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Isolation of Carbon Monoxide Dehydrogenase from *Acetobacterium woodii* and Comparison of Its Properties with Those of the *Clostridium thermoaceticum* Enzyme

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An oxygen-labile carbon monoxide dehydrogenase was purified to at least 98% homogeneity from fructose-grown cells of *Acetobacterium woodii*. Gel filtration and electrophoresis experiments gave molecular weights of 480,000 and 153,000, respectively, of the active enzyme. The molecular weights for the subunits are 80,000 and 68,000; the subunits occur in equal proportion. The small subunit of the *A. woodii* enzyme differs in size from that of the *Clostridium thermoaceticum* enzyme; however, the large subunits are similar. The specific activity of the *A. woodii* enzyme, measured at 30°C and pH 7.6, is 500 μmol of CO oxidized min^{-1} mg^{-1} with 20 mM methyl viologen as the electron acceptor. Analysis revealed (number per dimer) iron (9), acid-labile sulfide (12), nickel (1.4), and magnesium or zinc (1). This metal content is quite similar to that of the *C. thermoaceticum* enzyme (Ragsdale et al., J. Biol. Chem. **258**:2364–2369, 1983). The nickel as well as the iron-sulfur clusters are redox-active, as was found for the *C. thermoaceticum* enzyme (Ragsdale et al., Biochem. Biophys. Res. Commun. **108**:658–663, 1982). CO can reduce and CO_2 can oxidize the iron-sulfur clusters. The enzyme is inhibited by cyanide, but CO_2 in the presence of reduced methyl viologen or CO alone can reverse or prevent this inhibition. Several ferredoxins, flavodoxin, and rubredoxin and some artificial electron carriers were tested for their relative rates of reaction with the CO dehydrogenases from *A. woodii*, *C. thermoaceticum*, and *Clostridium formicoaceticum*. Rubredoxin was by far the most reactive acceptor and is proposed to be the primary natural electron carrier for the acetogenic CO dehydrogenases.

Acetobacterium woodii (9, 11), like many acetogenic bacteria (12, 21), contains a carbon monoxide dehydrogenase with high specific activity. Nickel has been shown or implied to be present in the CO dehydrogenases of *A. woodii* (11) as well as in those of *Clostridium thermoaceticum* (12, 15, 16), *Clostridium formicoaceticum* (12), and *Clostridium pasteurianum* (15). That nickel is a component of the *C. thermoaceticum* enzyme has been verified by purification of the enzyme (43) and by demonstration that the nickel component is redox active (44).

A tetrahydrofolate-corrinoid pathway seems to be involved in the synthesis of acetate from one carbon precursors, including CO, by the acetogenic bacteria (33; L. G. Ljungdahl, *in* D. L. Wise, ed., *Organic Chemicals from Biomass*, in press). The role of CO dehydrogenase in this pathway is not completely understood. The enzyme seems to be important in controlling the flow of electrons during reduction of CO_2 to acetate (11, 13). CO dehydrogenase has also been shown to be a component of enzyme systems that catalyze the formation of acetate from methyltetrahydrofolate, coenzyme A, and ATP with either pyruvate (17) or CO (24). A partially purified CO dehydrogenase fraction could also carry out the exchange of C-1 of acetyl coenzyme A with carbon monoxide (24). A scheme of Hu et al. (24) predicts that a corrinoid methyl-acceptor protein plus CO dehydrogenase should be necessary to carry out the exchange reaction. By using the homogeneous enzyme from *C. thermoaceticum*, it was found that CO dehydrogenase is unable to perform this exchange reaction by itself (43). It has also been postulated that CO dehydrogenase catalyzes the formation of a formate oxidation-level intermediate (17, 24, 35). This intermediate is proposed to be of central importance to the oxidation of pyruvate or CO, as well as to the formation of methyltetrahydrofolate (17, 24). Lynd et al. (35) suggest that formation of this intermediate could conserve ATP during synthesis of methyltetrahydrofolate. Formation of a nickel(III)-carbon species by reaction of the

purified CO dehydrogenase from *C. thermoaceticum* with CO has been demonstrated (44); however, the relation of the nickel-carbon species to the intermediate is unclear. Here we show that the purified enzyme from *A. woodii* forms a nickel(III)-carbon species which is identical to that found with the enzyme of *C. thermoaceticum*; however, it contains iron-sulfur centers that are different from the *C. thermoaceticum* enzyme.

Due to the apparent importance of CO dehydrogenases in the metabolism of acetogenic bacteria, as well as the general interest of the protein as a nickel-containing, iron-sulfur enzyme, we purified the enzyme from the mesophile, *A. woodii*, and compared its light-absorption and physical properties with those of the *C. thermoaceticum* enzyme (43). In this paper we also report on the kinetics of the purified CO dehydrogenases from *A. woodii*, *C. thermoaceticum*, and the partially purified enzyme from *C. formicoaceticum* with ferredoxin, flavodoxin, rubredoxin, and several artificial electron acceptors. A preliminary report of these results has been presented (S. W. Ragsdale, D. V. DerVartanian, and L. G. Ljungdahl, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, K32, p. 182).

MATERIALS AND METHODS

Cell material. *A. woodii* ATCC 29683 was generously supplied by R. S. Wolfe (Department of Microbiology, University of Illinois, Urbana, Ill.) and R. S. Tanner (Celanese Chemical Co., Inc., Corpus Christi, Tex.). *A. woodii* was cultured by the method of Balch et al. (4) either on a H_2-CO_2 (66/33) gas mixture or on fructose under a N_2-CO_2 (66/33) atmosphere. Yeast extract (2 g/liter; Difco Laboratories) was added to 10 liters or more of culture medium. Fructose-grown cells (10 liters) were transferred to a 400-liter fermentor (Fermacell; New Brunswick Scientific Co.). The pH was maintained at 6.8. Purity of the cultures was checked by microscopic examination and by product analysis with a Varian model 2700 gas chromatograph. Acetate was the only fermentation product detected. Cells in exponential growth phase (normally 2 g/liter) were harvested with a Sharples AS16P centrifuge, immediately frozen, and stored at −20°C. The nickel content of the medium was approximately 200 nM.

C. formicoaceticum ATCC 23439, described by Andreesen et al. (1), was maintained and grown by using the medium of Moore et al. (39). The conditions of growth and harvest of cells have been described previously (8).

C. thermoaceticum DSM 521 was grown at 58°C as described earlier (32).

Analytical methods. Protein was determined by the rose bengal dye-binding assay of Elliott and Brewer (18), except that 1 mg of rose bengal per ml was used. Ovalbumin was the protein standard. Iron was determined by plasma emission spectroscopy (25) and by the method of Doeg and Ziegler (14). Acid-labile sulfide was determined by the method of Rabinowitz (42).

Gel filtrations for purification and determination of molecular weight and Stokes radius (2) were performed with a tandem Bio-Gel A-1.5m or a Bio-Gel A-1.5m-Ultrogel AcA 22 (both 2 by 85 cm) column setup. Standards (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc.) for molecular weight and Stokes radius determinations in gel filtration and Ferguson-type gel electrophoresis experiments were: thyroglobulin (669,000, 85 Å), ferritin (440,000, 61 Å), catalase (232,000, 52.2 Å), and aldolase (156,000, 47.4 Å). Void and total volumes were estimated by using blue dextran and vitamin B_{12}, respectively.

Standard Tris-glycine alkaline electrophoresis (10) was modified by the method of Yamamoto et al. (49). Tris-barbital (22) and sodium dodecyl sulfate (SDS) gel electrophoreses (48) were performed according to standard procedures. Gels were stained for protein (45) or activity (43) and scanned by using a gel scanner and model 1200 spectrophotometer (both from Gilford Instrument Laboratories, Inc.). Tris-glycine electrophoresis was also run on CO dehydrogenase with gels prepared at 3.7, 5.6, 7.4, and 9.4% acrylamide. The slopes of the log R_f versus percent acrylamide plots were compared with those of standard proteins (above) (23).

