Two Membrane-Associated NiFeS-Carbon Monoxide Dehydrogenases from the Anaerobic Carbon-Monoxide-Utilizing Eubacterium Carboxydothermus hydrogenoformans

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Two monofunctional NiFeS carbon monoxide (CO) dehydrogenases, designated CODH I and CODH II, were purified to homogeneity from the anaerobic CO-utilizing eubacterium Carboxydothermus hydrogenoformans. Both enzymes differ in their subunit molecular masses, N-terminal sequences, peptide maps, and immunological reactivities. Immunogold labeling of ultrathin sections revealed both CODHs in association with the inner aspect of the cytoplasmic membrane. Both enzymes catalyze the reaction CO + H2O → CO2 + 2 e− + 2 H+. Oxidized viologen dyes are effective electron acceptors. The specific enzyme activities were 15,756 (CODH I) and 13,828 (CODH II) μmol of CO oxidized min−1 mg−1 of protein (methyl viologen, pH 8.0, 70°C). The two enzymes oxidize CO very efficiently, as indicated by kcat/Km values at 70°C of 1.3 · 105 M−1 s−1 (CODH I) and 1.7 · 105 M−1 s−1 (CODH II). The apparent Km values at pH 8.0 and 70°C are 30 and 18 μM CO for CODH I and CODH II, respectively. Acetyl coenzyme A synthase activity is not associated with the enzymes. CODH I (125 kDa, 62.5-kDa subunit) and CODH II (129 kDa, 64.5-kDa subunit) are homodimers containing 1.3 to 1.4 and 1.7 atoms of Ni, 20 to 22 and 20 to 24 atoms of Fe, and 22 and 19 atoms of acid-labile sulfur, respectively. Electron paramagnetic resonance (EPR) spectroscopy revealed signals indicative of [4Fe-4S] clusters. Ni was EPR silent under any conditions tested. It is proposed that CODH I is involved in energy generation and that CODH II serves in anabolic functions.

Bacteria which utilize CO as a growth substrate include the aerobic carboxidotrophs and the anaerobic acetogens, sulfate-reducers, methanogens, and phototrophs (16, 34, 42, 44, 47). Carboxydothermus hydrogenoformans is a strictly anaerobic, thermophilic, gram-positive eubacterium which was isolated from a volcanic hot spring (55). Phylogenetically, C. hydrogenoformans falls into the group of the low-G+C subphylum of the gram-positive bacteria and shows highest 16S rRNA gene sequence homology to Thermoterrabacterium (52). C. hydrogenoformans utilizes CO under chemolithoautotrophic conditions (55). The bacterium couples the oxidation of CO to CO2 (E0′ = −0.52 V) to the reduction of protons to H2 (E0′ = −0.41 V) in the energy-conserving reaction CO + H2O → CO2 + H2, ΔGθ° = −20 kJ mol−1 (54, 55). The metabolism of C. hydrogenoformans is strictly fermentative because the bacterium is obligately anaerobic and utilizes protons as the characteristic intracellular electron acceptor. On the basis of the formation of H2 as the ultimate fermentation product by the bacterium, we propose the terms “hydrogenogenic,” “hydrogenogens,” and “hydrogenogenesis” to refer to the type of metabolism, the physiological group, and the process of H2 formation, respectively. Although C. hydrogenoformans is not a phototroph, it is in many ways similar to the phototrophic bacteria Rhodospirillum rubrum and Rhodocyclus gelatinosus, which utilize CO anaerobically in the dark (34, 58, 59). C. hydrogenoformans is also able to ferment pyruvate to acetate and H2 (56).

