity, the presence of trypsin caused no differences in CODH activity, as measured by $^{14}CO_2$ formation over a period of several hours. This observation led to experiments (Fig. 4) in which the effects of various proteases on remaining activity were shown. CODH activity was resistant to proteolytic digestion by trypsin and chymotrypsin, but exposure of CODH to the nonspecific protease subtilisin quickly resulted in diminished activity. The resistance to trypsin digestion may be a useful method for the removal of contaminating proteins.

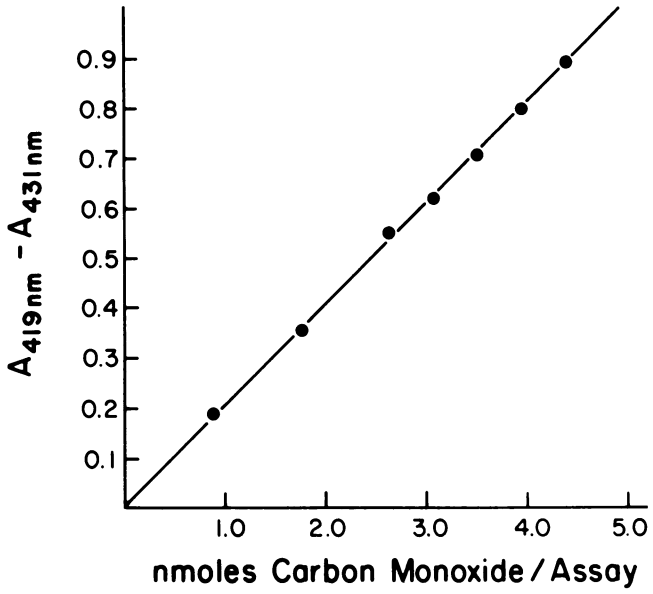


FIG. 2. Standard curve for the assay of dissolved CO with Hb. Assays were run as described in the text. $A_{419nm}$ and $A_{431nm}$, Absorbance at 419 and 431 nm, respectively.

To determine whether CODH from *R. rubrum* is soluble or membrane bound, a crude extract of *R. rubrum* was centrifuged at 144,000 × *g* for 2 h. More than 95% of the activity was recovered in the supernatant fraction, indicating that *R. rubrum* CODH is a soluble enzyme in vitro.

**Reactants and products of CODH.** The reaction carried out by this enzyme is believed to be $CO + H_2O \rightarrow CO_2 + 2H^+ + 2e-$. Evidence that CO is a substrate comes from the strict dependence of MV reduction on the presence of CO. In addition (Fig. 5), no loss of CO occurred, as measured by the Hb assay, until MV was added to the reaction mixture, after which a rapid loss of dissolved CO was seen. This result indicates that, in the absence of an electron acceptor, the reaction does not proceed and the reducing equivalents formed are not used to reduce protons to $H_2$ directly on the enzyme.

The possibility that $H_2$ was produced by preparations of CODH was also checked and ruled out by results of gas chromatography. No detectable formation of $H_2$ was observed during the reaction of CO with partially purified CODH in the absence of an electron acceptor, again supporting the conclusion that this enzyme will not function in the absence of an electron acceptor. $CO_2$ was shown to be the product of the reaction by using $^{14}CO$ as substrate. Radioac-

TABLE 1. Purification and yield of CODH from *R. rubrum*

| Step | Protein Concn (mg/ml) | Total vol (ml) | Sp act[a] | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|
| Crude extract | 22.36 | 1,260 | 0.152 | 1 | 100 |
| Centrifuged extract | 10.25 | 1,260 | 0.293 | 1.93 | 88 |
| DEAE-cellulose | 18.0 | 300 | 0.547 | 3.60 | 69.1 |
| Heat treatment | 2.64 | 260 | 3.662 | 24.1 | 58.6 |
| Hydroxylapatite | 4.30 | 9.2 | 14.48 | 95.3 | 13.4 |
| Preparative gel | 0.55 | 6.4 | 95.4 | 627.6 | 7.8 |

[a] Specific activity is expressed as micromoles of MV reduced per minute per milligram of protein.