The electron paramagnetic resonance (EPR) experiments were performed with a Varian E-109 spectrometer interfaced with a Hewlett-Packard HP-85 microcomputer. Measurements at liquid helium temperatures were conducted with an Air Products APD-E automatic temperature controller. Other EPR experimental details are found in the figure legends.

Bio-Gel A-1.5m was obtained from Bio-Rad Laboratories. AcA 22 was purchased from LKB Instruments, Inc., and carbon monoxide (99.99% Matheson purity) was from Matheson Scientific, Inc. DE32-cellulose was from Whatman, Inc. A type B anaerobic chamber (Coy Laboratory Products) was used in purification of the enzymes.

Kinetic analyses. Carbon monoxide dehydrogenase, hydrogenase, and formate dehydrogenase activities were all determined with 10 mM methyl viologen (MV) as the electron acceptor (9) by using $13.9 \times 10^3\ M^{-1}\ cm^{-1}$ as the 604-nm extinction coefficient (20). One unit of enzyme activity is defined as 2 μmol of MV reduced min^{-1} which is equivalent to 1 μmol of CO oxidized min^{-1} and specific activity as units mg^{-1}. Adjustment of the pH of all buffers was done at room temperature (25°C). The regular assay for CO dehydrogenase contained, in a total volume of 0.6 ml, 0.1 M Tris-hydrochloride (pH 7.6)–3.2 mM dithiothreitol, plus the electron carrier. The reaction mixture was bubbled with carbon monoxide for 5 min in serum-stoppered cuvettes at 55°C for the *C. thermoaceticum* enzyme or 35°C for the *A. woodii* or the *C. formicoaceticum* enzyme. The reaction mixture was then incubated for 5 min at 50°C for the *C. thermoaceticum* enzyme and at 30°C for the *C. formicoaceticum* and *A. woodii* enzymes before initiating the reaction with enzyme.

For the electron carrier experiments, the regular assay mixture was utilized. Reactions were begun by the addition of 26 ng of carbon monoxide dehydrogenase from *C. thermoaceticum* or *A. woodii* or 640 ng of enzyme from *C. formicoaceticum*. The electron carriers tested with the *A. woodii* and *C. formicoaceticum* enzymes were rubredoxin, flavodoxin, and ferre-

doxin from *C. formicoaceticum*, methylene blue, benzyl viologen, and MV. All of these electron carriers, plus ferredoxins I and II from *C. thermoaceticum*, were tested in the assay of the *C. thermoaceticum* CO dehydrogenase. Methylene blue was rapidly reduced by the dithiothreitol in the regular assay mixture. Therefore, in the comparison of methylene blue, benzyl viologen, and MV at 50 μV concentrations, dithiothreitol was omitted from the assay mixture. Reduction of the electron carriers was tested before each reaction was begun and then was subtracted from the total rate. Linearity of the reaction as a function of the enzyme concentration was tested with each electron carrier.

Apparent kinetic parameters were obtained by using a Basic modification of the Fortran "Curfit" program of Bevington (5). This iterative procedure is a nonlinear, least-squares fit to the one-substrate Michaelis-Menten equation. With all assays, an atmosphere of 100% CO was used. The extinction coefficients used in calculation of initial velocities are found in the legend to Fig. 6.

In the inhibition studies, enzyme was diluted into buffer containing Tris-hydrochloride (50 mM, pH 7.6), dithionite (2 mM), MV (0.2 mM), and ovalbumin (5 mg ml^{-1}). At time zero, potassium cyanide was added and, at the times indicated, either CO was bubbled through or sodium bicarbonate was added. The final concentration of enzyme in the assay mixtures was 0.1 mg ml^{-1}, and the final concentration of potassium cyanide was 20 μM. See the legend to Fig. 5 for more details.

Exchange of [1-^{14}C]acetyl coenzyme A with CO was carried out exactly as outlined in Hu et al. (24) with 0.1 mg (50 U) of CO dehydrogenase from *A. woodii*.

Enzyme purifications. (i) *A. woodii* CO dehydrogenase. All steps were carried out at 10°C approximately as outlined in Ragsdale et al. (43) for the purification of CO dehydrogenase from *C. thermoaceticum*, except that the heat-treatment step was omitted. The standard buffer contained 50 mM Tris-hydrochloride (pH 7.3) and 2 mM sodium dithionite. MV, 0.2 mM, was included in the gel filtration buffers. Buffers were pH adjusted at 25°C.

(i) The heterotrophically grown cells (80 g) were suspended in basic buffer containing 0.2 mM MV, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg of DNase I per ml, and lysed at 13,500 to 15,000 lb/in^2 with a French press under an O_2-free CO_2 atmosphere. The broken cell suspension was then centrifuged at 27,000 rpm in stainless steel centrifuge tubes with a type 35 rotor (Beckman Instruments, Inc.).

(ii) Ammonium sulfate was added (258 g/liter solution), and the suspension was centrifuged at 20,000 rpm for 20 min. To the supernatant was added 90 g of ammonium sulfate per liter, the suspension was centrifuged as before, and the pellet was dissolved in standard buffer. The salt concentration was then decreased 50-fold by concentration and dilution with an Amicon XM-100 ultrafiltration membrane.

(iii) This dialyzed protein solution was then applied to a DE32-cellulose column (5.5 by 13 cm) and washed with 200 mM Tris-hydrochloride. A 1,600-ml linear gradient was run between 200 and 400 mM Tris-hydrochloride (pH 7.6).

(iv) The active fractions, eluting at 0.29 M Tris-hydrochloride, were applied to a Bio-Gel hydroxylapatite column (200 ml) which had been previously equilibrated with the standard buffer. After washing with the standard buffer, a 1,600-ml linear gradient was run with the standard buffer containing 0 to 0.1 M potassium phosphate (pH 6.8). The active enzyme eluted at 70 mM potassium phosphate.

(v) The fractions with a specific activity greater than 185 were combined and concentrated to 6 ml. The enzyme was applied to the tandem gel filtration columns and run at a flow rate of 29 ml/h. The specific activity of the resulting enzyme peak was 500.

(ii) *C. formicoaceticum* CO dehydrogenase. The enzyme from *C. formicoaceticum* was partially purified by the same procedure as the *A. woodii* enzyme. The enzyme was apparently 25% pure as judged by Tris-glycine alkaline electrophoresis.

(iii) *C. thermoaceticum* CO dehydrogenase. The enzyme from *C. thermoaceticum* was isolated to apparent homogeneity as outlined earlier (43). The specific activity was 600 U mg^{-1}.

(iv) *C. formicoaceticum* electron carrier proteins. The electron carrier proteins were all isolated as described in another report (S. W. Ragsdale and L. G. Ljungdahl, submitted for publication). Ferredoxin, flavodoxin, and rubredoxin were all judged to be homogeneous by absorbance peak ratios, sedimentation velocity and equilibrium, and polyacrylamide gel electrophoresis.

RESULTS

Purification and properties of *A. woodii* CO dehydrogenase. A representive purification is shown in Table 1. The final specific activity varied among different preparations from 400 to 500 U mg^{-1}, which we attribute to differences in metal content. By assuming a 13% recovery, approximately 3% of the soluble cell protein exists as CO dehydrogenase. The enzyme with a specific activity of 500 is apparently 98% pure by densitometric scanning of the SDS gels and is apparently pure by alkaline electrophoresis. The specific activity of the protein was constant across gel filtration and DEAE chromatographic peaks, indicating homogeneity.