Although carbon monoxide dehydrogenases (CODHs) formally catalyze the same reaction (CO + H2O → CO2 + 2 e− + 2 H+), different types of enzymes serving different metabolic functions operate in the various groups of CO-oxidizing bacteria (15, 16, 42, 44, 47). Aerobic CODHs are MoFeS flavoproteins containing [2Fe-2S] clusters, while anaerobic CODHs are NiFeS proteins containing [4Fe-4S] clusters. The high-resolution crystal structures of the MoFeS CODHs from Oligotropha carboxidovorans (12, 26, 43) or Hydrogenophaga pseudoﬂava (27, 43) show a dimer of two heterotrimers in an (LMS)2 subunit structure. Each heterotrimer is composed of a molybdoprotein (L subunit), a flavoprotein (M subunit), and an iron-sulfur protein (S subunit). The molybdoprotein carries the active site, which contains a 1:1 molar complex of molybdopterin cytosine dinucleotide and a molybdenedium atom. The iron-sulfur protein contains the type I and type II [2Fe-2S] centers. The flavoprotein contains the flavin adenine dinucleotide (FAD) cofactor and shows a new flavin-binding type (25, 26). The NiFeS CODHs are either monofunctional or bifunctional (15, 16, 47). The latter are associated and operate in a complex with acetyl coenzyme A (acetyl-CoA) synthase (ACS). The monofunctional CODH from the phototrophic bacterium R. rubrum (6, 7) is inducible in the dark under anaerobic conditions in the presence of CO, shows a micromolar Km for CO (6, 7, 34), and contains a proposed nickel-iron-sulfur cluster (cluster C) (30, 32, 53) and a conventional [4Fe-4S] cluster (cluster B) (32, 47). Radiolabeling studies suggested a catalytically essential nonsubstrate CO ligand (CO2) to the Fe atom in the putative [Fe-Ni] center of cluster C (31). Acetogens and

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5134
methylene glycol; 500 NaCl; 500 Na-tigloylcollate; 500 yeast extract; 1 resazurin; 2 NCl 2; 6 H2O; 10 ammonium ferric(III) citrate; 1 FeSO4 7H2O; 100 mM MgSO4; 5 MnSO4; 0.1 H2SO4; 0.1 CuSO4; 0.1 CuSO4·5H2O; 0.1 Na2MoO4; 0.2 H2SO4; 0.2 Na2SeO3; 0.5 H2O; 0.1 KI[SO4]2·12H2O; 1 mL of vitamin solution (61). The fermentors were continuously supplied with 0.5 liters of CO min−1 and stirred at 500 rpm. Bacteria were harvested by centrifugation under N2 and kept frozen at −20°C under N2 until use.

Anaerobic procedures. All procedures for the preparation of cell extracts, cell fractions, and enzyme purifications were carried out anaerobically under a flow of N2 or in an anaerobic glove box chamber (model 1024 anaerobic system; Forma Scientific, Marietta, Ohio) under an atmosphere of pure N2. All buffers were repeatedly degassed by evacuation, flushed with N2, supplied with 2 mM Na-dithionite, and maintained under a slight overpressure of N2.

Preparation and purification of CODH. For enzyme purifications, C. hydrogenoformans was harvested at an approximate optical density at 436 nm of 2. About 100 g of bacterial cell mass was suspended in 200 mL of 50 mM Tris-HCl (pH 8.0) containing 2 mM Na-dithionite (buffer A). 0.1 mg of lysyme per mL, 0.05 mg of DNase I per mL, and 0.2 mM phenylmethylsulfonyl fluoride and incubated for 30 min at 37°C with gentle stirring. The protoplasts thus obtained were subjected to osmotic lysis followed by low-spin centrifugation. The resulting cell-free extracts were subjected to ultracentrifugation for 2 h at 120,000 × g, yielding cytoplasmic and membrane fractions. Intact protoplasts were prepared from bacteria suspended in the above buffer and supplied with 0.6 M sucrose. Cytoplasmic fractions (250 mL) were subjected to anion exchange chromatography (Macro-Prep High Q; Bio-Rad) on columns (dimensions, 17 by 5 cm) equilibrated with buffer A. Elution was with 700 mL of buffer A followed by 2,800 mL of a linear gradient of 0 to 1 M NaCl in buffer A. Fractions with CODH activity were pooled, supplemented with 1.3 M ammonium sulfanate, and gently stirred for 30 min, and the precipitated protein was removed by low-spin centrifugation. The supernatant was loaded onto a hydrophobic interaction chromatography on Butyl-Sepharose 4 Fast Flow (Pharmacia). Columns (dimensions, 20 by 5 cm) were equilibrated with 1.2 M ammonium sulfanate in buffer A, and eluted with 800 mL of equilibration buffer followed by 2,400 mL of a decreasing linear gradient of 1.2 to 0 M ammonium sulfanate in buffer A. Two separate peaks showing CODH activity appeared which were designated CODH I and CODH II and purified separately. CODH I was subjected to hydrophobic interaction chromatography on butyl-Sepharose 4 Fast Flow (Pharmacia). Fractions (columns, 20 by 5 cm) were equilibrated with 0.7 M ammonium sulfanate in buffer A, and proteins were desorbed with 200 mL of equilibration buffer followed by 800 mL of a decreasing linear gradient of 0.7 to 0 M ammonium sulfanate in buffer A. CODH I or CODH II were desalted by gel filtration on Sephadex G-25 in buffer A, subjecting separately to anion exchange chromatography on Source 30 Q (Pharmacia; column dimensions, 12 by 2.6 cm), equilibrated with buffer A, and eluted with 130 mL of buffer A followed by 640 mL of a linear gradient of 0 to 0.6 M NaCl in buffer A. CODH I or CODH II were then subjected to gel filtration (Sephacryl S-200; Pharmacia; column dimensions, 60 by 2.6 cm) employing buffer A. Preparations of purified CODH I and CODH II were frozen in liquid N2 and kept at −20°C under N2 until use.