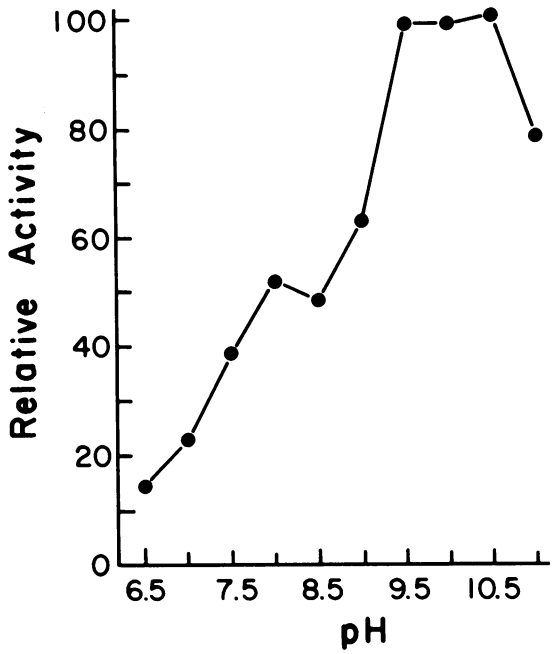


FIG. 3. Effect of pH on CODH activity. Assays were performed as described in the text, except the buffer was adjusted to the indicated pH. The buffer system included 50 mM each of morpholineethanesulfonic acid, MOPS, Tris, and lysine. The pH was adjusted to the desired value with NaOH or HCl.

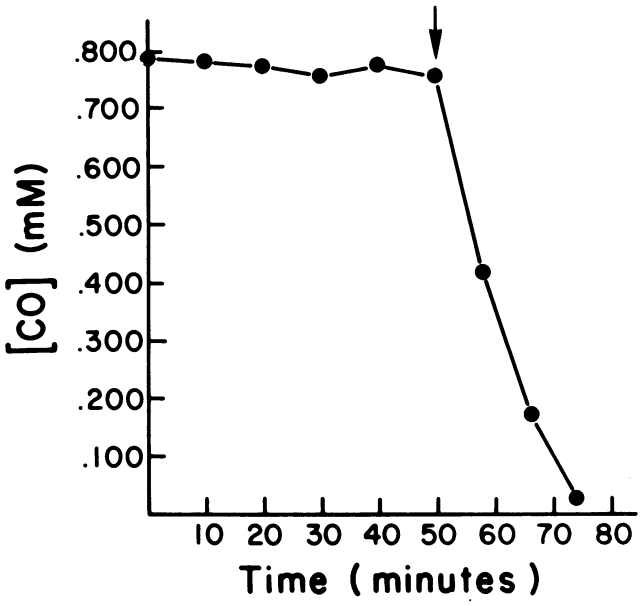


FIG. 5. Measurement of dissolved CO levels during reaction. Assays were run in a serum-stoppered glass syringe with no gas phase. Assays were initiated by the injection of CO-saturated CODH in 50 mM lysine buffer (pH 10.0). Samples of 3 μl were removed at the indicated times and assayed for dissolved CO content by the Hb assay. MV was added (arrow) to a final concentration of 5 mM.

tivity due to $^{14}CO_2$ was observed only in the complete reaction mixture with native enzyme. Substitution of boiled or $O_2$-inactivated enzyme in place of native enzyme or elimination of the electron acceptor resulted in background levels of counts, indicating that formation of the product $CO_2$ did not occur.