Attempts to purify the *C. formicoaceticum* enzyme to homogeneity were unsuccessful. It appears that this enzyme is more labile than

TABLE 1. Purification of CO dehydrogenase from 80 g of *A. woodii* heterotrophically grown cells

Step	Units[a]	mg	SA[b]	% Recovery
Crude extract	106,400	6,420	17	100
Ammonium sulfate fractionation	91,300	3,430	27	86
DE32-cellulose	38,300	580	66	36
Bio-Gel hydroxylapatite	17,300	90	190	16
Gel filtration	13,500	27	500	13

[a] Units are the micromoles of CO oxidized per minute equivalent to 2 μmol of MV reduced min^{-1}.
[b] SA, Specific activity (units per milligram).

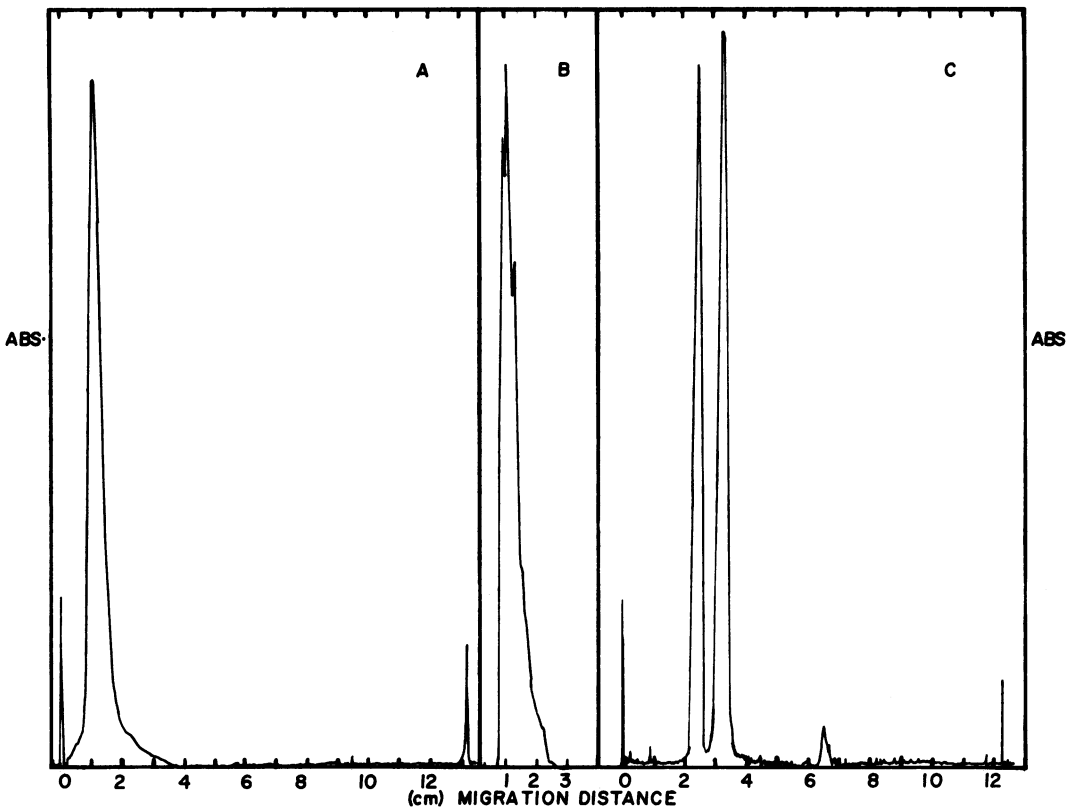

FIG. 1. Densitometric scans of gel electrophoresis of different preparations of purified carbon monoxide dehydrogenase from *A. woodii*. (A) Enzyme (20 μg) was run in gels (4 by 150 mm). Electrophoresis was run under anaerobic conditions in Tris-glycine buffer, pH 8.9 (9), containing 2 mM dithiothreitol. The gel was prerun for 2 h with buffer containing 2 mM thioglycolate. The scan was run at 595 nm after staining for protein. The activity band, obtained as described in Fig. 1B, corresponded to the protein band. (B) Enzyme (10 μg) was run according to Fig. 1A. The gel was incubated with carbon monoxide in the presence of 50 mM KP_i–1 mM benzyl viologen until the blue activity band appeared. Then 1 mg ml^{-1} triphenyl tetrazolium chloride was added to form a permanent stain. The scan was carried out at 533 nm. (C) SDS-gel electrophoresis. A total of 20 μg of protein was loaded onto gels (4 by 150 mm). For molecular weight determination, the following standards were used: phosphorylase b, 92,500; bovine serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; and lysozyme, 17,440. The gel was scanned at 595 nm. ABS, absorbance. The spike at 0 cm marks the beginning of the gel, and the spikes at 12.5 or 13 cm mark the position of the tracking dye.

those from *C. thermoaceticum* and *A. woodii*. We therefore used a partially purified fraction to study the interaction of the *C. formicoaceticum* electron carriers with this enzyme.

Determination of the native molecular weight gave results that suggest that the *A. woodii* enzyme can exist as a dimer or hexamer. Gel electrophoresis with a Tris-barbital (pH 8.0) or Tris-glycine (pH 8.9) system gives from one to several protein bands, all of which stain for activity (Fig. 1A and B). Only one enzyme peak, however, is detected by the chromatographic procedures during the purification procedure. When three major bands were detected (Fig. 1B), we analyzed the molecular weights of the active enzyme bands.

The log R_f versus percent acrylamide plot (23) gave parallel lines (average slope, 0.165 ± 0.009), demonstrating that the different forms of the *A. woodii* CO dehydrogenase are charge isomers with a molecular weight of 153,000 ± 10,000. Gel filtration of the enzyme indicates that the molecular weight is 480,000 and that the Stokes radius is 70.2 Å. SDS electrophoresis of the freshly prepared enzyme yielded two bands of equal staining intensity corresponding to subunit molecular weights of 80,000 and 68,000 (Fig. 1C). Electrophoretic techniques, therefore, indicate a dimeric form of the enzyme ($\alpha\beta$), whereas gel filtration experiments indicate a hexameric $(\alpha\beta)_3$ enzyme structure. In SDS electrophoresis, only two protein bands were found whether

TABLE 2. Properties of acetogenic CO dehydrogenases[a]

Species	Mol wt	Subunit structure	Subunit size	Mol metal content/ mol hexamer for the following metals:			Labile sulfide content	K_m (mM) for MV	Sp act	% Soluble cell protein
				Ni	Fe	Zn				
C. thermoaceticum	440,000	$(\alpha\beta)_3$, $\alpha\beta$	77,600 70,900	6	32	3	42	3	675[b]	2
A. woodii	460,000	$(\alpha\beta)_3$, $\alpha\beta$	80,000 68,000	5	27	Variable	36	12	500[c]	3

[a] Data on the *C. thermoaceticum* enzyme was taken from reference 43.
[b] Determined at 50°C (pH 7.6) with 10 mM MV as the electron acceptor.
[c] Determined at 30°C (pH 7.6) with 20 mM MV as the electron acceptor.

the enzyme showed one (Fig. 1A) or several (Fig. 1B) electrophoretic forms. In a preliminary report on this enzyme, we reported three subunits (S. W. Ragsdale, D. V. DerVartanian, and L. G. Ljungdahl, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, K32, p. 182). However, the third band was apparently a breakdown product, since fresh enzyme, isolated with protease inhibitor in the initial cell suspension, consistently gave only two subunits. Interestingly, when the *A. woodii* and the *C. thermoaceticum* CO dehydrogenases were electrophoresed together and in parallel, the large subunits comigrated, whereas the *A. woodii* small subunit was clearly of lower molecular weight than that of *C. thermoaceticum*.