Enzyme assays. CODH activity was assayed at 70°C, which is the optimal growth temperature for C. hydrogenoformans (55), by following the CO-dependent reduction of oxidized methyl viologen (MV) in a spectrophotometer employing an ε of 9.7 mM−1 cm−1 (6). For the assays, 1 mL of the growing culture (20 mL of medium) and 2 mM dichloroindophenolate (DTE) in buffer (50 mL of 0.1 M Hepes-NaOH [pH 8.0]) were flushed with CO in screw-cap cuvettes sealed with a rubber septum. Reactions were initiated by injecting enzyme with a syringe. H2 oxidation activity was assayed under the same conditions except that CO was replaced by H2. One unit of CO or H2 oxidation activity is defined as the reduction of 2 μmol of MV min−1, which is equivalent to 1 μmol of CO or H2 oxidized min−1.

H2 evolution activity was assayed in a gas chromatograph by headspace analysis of the H2 produced with reduced MV or CO as the source of reducing equivalents for the reduction of protons. Assays were carried out at 70°C in 40-mL serum-stoppered vials which were kept shaken at 120 rpm. Assays with reduced MV were composed of 10 mL of buffer B, 2 mM MV, and 60 mM Na-dithionite under a gas atmosphere of N2. Assays with CO were composed of 10 mL of 50 mM buffer B, and 2 mM DTE under a gas atmosphere of pure CO. The gas chromatograph (model CP 9000; Chrompack, Middelburg, The Netherlands) was equipped with a thermal conductivity detector (model 903 A), a HayeSep Q column (2.5 m), and a molecular sieve 13Å column (1.8 m). The temperatures (in degrees Celsius) were 40 (oven), 80 (injection port), and 200 (detector), respectively. The carrier gas was N2 at a flow rate of 30 mL min−1. One unit of H2 evolution activity is defined as 1 μmol of H2 produced min−1.

The [1-14C]acetyl-CoA–CO exchange activity in cell-free extracts or of purified CODHs was assayed at 30, 50, and 70°C following published procedures (13, 49). ACS activity was examined by following acetate formation from 5-methyltetrazolium (MTT) and hydrogen gas. Reduction of oxidized methyl viologen (MV) in a spectrophotometer employing the following conditions: gas chromatograph (model 430; Packard Instrument Company, Downers Grove, Ill.); flame ionization detector (model 901); column (Porapak Q, 50/80 mesh, 2.5 m); 175°C (oven); 200°C (injection port and detector). The carrier gas was N2 (30 mL min−1). Samples of 0.5 mL were acidified with 10 μL of concentrated aqueous HCl, precipitated protein was removed by low-spin centrifugation, and 2 μL of the supernatant was injected into the column.