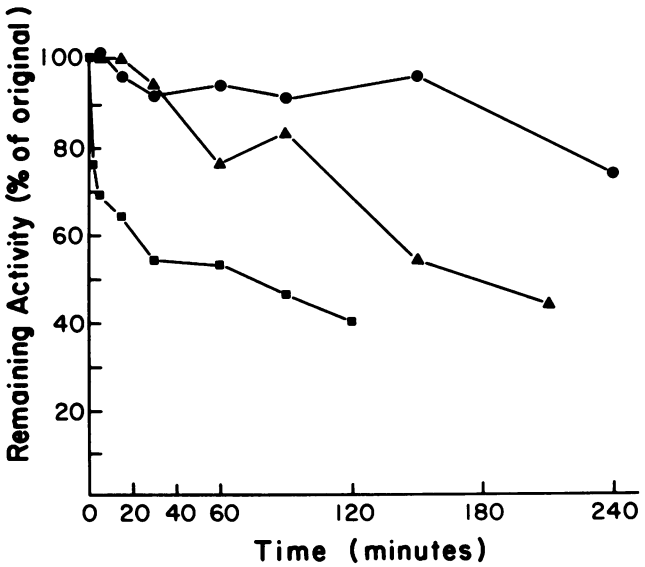


FIG. 4. Effect of protease on CODH activity. Freshly prepared anaerobic solutions of each protease (1 mg/ml) in 50 mM MOPS buffer (pH 7.5) containing 1 mM $Na_2S_2O_4$ were added to partially purified CODH (preparative gel; specific activity, 64 μmol of MV reduced $min^{-1}$ $mg^{-1}$) at time zero. Each protease was present at a final concentration of 5% (wt/wt). Remaining activity was determined by assaying 2-μl samples at the indicated times by the MV reduction assay. Symbols: ●, trypsin; ▲, chymotrypsin; ■, subtilisin.

Evidence that the in vitro reaction is indeed a two-electron oxidation of CO was obtained with the Hb assay. CO-saturated buffer containing a range of MV concentrations was injected into a serum-stoppered glass syringe with no gas phase. After the initial dissolved CO level was measured, CODH and excess CO was injected into the syringe and allowed to react to completion, and the CO level was again measured. A plot of the observed decrease in dissolved CO versus the initial MV concentration indicated a ratio of MV reduced to CO oxidized of 2.32 (Fig. 6).

CODH is able to utilize several different electron acceptors in vitro. MV and benzyl viologen function quite well, with MV supporting the highest in vitro rate of any electron acceptor tested. Table 2 shows a comparison of the extent of reaction ($^{14}CO_2$ assay) supported by various electron acceptors with MV as 100%. The in vivo electron acceptor is not known.

**Kinetic constants.** The $K_m$ for the electron acceptor MV was determined by varying the concentration of MV under saturating CO conditions and observing changes in the initial velocity. A double reciprocal plot of the data indicated a $K_m$ of 0.80 mM. The $K_m$ for CO was determined with the Hb assay to measure substrate concentration. The sample cuvettes containing lysine buffer were flushed with CO or $N_2$, followed by injection of small amounts of CO for subsaturating conditions. After mixing, 5-μl samples were removed and assayed to determine the dissolved CO concentration. A 20-μl volume of 500 mM MV under $N_2$ was then injected into each sample cuvette and shaken. The cuvettes were prereduced with a trace of dithionite before injection of CODH, and the initial velocity of substrate reduction was determined. A double reciprocal plot of the data indicated a $K_m$ for CO of 0.11 mM. In these experiments, MV was added