The purified *A. woodii* enzyme is best stored anaerobically at approximately 4 to 10°C in buffer containing dithionite and MV under an atmosphere of N_2-H_2. When the enzyme was incubated under a CO atmosphere for 2 days, 50% of the activity was lost. Addition of glycerol

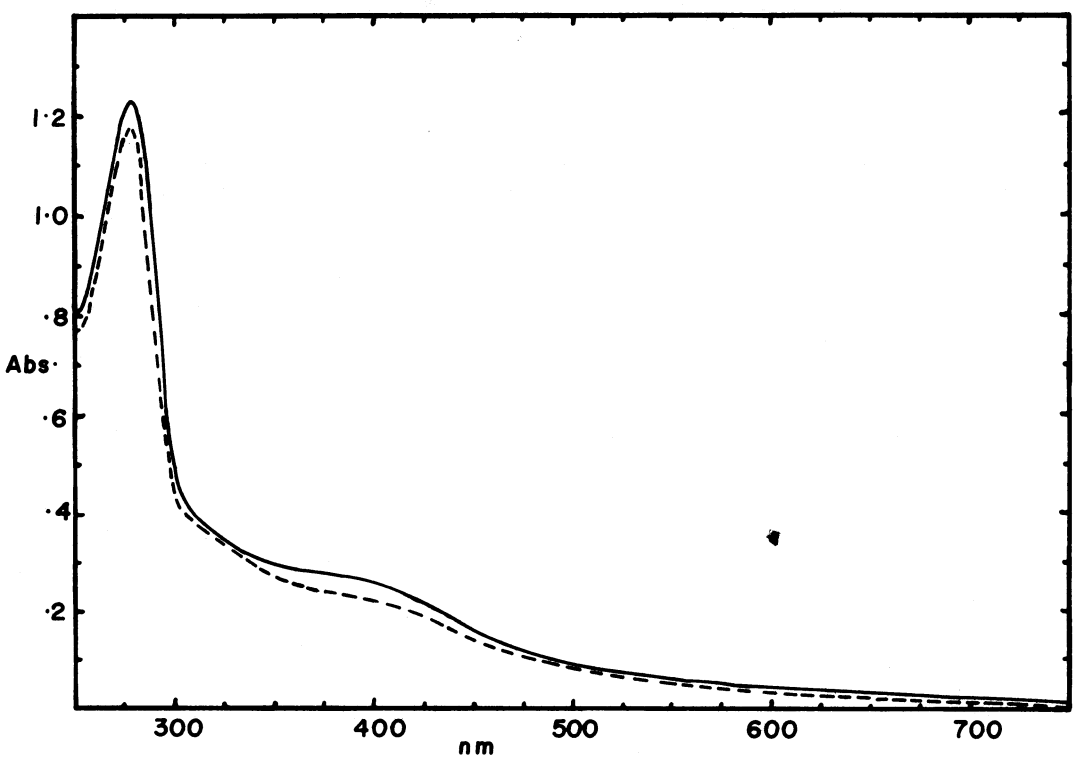

FIG. 2. Absorbance (Abs.) spectrum of CO dehydrogenase from *A. woodii*. CO dehydrogenase (1.0 mg ml^{-1}) was in oxygen-free, 50 mM Tris hydrochloride (pH 7.6). —, Enzyme as isolated after gel filtration to remove all reducing agent; ---, enzyme bubbled with CO for 5 min.

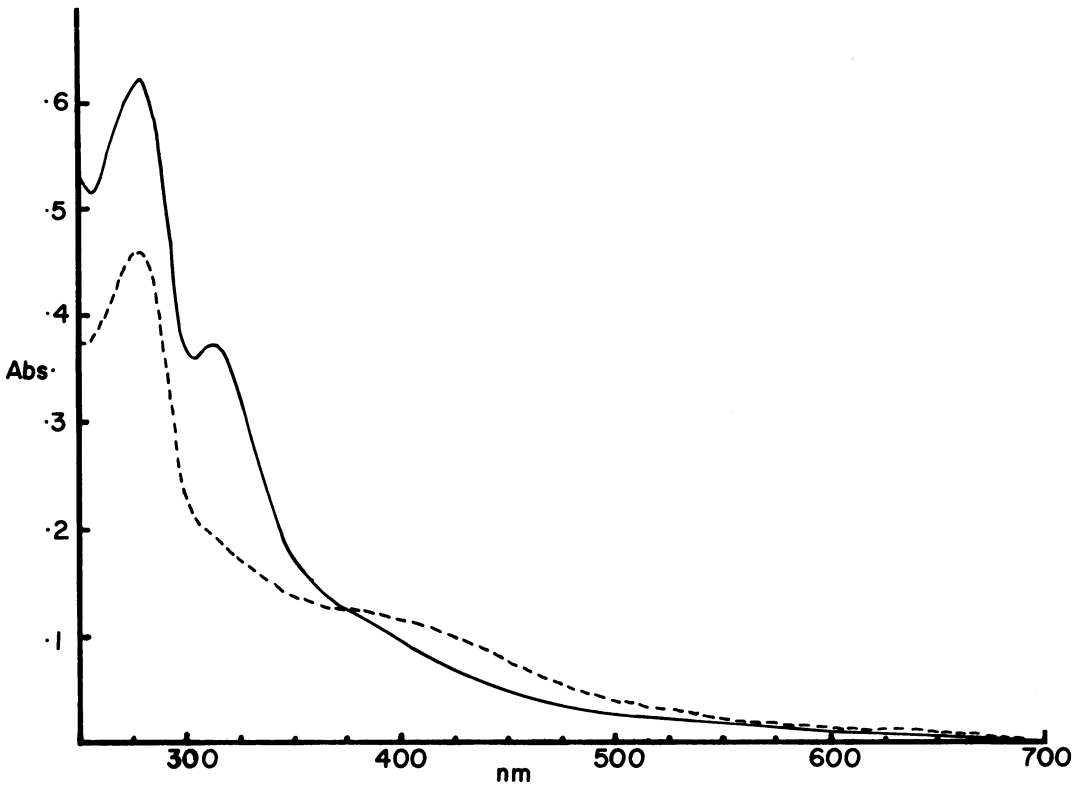

FIG. 3. Absorbance (Abs.) spectrum of CO dehydrogenase from *A. woodii*. CO dehydrogenase (0.5 mg ml^{-1}) was in anaerobic 0.5 M Tris-hydrochloride (pH 7.6), and a trace of dithionite. —, Enzyme after elution from DEAE-cellulose to remove most of dithionite; ---, enzyme bubbled with CO_2 for 5 min.

to a 20% final concentration did not affect the enzyme activity under any conditions. Freezing caused a loss of most of the enzyme activity whether the atmosphere was N_2-H_2 or CO. Oxygen causes inactivation of the enzyme, which cannot be reversed by treatment with reducing agents or carbon monoxide in the presence or absence of the electron carrier. The enzyme can be stored for at least 1 month without an apparent loss of activity.

Metals and acid-labile sulfide content. Plasma emission spectroscopy of different preparations of the homogeneous *A. woodii* enzyme with a specific activity of 500 revealed that the enzyme contains (number per mole of dimer) nickel (1.4 ± 0.1), iron (9 ± 2), magnesium (1.0 ± 0.6), zinc (0.5 ± 0.5), and highly variable amounts of calcium (1 to 4.0). When zinc is present, magnesium is absent and vice versa. Analysis reveals 12 acid-labile sulfides per dimer. No molybdenum or copper was present in the *A. woodii* enzyme. Table 2 compares the metal content of the *A. woodii* enzyme with that of the *C. thermoaceticum* enzyme. The same metal content was obtained when the enzyme was dialyzed against Tris-hydrochloride (50 mM)–EDTA (0.5 mM) or Tris-hydrochloride (50 mM) without EDTA.