Electron acceptor specificity. The electron acceptor specificity of CO oxidation catalyzed by CODH was tested spectrophotometrically under the conditions of the CODH activity assay with the exception that DTE was omitted from the reaction mixture. The following compounds (100 μM concentrations of each) were examined (extinction coefficient units are per millimolar per centimeter) (11): BV, ε504 = 8.7; NAD+, ε340 = 6.2; NADPH, ε340 = 11.5; flavin mononucleotide (FMN), ε258 = 12.2; 1-phenyl-2-(4-iodophenyl)-3-(4-nitrophenyl)-2H-benzoquinoline (Q) (20 μM 1-methoxy-phenazine methosulfate (MPS), ε350 = 18.3 (55); ubiquinone Q10, ε270 = 14.7; methylene blue, ε371 = 31.7; phenazine methosulfate (PMS), ε350 = 25.0; 2,6-dichlorophenol-indophenol (DCPIP), ε660 = 16.1; horse heart cytochrome c, ε550 = 29.5.

Kinetic measurements. The Km for CO was determined by varying the CO concentration in the reaction mixture under MV saturation (20 mM). The different CO concentrations were established by adding appropriate amounts of CO-saturated reaction mixture composed of buffer B, 20 mM MV, and 2 mM DTE to assays containing the same reaction mixture saturated with N2. At 70°C and 1 atm pressure, the CO concentration in CO-saturated reaction mixtures was taken to be 645 μM (38). The actual starting CO concentration was calculated from the final concentration of reduced MV, considering that 1 μmol of CO reduces 2 μmol of MV. The Km for MV was determined in a CO-saturated reaction mixture composed of 50 mM buffer B, 2 mM DTE, and different concentrations of MV.

Peptide mapping by limited proteolysis. Purified CODH in 50 mM Tris-HCl (pH 8.0) containing 0.5% (wt/vol) sodium dodecyl sulfate (SDS) was denatured by boiling for 2 min. Assay mixtures were diluted to 0.1% SDS by employing 100 mM acetic acid buffer (pH 4.0) (pepsin digestion) or 100 mM Tris-HCl (pH 8.0) (a-chymotrypsin digestion). Limited proteolysis was carried out at 25°C (pepsin) or 37°C (a-chymotrypsin) at protein-to-protease ratios (by mass) of 4:1 for pepsin and 80:1 for a-chymotrypsin. Proteolytic fragments were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 12 or 15% (wt/vol) running gels.
**TABLE 1. Purification of CODH I and CODH II from CO-grown C. hydrogenoformans**

<table>
<thead>
<tr>
<th>Purification state</th>
<th>CODH fraction</th>
<th>Total protein (mg)</th>
<th>Total activity (kU)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free extract (step 1)</td>
<td>I + II</td>
<td>13,086</td>
<td>15,301</td>
<td>1,169</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Cytoplasmic fraction (step 2)</td>
<td>I + II</td>
<td>6,260</td>
<td>10,814</td>
<td>1,728</td>
<td>71</td>
<td>1.5</td>
</tr>
<tr>
<td>Macro-Prep High Q (step 3)</td>
<td>I + II</td>
<td>2,448</td>
<td>11,856</td>
<td>4,884</td>
<td>78</td>
<td>4.2</td>
</tr>
<tr>
<td>Source 15 ISO (step 4)</td>
<td>I</td>
<td>643</td>
<td>6,253</td>
<td>9,725</td>
<td>41</td>
<td>8.3</td>
</tr>
<tr>
<td>Butyl-Sepharose 4 Fast Flow (step 5)</td>
<td>I</td>
<td>427</td>
<td>5,228</td>
<td>12,243</td>
<td>34</td>
<td>10.5</td>
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<tr>
<td>Source 30 Q (step 6)</td>
<td>I</td>
<td>366</td>
<td>4,601</td>
<td>13,692</td>
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<td>11.7</td>
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<td>Sephacryl S-200 (step 7)</td>
<td>I</td>
<td>279</td>
<td>4,399</td>
<td>15,756</td>
<td>29</td>
<td>13.5</td>
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<tr>
<td>Source 15 ISO (step 8)</td>
<td>II</td>
<td>249</td>
<td>2,963</td>
<td>11,890</td>
<td>19</td>
<td>10.2</td>
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<tr>
<td>Source 30 Q (step 9)</td>
<td>II</td>
<td>190</td>
<td>1,883</td>
<td>9,919</td>
<td>12</td>
<td>8.5</td>
</tr>
<tr>
<td>Sephacryl S-200 (step 10)</td>
<td>II</td>
<td>142</td>
<td>1,964</td>
<td>13,828</td>
<td>13</td>
<td>11.8</td>
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</tbody>
</table>