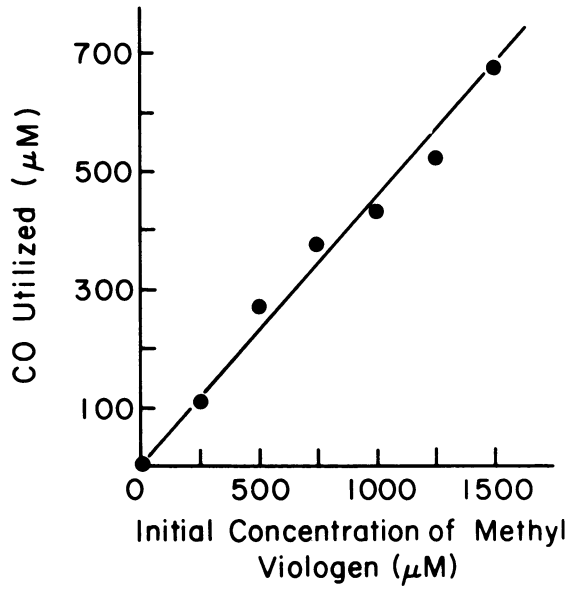


FIG. 6. Stoichiometry of CO and MV utilization by CODH. Reactions were run in a 1.0-ml serum-stoppered glass syringe containing 500 μl (final volume) of CO-saturated 50 mM lysine buffer (pH 10.0) plus 0 to 1,500 μM MV. The dissolved CO level of each reaction mixture was measured before and 10 min after addition of excess CODH. The manipulations and CODH preparation used in experiments shown here led to a CO utilization of 33.8 μM over 10 min in the absence of MV, and this value was subtracted from all other values. The best-fit line gives a ratio of MV reduced per CO oxidized of 2.32.

TABLE 2. Electron acceptors utilized by CODH[a]

| Electron acceptor | Extent of reaction (%) |
|---|---|
| MV | 100 |
| Benzyl viologen[b] | 95 |
| Methylene blue | 0.9 |
| DCPIP | 0 |
| NAD | 0 |
| NADP | 0 |
| Riboflavin | 23.7 |
| Cytochrome *c* | 0 |

[a] All electron acceptors were present at a concentration of 5 mM, except cytochrome *c*, which was present at 1 mM. Reactions were run at pH 10.0 under a 20% CO–80% $N_2$ gas phase containing $10^5$ cpm of $^{14}CO$. Reactions were run for 20 h before termination with $H_2SO_4$ and trapping of $^{14}CO_2$. A total of 9,070 cpm was trapped when MV was used as substrate, which was set to equal 100%.

[b] Activity with benzyl viologen was compared by spectrophotometric assay.

after the measurement of the CO level, owing to the interfering absorbance of its reduced form at this high concentration in the Hb assay.

**Inhibitors of CODH.** The effect of various inhibitors and chelating agents on in vitro CODH activity, as measured by MV reduction, was investigated. Cyanide, a known inhibitor of CODH from other anaerobes, inhibits the *R. rubrum* enzyme. A cyanide concentration of 650 μM caused a 50% decrease in the rate of MV reduction after 1 min. The presence of cyanide caused an exponential decay in the rate of MV reduction throughout the time course of the standard assay. These results indicate that cyanide did not function as a reversible competitive inhibitor but rather as an inactivator of the enzyme which is irreversible during the time scale of the assay. EDTA and sodium azide had no effect at any level tested up to 10 mM. A final concentration of 10 mM α,α′-dipyridyl resulted in only 19% inhibition. The vitamin $B_{12}$ binding protein, intrinsic factor, showed no inhibition at any level tested up to 2 U per assay. One unit of intrinsic factor binds 1.0 ng of vitamin $B_{12}$ at pH 7.5 and 25°C.

Dimethyl gloxime, a nickel-specific chelator, was also tested as an inhibitor of CODH and gave 50 and 97% inhibition of CO-dependent MV reduction activity at 125 and 250 μM, respectively. Added $Ni^{2+}$, however, did not protect against this inhibition, so it is not possible to conclude that dimethyl glyoxime inhibits by binding enzyme-bound Ni.

**Whole-cell utilization of CO.** The utilization of CO by whole cells was studied by measuring changes in the gas phase above a cell suspension with gas chromatography. Figure 7 shows the utilization of CO and concomitant formation of $CO_2$ and $H_2$ over an 8-day period by cultures of *R. rubrum* in the light. In vials initially under 100% CO in the light, the $CO_2$ and $H_2$ levels after 8 days were 12- and 85-fold higher, respectively, than those in the controls run in the absence of CO. In similar vials which were kept in the dark, no measurable change in CO occurred, and the increase in $CO_2$ was only 1.8-fold above that of the control. No increase in the $H_2$ level was seen. Thus, in whole cells, a CO-dependent formation of $CO_2$ and $H_2$ was observed. This process appeared to require energy, however, as it did not occur in cells in the dark.