Light-absorption properties. The spectrum of the *A. woodii* enzyme is affected by the redox state. When the enzyme, isolated in the presence of dithionite as shown above, is prepared by gel filtration with anaerobic buffers but no reducing agent, it appears to be a ferredoxin-like protein with a distinct, 390-nm shoulder (Fig. 2). The enzyme can be reduced with carbon monoxide, as seen by the decrease in absorbance at 390 nm. Cyanide has no effect on the spectrum of the enzyme as isolated or in the presence of CO. CO_2 cannot increase the 390-nm absorbance of the gel-filtrated enzyme. Oxygen also has no effect on the spectrum of the oxidized enzyme.

When the enzyme is adsorbed to a DE-32 column and extensively washed with anaerobic buffer containing no reducing agent, apparently some dithionite remains bound to the column. Elution with 0.5 M Tris (pH 7.6) yields an enzyme with a 310-nm peak but no 390-nm shoulder (Fig. 3). Under these conditions, CO does not further decrease the 390-nm absorbance, but CO_2 or O_2 causes the 390-nm shoulder to appear. The 310-nm peak in Fig. 3 is due

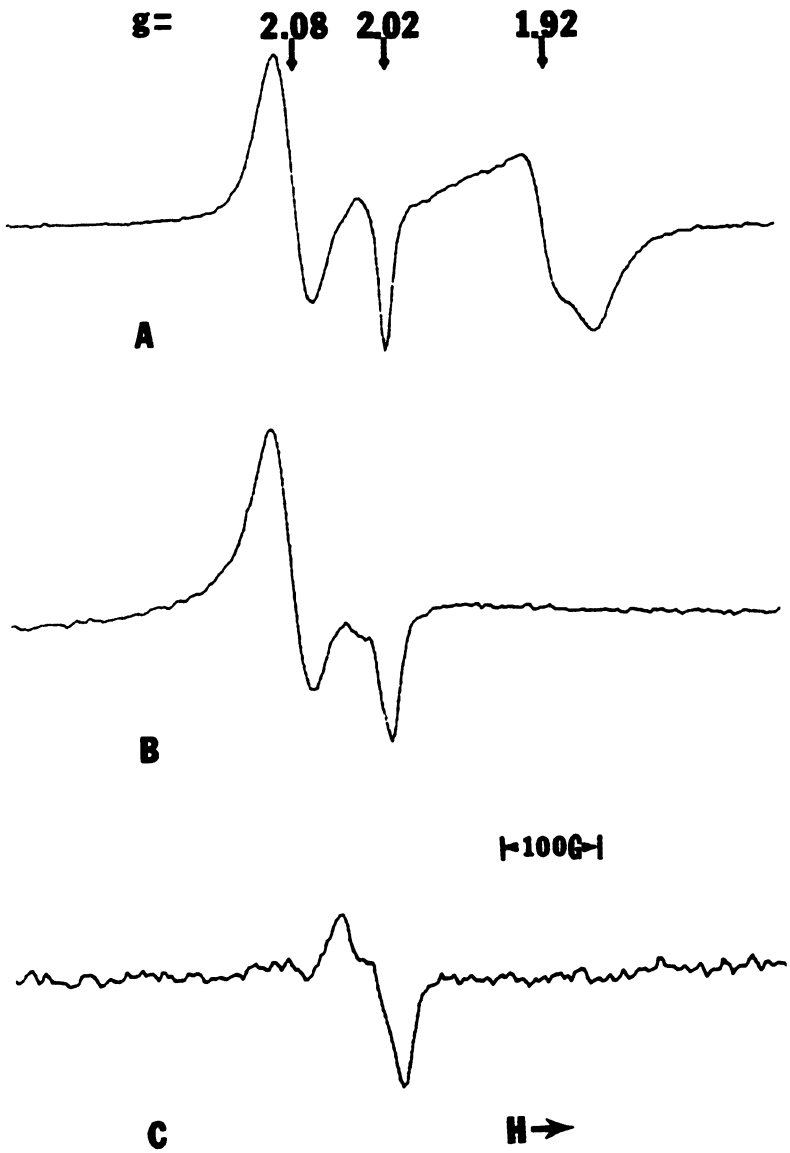

FIG. 4. EPR spectra of carbon monoxide dehydrogenase purified from *A. woodii*. (A) Purified, reduced CO dehydrogenase (30 μM in protein in 500 mM Tris buffer, pH 7.6), reacted with CO for 10 min under anaerobic conditions. EPR conditions: microwave power, 50 μW; microwave frequency, 9.162 GHz; scanning rate, 500 G per min; time constant, 0.1 s; temperature, 12 K; gain, 2×10^4. (B) EPR spectrum as in (A) except for the following changes in EPR conditions: microwave power, 10 mW; microwave frequency, 9.161 GHz; temperature, 77 K; gain, 1.25×10^4. (C) Purified, reduced CO dehydrogenase reacted for 10 min with 0.52 mM potassium ferricyanide under anaerobic conditions. EPR conditions as in (A) except that gain was equal to 4×10^4.

to dithionite, and any changes in the 310-nm peak in the presence of CO_2 or CO were also observed with a dithionite solution free of enzyme.

EPR spectroscopy. The EPR spectra of CO dehydrogenases from *A. woodii* and *C. thermoaceticum* are similar since both enzymes show a reduced "g = 1.94 type" (4Fe-4S) signal. However, it should be noted that the CO dehydrogenase from *A. woodii* differs in exhibiting a single reduced (4Fe-4S) cluster signal with a major g value at 1.92 (data not shown). Thus, a second reduced (4Fe-4S) cluster signal with g values at 1.86 and 1.75 found in the enzyme from *C. thermoaceticum* is absent in the *A. woodii* enzyme (cf. reference 44, Fig. 1). Quantitation,

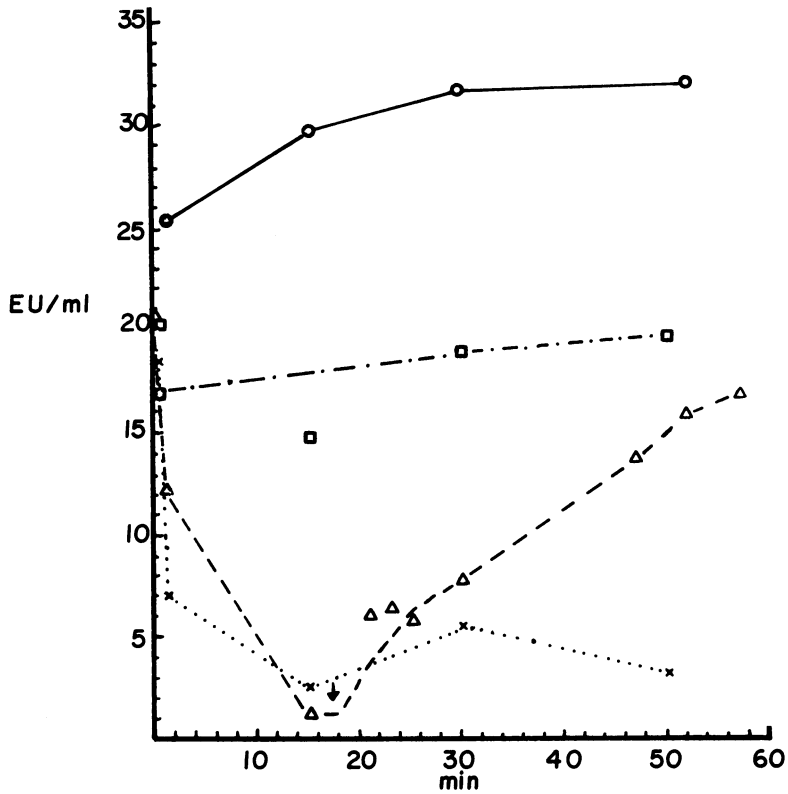

FIG. 5. Inhibition of the *A. woodii* enzyme with cyanide. Enzyme (0.1 mg ml^{-1}) was in 50 mM Tris-hydrochloride (pH 7.6)–2 mM sodium dithionite–0.2 mM MV–5 mg ml^{-1} ovalbumin in the presence or absence of 10 μl of 0.82 mM potassium cyanide (final concentration of cyanide is 20 μM). The cyanide solution was in the same buffer as the enzyme except without ovalbumin. Symbols: ○, enzyme with addition of 10 μl of cyanide and addition of 10 μl of 1 M sodium bicarbonate at time zero; □, enzyme with addition of 10 μl of buffer (no cyanide or bicarbonate); △, enzyme with addition of 10 μl of cyanide at time zero and then addition of 10 μl of 1 M sodium bicarbonate at time indicated by the arrow; ×, enzyme with addition of 10 μl of cyanide at time zero. EU/ml, Enzyme units per milliliter.

as described previously (44), of the single reduced (4Fe-4S) cluster found with reduced CO dehydrogenase from *A. woodii* accounts for 0.93 electrons per mol of hexameric enzyme; this value is consistent with one EPR-detectable, reduced (4Fe-4S) cluster. Reaction of the reduced enzyme with CO or CO_2 under anaerobic conditions results in the appearance of a new, intense EPR signal, with resonances at g = 2.08 and 2.02 (Fig. 4A) superimposed on the single, reduced (4Fe-4S) cluster signal with major g value at 1.92. The EPR resonances of the g = 2.08 and 2.02 signal may be studied without interference from the (4Fe-4S) cluster signal by raising the temperature of the EPR measurement. The signal of the reduced (4Fe-4S) cluster (g = 1.92) characteristically disappears at higher temperatures (above 25 K). The EPR parameters of the g = 2.08 and 2.02 signal at 77 K (Fig. 4B) strongly resemble those found under similar conditions with the CO dehydrogenase from *C. thermoaceticum* (44). Quantitation of the g = 2.08 and 2.02 EPR signal indicates that 2.17 electrons per mol of enzyme in the hexameric form are present. This value corresponds to 43% of the nickel content determined by plasma emission spectroscopy. We attribute this g = 2.08 and 2.02 EPR signal to a nickel(III)-carbon radical species based on EPR parameters of nickel (the only other transition metal detected in the enzyme) and on the resemblance of the resonance to the radical doublet intermediate observed by Babior et al. (3) in ethanolamine ammonia-lyase in the presence of 2-aminopropanol. This latter intermediate was proposed (3) to result from the interaction between Co(II) and a radical species. Since Co(II) is isoelectronic with Ni(III), it was suggested (44) that the new resonance at g = 2.08 and 2.02 was derived from Ni(III) reacting with a carbon radical species. In this regard it is important to note, in support of this letter assignment, that reaction of ^{13}CO (when compared with that of ^{12}CO) with reduced CO dehydrogenase from either *A. woodii*

or *C. thermoaceticum* results in a broadening of 7 gauss in the g = 2.08 and 2.02 resonance, proving that a carbon species formed from CO is involved in the resonance (Ragsdale et al., submitted). In addition, experiments with ^{61}Ni (nuclear spin [I] = 3/2) nuclear isotopic substitution in CO dehydrogenase have also established that nickel is involved in the same resonance (S. W. Ragsdale, L. G. Ljungdal, and D. V. DerVartanian, Biochem. Biophys. Res. Commun., in press).

When reduced CO dehydrogenase from *A. woodii* is reacted with potassium ferricyanide, oxidation of the (4Fe-4S) cluster and Ni(III)-carbon species occurs with loss of both EPR signals. In their place (Fig. 4C) is observed a new EPR signal at g = 2.01 which quantitates to 0.23 electrons per mole of hexameric enzyme. This EPR signal is consistent with either the oxidized state of a 3Fe cluster or a superoxidized state of a (4Fe-4S) cluster.

Kinetic properties. (i) Inhibition by cyanide. Treatment of the enzyme (0.1 mg ml^{-1}) with potassium cyanide (20 μM) caused inhibition that was prevented by prior incubation with CO or $NaHCO_3$ (Fig. 5). When the CN-inhibited enzyme was bubbled with CO or CO_2, the inhibition was reversed to give 90 to 100% of the original activity. However, the conditions for reversal of inhibition are quite rigorous. At 0°C no apparent inhibition was seen for over 1 h of incubation, but the inhibition occurred quite rapidly at 25°C. Reversal of inhibition by CO_2 could only be accomplished with enzyme maintained in a reduced state with dithionite plus MV. Whenever MV was excluded from the mixture, reversal of inhibition could only be seen with CO. CO_2 apparently has a stimulatory effect on the enzyme since the velocity of the CO_2-treated enzyme is approximately 30% higher than that of the nontreated enzyme; however, prolonged incubation (e.g., several days) under CO_2 causes a loss of enzyme activity.

(ii) Formate dehydrogenase and hydrogenase activity. The *A. woodii* enzyme catalyzes a slight formate-dependent reduction of MV. By using 20 mM MV, the rate of formate oxidation was 2.4% of the rate of CO oxidation. Absolutely no H_2-dependent MV reduction was seen.

(iii) Reduction of electron carriers with CO dehydrogenase from *C. formicoaceticum*, *C. thermoaceticum*, and *A. woodii*. Table 3 compares the rates of the different electron acceptors relative to rubredoxin in their ability to accept electrons from CO dehydrogenase. Figures 6A and B show the velocity versus electron carrier concentration plots by using the Michaelis-Menten model to calculate the lines corresponding to the experimental points. Approximately the same types of curves are obtained for the *C. thermoaceticum* enzyme (Fig. 6B) as with the *C. formicoaceticum* enzyme (Fig. 6A) with the *C. formicoaceticum*-derived electron carriers. A similar kinetic plot is also obtained by using the *A. woodii* enzyme with these electron carriers. The progress curves ($-dA/dt$, where A is absorbance and t is time) for each electron carrier had a constant slope in at least the initial 20 s of reaction, but the slope decreased as the reaction approached equilibrium or until oxidized rubredoxin was exhausted (data not shown). Oxidized ferredoxin was still present after the reaction was complete, but rubredoxin was completely reduced. CO dehydrogenase, free of dithionite by chromatography on a G-25 column, still catalyzed the reduction of all electron acceptors shown to be active. It was essential, however, to keep dithionite with all the CO dehydrogenases to maintain stability. The reactions were dependent on CO, and none of the electron carriers were reduced by H_2 or N_2 when substituted for CO in the assay mixture. The reaction velocity was linear with both electron carrier and enzyme concentration.

Rubredoxin is the most efficient of the electron carriers tested. At a concentration of 50 μM, the velocities (unit per milligram) of the *A. woodii* enzyme with MV, ferredoxin, and rubredoxin were 3, 14, and 58, respectively. The

TABLE 3. Initial velocity measurements of carbon monoxide dehydrogenase activity with different electron carriers[a]

Carrier	% Activity relative to rubredoxin for the following organisms[b]:		
	A. woodii[c]	*C. thermoaceticum*[c]	*C. formicoaceticum*[c]
Methylene blue	27	84	67
Rubredoxin	100	100	100
Flavin adenine dinucleotide	20	nd[d]	nd
Flavin mononucleotide	16	nd	nd
NAD and NADP	0	0	0
Flavodoxin	2	10	10
Ferredoxin	25	12	23
Benzyl viologen	15	11	33
MV	6	4	23

[a] The assays were carried out as described in the legend to Fig. 4.