**Nickel uptake by *R. rubrum*.** Nickel has been shown to occur in the CODHs from several bacteria (9, 10), and nickel

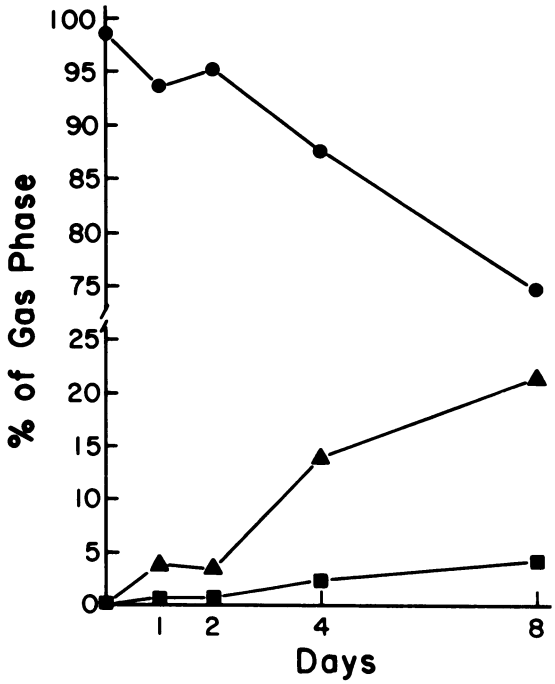


FIG. 7. CO-dependent $CO_2$ and $H_2$ formation in phototrophically grown cells. The gas phase above late-log-phase cells was assayed at the times shown for CO (●), $H_2$ (▲), and $CO_2$ (■). Serum-stoppered vials (25 ml) originally contained 10 ml of cell suspension under 100% CO. An initial gas phase of either 100% He and 100% $N_2$ was used in control vials for measurement of $CO_2$ and $H_2$, respectively.

is specifically taken up and incorporated into the CODH of *C. thermoaceticum* (10). The ability of *R. rubrum* to take up $^{63}Ni$ from the medium was studied. To a 500-ml culture of growing cells, 5 μCi of $^{63}Ni$ (0.07 μmol) was added to give a final nickel concentration of 0.14 μM. The culture was followed for growth by measurement of absorbance at 600 nm, $^{63}Ni$ incorporated into the cells, and $^{63}Ni$ remaining in the medium after cells were removed by centrifugation. The $^{63}Ni$ associated with cells was measured by collecting 0.5-ml samples of cells on Metricell GA8 membrane filters, followed by washing with 10 ml of medium containing 10 mM $NiCl_2$ to reduce nonspecific $^{63}Ni$ binding. The filters were then placed in scintillation fluid for counting. The results of this experiment indicate that, after 49 h of growth and a 4.7-fold increase in cell density, less than 3% of the radioactive nickel was found to be associated with the cells. The nickel remaining in the medium and the nickel per unit of cells remained essentially unchanged throughout the experiment. The slow increase in nickel per unit of cells during the first few hours and the lack of further increase during subsequent growth argues against any specific nickel uptake by these cells.

## DISCUSSION

CO-oxidizing enzymes were isolated from a variety of organisms and fell into two groups: (i) the oxygen-stable, molybdenum, FAD, and non-heme iron-containing enzymes from aerobes, and (ii) the oxygen-labile, nickel-containing enzymes from anaerobes. In this paper the properties of a CO-oxidizing system from the photosynthetic bacterium *R. rubrum* are discussed. This enzyme is similar to the enzymes from the anaerobic bacteria in many respects. It is extremely oxygen labile and thus resembles the CODHs from *C. thermoaceticum* and *Clostridium pasteurianum*. The enzyme is also soluble, as are the CODHs from other anaerobes, with the exception of the membrane-bound CODH from *R. gelatinosa* (35). At low pH the *R. rubrum* CODH does show some affinity for the pelletable fraction of the cell extract, but the bulk of the activity remains soluble. The enzyme is also quite heat stable. CO is not required during growth for induction of CODH activity. On the basis of these similarities to other anaerobic CODHs, it was presumed that the enzyme would contain nickel. Definitive evidence for or against the presence of nickel in this enzyme has proven elusive. The following initial evidence suggested an absence of nickel in the enzyme: the cells took up $^{63}Ni$ very poorly from the medium, the small amount of nickel found in the cell extract did not increase in specific activity as the enzyme was purified, the nickel chelator dimethyl glyoxime could not be shown to specifically inhibit CODH by its chelation activity, and in early experiments no radioactivity due to $^{63}Ni$ comigrated with CODH activity on native polyacrylamide gels. In contrast, recent experiments in which highly purified and concentrated CODH from $^{63}Ni$-grown cells was used have shown comigration of CODH activity and a small peak of $^{63}Ni$ counts on tube gels. We remain unconvinced at this time that nickel is present in stoichiometric amounts and that the observed counts are not simply due to a nonspecific association of $^{63}Ni$ with the protein.