[b] Carriers of 1 electron were at a concentration of 50 μM and carriers of 2 electrons were at a concentration of 25 μM.

[c] A total of 26 ng of *A. woodii* and *C. thermoaceticum* and 640 ng of *C. formicoaceticum* were used in each assay mixture. By using the standard assay, the specific activities of the enzymes were 450 (*A. woodii*, 30°C), 500 (*C. thermoaceticum*, 50°C), and 60 (*C. formicoaceticum*, 30°C).

[d] nd, Not determined.

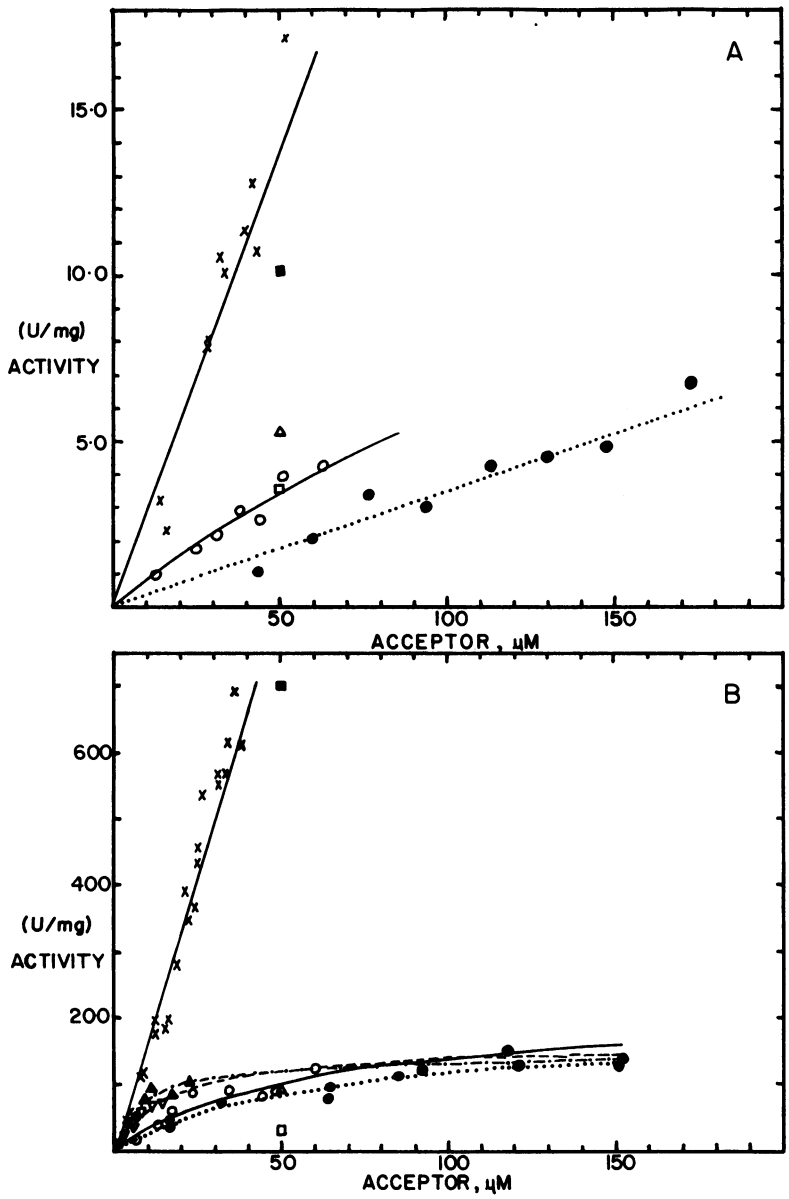

FIG. 6. Velocity of CO dehydrogenases with different artificial and natural electron carriers. Purified electron carriers were added to the assay mixture under an atmosphere of 100% carbon monoxide. Activity is defined as units per milligram (U/mg) (see text for details). Symbols and the extinction coefficients (M^{-1} cm^{-1}) used are: ×, rubredoxin, *C. formicoaceticum*, 7.3 × 10^3 (490 nm) (Ragsdale and Ljungdahl, submitted); ●, flavodoxin, *C. formicoaceticum*, 7.7 × 10^3 (444 nm for oxidized) (Ragsdale and Ljungdahl, submitted) and 6.2 × 10^3 (444 nm for oxidized minus semiquinone) (Ragsdale and Ljungdahl, submitted); ○, ferredoxin *C. formicoaceticum*, 17.6 × 10^3 (390 nm) (Ragsdale and Ljungdahl, submitted); ▲, ferredoxin I, *C. thermoaceticum*, 16.8 × 10^3 (390 nm) (50); ▽, ferredoxin II, *C. thermoaceticum*, 30.4 × 10^3 (390 nm) (19); □, MV, 13.9 × 10^3 (604 nm) (20); △, benzyl viologen, 12 × 10^3 (555 nm) (6); ■, methylene blue, 37.1 × 10^3 (615 nm) (37). The extinction coefficients for the *C. formicoaceticum* electron carriers were determined separately (Ragsdale and Ljungdahl, submitted). (A) A total of 640 ng of partially purified CO dehydrogenase from *C. formicoaceticum* was added to each assay performed at 30°C to begin the reaction. (B) A total of 26 ng of homogeneous CO dehydrogenase from *C. thermoaceticum* was added to each assay performed at 50°C.

kinetic pattern of the rubredoxin reaction with all three CO dehydrogenases is quite unexpected, however (Fig. 6). Even at a concentration of 50 μM, saturation is not seen. With flavodoxin, saturation is not seen even at a concentration of 50 μM. Ferredoxins from the mesophile, *C. formicoaceticum*, or the thermophile, *C. thermoaceticum*, were equal in activity, even though ferredoxin I (50) and *C. formicoaceticum* ferredoxin (Ragsdale and Ljungdahl, submitted) have one (4Fe-4S) cluster and ferredoxin II has two (4Fe-4S) clusters. As is found in other systems, flavodoxin is less efficient than ferredoxin in its ability to carry out electron transfer.

DISCUSSION

Table 2 compares some properties of the *C. thermoaceticum* and *A. woodii* CO dehydrogenases. The oxygen-sensitive dehydrogenases from both bacteria can be isolated to high specific activity by using approximately the same purification procedure. The *C. formicoaceticum* enzyme, however, is apparently more labile and was not purified to homogeneity. Approximately 3% of the *A. woodii* soluble cell protein is made up of CO dehydrogenase.

The subunit structure of the *A. woodii* enzyme is quite similar to that of *C. thermoaceticum* (43); however, by coelectrophoresis, we found that the *A. woodii* small subunit is of lower molecular weight than that of *C. thermoaceticum*, and the large subunits are of approximately equal molecular weight. Since the gel filtration molecular weight is about 480,000, the *A. woodii*, like the *C. thermoaceticum* (43), enzyme can exist as a hexamer with the structure $(\alpha\beta)_3$. Electrophoretic techniques suggest that the enzymes from *C. thermoaceticum* (43) and *A. woodii* also can exist as dimers. Gel filtration and gel electrophoresis should give similar results (for example, see reference 7). Since electrophoresis was run at one pH unit higher than gel filtration, the dimeric form may be the more stable form at higher pH values.

The *A. woodii*, but not the clostridial, CO dehydrogenase can exist in several active forms which differ in charge. When *C. thermoaceticum* and *C. formicoaceticum* crude extracts are electrophoresed under identical conditions to the *A. woodii* enzyme, the clostridial enzymes migrate as a single activity band. It is likely that the forms of the *A. woodii* enzyme differ in metal content; however, further studies will be required to demonstrate why the *A. woodii*, but not the clostridial, enzyme can exist as charge isomers.

The metal content of the *C. thermoaceticum* and *A. woodii* enzymes is quite similar (Table 2). Nickel is present in concentrations that suggest 2 mol of Ni per mol of dimeric enzyme. *C. pasteurianum* (15) and *C. formicoaceticum* (12) CO dehydrogenases also apparently contain nickel. Clearly, a major difference between the pseudomonas-type and the acetogenic-type CO dehydrogenases is that the pseudomonas enzymes do not contain nickel (27, 36) but molybdenum (36). Molybdenum is absent from the purified CO dehydrogenases of *A. woodii* and *C. thermoaceticum*. Jack bean urease, several hydrogenases, as well as the methanogenic cofactor, F_{430}, contain nickel (46). Nickel has been found to be redox active in a number of hydrogenases (29, 30, 40, 46). The *C. thermoaceticum* (44) and *A. woodii* CO dehydrogenases, on reaction with carbon monoxide, form an EPR-detectable species which is postulated to be a nickel(III)-carbon radical. Thus, the enzyme from *A. woodii*, as well as from *C. thermoaceticum*, exhibits redox-active nickel.

In the *A. woodii* and the *C. thermoaceticum* enzymes, iron and acid-labile sulfide are in a concentration that suggests 2 to 3 (4Fe-4S) clusters per mole of dimeric enzyme. EPR spectroscopy directly demonstrates the presence of iron-sulfur clusters of the (4Fe-4S) type; however, a 3Fe center may also be present in the *A. woodii* enzyme. CO can reduce and CO_2 can oxidize these centers, but no other light-absorption changes are seen in the 300- to 750-nm region. The absorption ratio (390/277 nm) of the oxidized *A. woodii* and *C. thermoaceticum* enzymes is approximately 0.2. The pseudomonas CO dehydrogenases also contain iron and inorganic sulfur (36). Zinc is present in the *Pseudomonas carboxydovorans* (36), as well as the *C. thermoaceticum* and *A. woodii*, CO dehydrogenases. Both of the pseudomonas CO dehydrogenases (27, 36) contain flavin; however, there is no evidence for flavin in either the *A. woodii* or *C. thermoaceticum* enzymes.

Cyanide has no effect on the light-absorption spectrum of the oxidized, reduced, CO−, or CO_2-treated enzyme. However, it inhibits both the *A. woodii* and the *C. thermoaceticum* (43) enzymes, and the inhibition is reversed and prevented by CO. In the presence of reduced electron carrier (e.g., MV), CO_2 has the same effect as CO on the cyanide-treated *A. woodii* enzyme. Therefore, we feel that the production of CO from CO_2 under these conditions is responsible for the reversal or prevention of cyanide inhibition by CO_2.

In contrast to the *C. thermoaceticum* (43) enzyme, the *A. woodii* CO dehydrogenase contains a slight formate:MV oxidoreductase activity. Hydrogenase activity was still present in the purified CO dehydrogenase of *P. carboxydovorans* (38), but not in the enzymes from *A. woodii* or *C. thermoaceticum*. Neither the *C. thermoaceticum* nor the *A. woodii* enzymes alone can

carry out exchange of the C-1 of acetyl coenzyme A with carbon monoxide.

The *A. woodii* enzyme, as well as the clostridial CO dehydrogenases, utilize rubredoxin (Rd) as the best acceptor of electrons from CO. Kim and Hegeman (28) propose that a quinone is the electron acceptor in the pseudomonas enzyme. The standard redox change ($\Delta E^{\circ\prime}$) for the reaction $CO + H_2O \rightarrow H_2 + CO_2$ should be +0.104 V, when calculated using a standard free-energy change of −4.8 kcal/mol of CO (47). The potential for the reaction $CO + 2Rd_{ox} + H_2O \rightarrow 2H^+ + CO_2 + 2Rd_{red}$ should therefore be +0.410 V by using −0.059 V (34) and −0.420 V as the redox potentials for the Rd_{ox}/Rd_{red} and $H_2/2H^+$ couples. Substitution of ferredoxin for 2 rubredoxin in the reaction gives a $\Delta E^{\circ\prime} = +0.168$ V by using −0.36 (19) as the redox potential for ferredoxin. Reduction of rubredoxin is clearly more thermodynamically favorable than reduction of ferredoxin or flavodoxin. This was confirmed when the assays were run to equilibrium. Since our electron carrier comparisons were initial velocity experiments, kinetic and thermodynamic considerations seem to favor the reduction of rubredoxin. Since thermodynamics predict that reduced ferredoxin should be best in reduction of CO_2 to CO, the reverse reaction of CO dehydrogenase with the different electron carriers should also be studied.

The fate of reduced rubredoxin is not known. Pyridine nucleotide-dependent rubredoxin oxidoreductases have been isolated from *C. thermoaceticum* (S.-S. Yang and L. G. Ljungdahl, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, K135, p. 208), *C. acetobutylicum* (41) and *Desulfovibrio gigas* (31). NADH:ferredoxin oxidoreductase is also widespread among anaerobic bacteria (26). One would expect that the following sequence of reactions may be feasible in the acetogenic bacteria (Fig. 7):

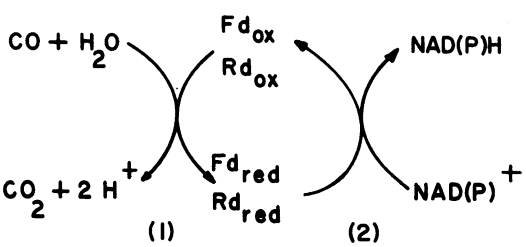

FIG. 7. Proposed coupling of CO dehydrogenase with NAD(P):rubredoxin (Rd) (or ferredoxin [Fd]) oxidoreductase.

In this sequence, CO dehydrogenase (reaction 1) couples with ferredoxin (or rubredoxin) reductase (reaction 2). Yang (Ph.D. thesis, University of Georgia, Athens, 1977), however, studied the reactions of rubredoxin oxidoreductase in *C. thermoaceticum* and was unable to reduce NAD^+ or $NADP^+$ with reduced rubredoxin from *C. thermoaceticum*. We have recently been successful in reducing NAD^+ with carbon monoxide by coupling ferredoxin, CO dehydrogenase, and a partially purified oxidoreductase fraction (data not shown). Rubredoxin may replace ferredoxin when coupled with the correct oxidoreductase, since in the above scheme, rubredoxin and ferredoxin act as catalytic intermediates. In the acetogenic bacteria, reduced pyridine nucleotides would then be used to reduce CO_2 to acetate, as well as to supply reducing equivalents needed in growth.

In the acetogenic bacteria, CO dehydrogenase may be involved in the formation of an intermediate in the synthesis of either the methyl or carboxyl group of acetate. The reduction of CO to the methyl group of acetate seems to proceed through CO_2 and free formate (43). An intermediate in the oxidation of CO to CO_2 may be the EPR-detectable, Ni-carbon radical which is formed from CO and CO dehydrogenase. A physiological role for this intermediate has not been shown; however, a C-1 intermediate may form the carboxyl of acetate upon reaction with methyltetrahydrofolate (24). Hu et al. (24) have proposed that this same C-1 intermediate may be formed from pyruvate also. Detailed studies in which pyruvate:ferredoxin oxidoreductase and CO dehydrogenase are coupled should clarify the nature of this intermediate.

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