The enzyme has been characterized with respect to its substrates and products. Whole cells of *R. rubrum* will oxidize CO, and the products found in the gas phase are $CO_2$ and $H_2$. By using $^{14}CO$, it was possible to show that $^{14}CO_2$ was obtained. The reaction was found to proceed more rapidly in the light than in the dark. This result is in contrast to the work with *R. gelatinosa* strain 1 by Uffen (30), in which CO was better oxidized in the dark. The production of $H_2$ by cells in the light in the presence of CO is of interest. Although the purification of hydrogenase from photoheterotrophically grown *R. rubrum* has been reported (1), there is not convincing evidence in the literature for hydrogen evolution by phototrophically grown *R. rubum* that is not attributable to the action of nitrogenase. Cells used in the experiments shown in Fig. 7 were grown in the presence of $NH_4^+$ to repress nitrogenase and were shown to lack nitrogenase activity by the acetylene reduction technique. In our laboratory and in other laboratories, stock cultures of *R. rubrum* are maintained in stoppered bottles in the light, and no buildup of hydrogen pressure is observed. The purified CODH was not capable of $H_2$ evolution or $H_2$ oxidation; thus, it seems likely that hydrogen is being evolved via some hydrogenase which is either induced or activated in the presence of CO. Under photoautotrophic conditions, *R. rubrum* is able to utilize $H_2$ as an electron donor for $CO_2$ fixation (2). It is not clear, however, whether this is due to a reversible hydrogenase or an uptake hydrogenase. A hydrogenase activity similar to uptake hydrogenase has been reported in *R. rubrum* (3). There are no reports of phototrophically grown *R. rubrum* with $H_2$ as a reductant to drive nitrogenase in vivo, and we have been unable to achieve this result (Ludden, unpublished results). This last observation is in contrast to the ability of *Rhodopseudomonas capsulata* to use $H_2$ as an electron donor to nitrogenase (15, 36). The ability of CO to evoke hydrogen evolution may help in understanding the role of hydrogenase in *R. rubrum*.

The spectral changes that occur upon CO binding to hemoglobin have been used to measure either added CO or CO levels present in blood samples (19, 28, 34). Lang et al. (19) have presented a method to measure CO in liquid or gas samples, using whole blood. These methods were either not applicable in our experiments or suffered from nonlinearity of response. A method is presented here which is highly sensitive and shows a linear response to added CO. This assay allows accurate determination of the $K_m$ for CO and also provides an additional method to follow the progress of the CODH reaction.

## ACKNOWLEDGMENTS

This work was supported by funds from the College of Agriculture and Life Sciences and the Graduate School of the University of Wisconsin, Madison. P.W.L. is a Steenbock Career Development Award winner, and D.B. is supported by Cellular and Molecular Biology Training grant 2 T32 GM 07215 from the National Institutes of Health.

## LITERATURE CITED

1. **Adams, M. W. W., and D. O. Hall.** 1977. Isolation of the membrane bound hydrogenase from *Rhodospirillum rubrum*. Biochem. Biophys. Res. Commun. **77**:730–736.
2. **Anderson, L., and R. C. Fuller.** 1967. Photosynthesis in *Rhodospirillum rubrum*. III. Metabolic control of reductive pentose phosphate and tricarboxylic acid cycle enzymes. Plant Physiol. **42**:497–502.
3. **Bose, S. K., H. Gest, and J. G. Ormerod.** 1961. Light activated hydrogenase activity in a photosynthetic bacterium: a permeability phenomenon. J. Biol. Chem. **236**:PC13–PC14.
4. **Daniels, L., G. Fuchs, R. K. Thauer, and J. G. Zeikus.** 1977. Carbon monoxide oxidation by methanogenic bacteria. J. Bacteriol. **132**:118–126.
5. **Dashekvicz, M. P., and R. L. Uffen.** 1979. Identification

of a carbon monoxide-metabolizing bacterium as a strain of *Rhodopseudomonas gelatinosa* (Molisch) van Niel. Int. J. Syst. Bacteriol. **29**:145–148.
6. **Diekert, G., and M. Ritter.** 1982. Nickel requirement of *Acetobacterium woodii*. J. Bacteriol. **151**:1043–1045.
7. **Diekert, G. B., and R. K. Thauer.** 1978. Carbon monoxide oxidation by *Clostridium thermoaceticum* and *Clostridium formicoaceticum*. J. Bacteriol. **136**:597–606.
8. **Diekert, G., and R. K. Thauer.** 1980. The effect of nickel on carbon monoxide dehydrogenase formation in *Clostridium thermoaceticum* and *Clostridium formicoaceticum*. FEMS Microbiol. Lett. **7**:187–189.
9. **Drake, H. L.** 1982. Occurrence of nickel in carbon monoxide dehydrogenase from *Clostridium pasteurianum* and *Clostridium thermoaceticum*. J. Bacteriol. **149**:561–566.
10. **Drake, H. L., S.-I. Hu, and H. G. Wood.** 1980. Purification of carbon monoxide dehydrogenase, a nickel enzyme from *Clostridium thermoaceticum*. J. Biol. Chem. **255**:7174–7180.
11. **Drake, H. L., S.-I. Hu, and H. G. Wood.** 1981. Purification of five components from *Clostridium thermoaceticum* which catalyze synthesis of acetate from pyruvate and methyltetrahydrofolate. J. Biol. Chem. **256**:11137–11144.
12. **Genthner, B. R. S., and M. P. Bryant.** 1982. Growth of *Eubacterium limosum* with carbon monoxide as the energy source. Appl. Environ. Microbiol. **43**:70–74.
13. **Hegeman, G.** 1980. Oxidation of carbon monoxide by bacteria. TIBS **5**:256–259.
14. **Hirsch, P.** 1968. Photosynthetic bacterium growing under carbon monoxide. Nature (London) **217**:555–556.
15. **Hongyu, S., C. Hancai, W. Monggan, C. Bingjian, and Y. Baolin.** 1980. Relationship between hydrogenase and nitrogenase in hydrogen metabolism of photosynthetic bacterium *Rhodopseudomonas capsulata*. Sci. Sin. **23**:252–260.
16. **Hu, S.-I., H. L. Drake, and H. G. Wood.** 1982. Synthesis of acetyl coenzyme A from carbon monoxide, methyltetrahydrofolate, and coenzyme A by enzymes from *Clostridium thermoaceticum*. J. Bacteriol. **149**:440–448.
17. **Kim, Y. M., and G. D. Hegeman.** 1981. Purification and some properties of carbon monoxide dehydrogenase from *Pseudomonas carboxydohydrogena*. J. Bacteriol. **148**:904–911.
18. **Kluyver, A. J., and Ch. G. T. P. Schnellen.** 1947. On the fermentation of carbon monoxide by pure cultures of methane bacteria. Arch. Biochem. **14**:57–70.
19. **Lang, W., H. U. Wolf, and R. Zander.** 1979. A sensitive continuous and discontinuous photometric determination of oxygen, carbon dioxide, and carbon monoxide in gases and fluids. Anal. Biochem. **92**:255–264.
20. **Ludden, P. W., and R. H. Burris.** 1978. Purification and properties of nitrogenase from *Rhodospirillum rubrum*, and evidence for phosphate, ribose and an adenine-like unit covalently bound to the iron protein. Biochem. J. **175**:251–259.
21. **Lynd, L., R. Kerby, and J. G. Zeikus.** 1982. Carbon monoxide metabolism of the methylotrophic acidogen *Butyribacterium methylotrophicum*. J. Bacteriol. **149**:255–263.
22. **Meyer, O.** 1982. Chemical and spectral properties of carbon monoxide: methylene blue oxidoreductase. J. Biol. Chem. **257**:1333–1341.
23. **Meyer, O., and H. G. Schlegel.** 1980. Carbon monoxide: methylene blue oxidoreductase from *Pseudomonas carboxydovorans*. J. Bacteriol. **141**:74–80.
24. **Meyer, O., and H. G. Schlegel.** 1983. Biology of aerobic carbon monoxide-oxidizing bacteria. Annu. Rev. Microbiol. **37**:277–310.
25. **Ormerod, J. G., K. S. Ormerod, and H. Gest.** 1961. Light-dependent utilization of organic compounds and photoproduction of molecular hydrogen by photosynthetic bacteria; relationships with nitrogen metabolism. Arch. Biochem. Biophys. **94**:449–463.
26. **Ragsdale, S. W., J. E. Clark, L. G. Ljungdahl, L. L. Lundie, and H. L. Drake.** 1983. Properties of purified carbon monoxide dehydrogenase from *Clostridium thermoaceticum*, a nickel, iron-sulfur protein. J. Biol. Chem. **258**:2364–2369.
27. **Ragsdale, S. W., L. G. Ljungdahl, and D. V. DerVartanian.** 1982. ERP evidence for nickel-substrate interaction in carbon monoxide dehydrogenase from *Clostridium thermoaceticum*. Biochem. Biophys. Res. Commun. **108**:658–663.
28. **Sawicki, C. A., and A. H. Gibson.** 1979. A photochemical method for rapid and precise determination of carbon monoxide levels in blood. Anal. Biochem. **94**:440–449.
29. **Thauer, R. K., G. Fuchs, B. Käufer, and U. Schnitker.** 1974. Carbon monoxide oxidation in cell free extracts of *Clostridium pasteurianum*. Eur. J. Biochem. **45**:343–349.
30. **Uffen, R. L.** 1976. Anaerobic growth of a *Rhodopseudomonas* species in the dark with carbon monoxide as sole carbon and energy substrate. Proc. Natl. Acad. Sci. U.S.A. **73**:3298–3302.
31. **Uffen, R. L.** 1981. Metabolism of carbon monoxide. Enzyme Microb. Technol. **3**:197–206.
32. **Uffen, R. L.** 1983. Metabolism of carbon monoxide by *Rhodopseudomonas gelatinosa*: cell growth and properties of the oxidation system. J. Bacteriol. **155**:956–965.
33. **Van Assendelft, O. W.** 1970. Spectrophotometry of haemoglobin derivatives, p. 55–61. Charles C Thomas, Publisher, Springfield, Ill.
34. **Van Kampen, E. J., H. C. Volger, and W. G. Zijlstra.** 1954. Spectrophotometric determination of carboxyhemoglobin in human blood and the application of this method to the estimation of carbon monoxide in gas mixtures. Proc. K. Ned. Akad. Wet. Ser. C **57**:320–331.
35. **Wakim, B. T., and R. L. Uffen.** 1983. Membrane association of the carbon monoxide oxidation system in *Rhodopseudomonas gelatinosa*. J. Bacteriol. **153**:571–573.
36. **Wall, J. D., P. F. Weaver, and H. Gest.** 1975. Genetic transfer of nitrogenase-hydrogenase activity in *Rhodopseudomonas capsulata*. Nature (London) **258**:630–631.
37. **Yagi, T.** 1958. Enzymic oxidation of carbon monoxide. Biochim. Biophys. Acta **30**:194–195.
38. **Yagi, T.** 1959. Enzymic oxidation of carbon monoxide II. J. Biochem. **46**:949–955.