* One unit is defined as 1 nmol of CO oxidized min⁻¹ with 20 mM oxidized MV as electron acceptor at 70°C and pH 8.0.

**RESULTS**

CO oxidation in subcellular fractions. Under the conditions detailed in Materials and Methods, *C. hydrogenoformans* grew with a generation time of 4.9 h and a yield of 1.2 mg of protein per ml. During growth, the specific CO oxidation activity in cell-free extracts increased continuously from 20 to 1,800 U mg protein⁻¹. About 71% of the total CO oxidation activity in extracts appeared to be soluble in the cytoplasmic fraction (Table 1). Less than 1% of the total CO oxidation activity showed up outside protoplasts. CODH is apparently only loosely associated with the cytoplasmic membrane, since washing of the membranes with buffer mobilized about 50% of the membrane-associated CODH fraction. The H₂-evolving hydrogenase (74.7 U mg of membrane protein⁻¹) of *C. hydrogenoformans* is entirely membrane bound and showed only a little H₂ oxidation activity (0.9 U mg of membrane protein⁻¹).

Identification of two distinct CODHs. We managed to establish a protocol for the purification of two CODHs from *C. hydrogenoformans* (Table 1). Upon anion exchange chromatography (step 3) CODH activity eluted as a single peak at 0.25 M NaCl. Upon hydrophobic interaction chromatography (steps 4 and 8) two distinct peaks of CODH activity appeared, one at 0.6 M ammonium sulfate (designated CODH I) and a second one at 0.35 M ammonium sulfate (designated CODH II). CODH I and CODH II were purified separately (Table 1). Upon hydrophobic interaction chromatography (step 5) CODH I eluted at 0.05 M ammonium sulfate. Upon anion exchange chromatography (steps 6 and 9) CODH I and CODH II eluted at 0.17 and 0.14 M NaCl, respectively. CODH I was purified 14-fold with a specific activity of 15,756 U mg of protein⁻¹ and a yield of 29%. CODH II was purified 12-fold.
with a specific activity of 13,828 U mg of protein$^{-1}$ and a yield of 13%. Both enzymes had the same isoelectric point (pI) of 5.5.

The two CODHs have been purified to homogeneity, as shown by the single bands obtained after native and SDS-PAGE (Fig. 1). They are homodimers with subunit masses of 62.5 kDa (CODH I) and 64.5 kDa (CODH II) (Fig. 1B), which can be deduced by considering the following. Gel filtration of CODH I revealed a Stokes radius of 4.370 nm, corresponding to a molecular mass of 118,768 Da. The corresponding values of CODH II were 4.328 nm and 116,869 Da. CODH I and CODH II showed slightly different mobilities upon native PAGE, corresponding to molecular masses of 135 and 142 kDa, respectively (Fig. 1A). The lowered mobility of CODH I might reflect an increased structural flexibility or a different Stokes radius of the native protein (Fig. 1A).

The subunit amino-terminal sequences of CODH I (SNWKNSVDDAVDYLPIAKKAG) and CODH II (AKQN LXXKTDRAVQQMLDKAK) as determined by Edman degradation characterize the two enzymes as distinct proteins. The sequences indicate that the methionyl residue at each N terminus was excised. The proteolytic peptide patterns of the CODHs indicate different primary structures (Fig. 1C). The N terminus of a 49-kDa chymotryptic peptide of CODH II (Fig. 1C) was VTVVLPPSRV. Polyclonal IgG antibodies raised against CODH I were specific for CODH I and did not react with CODH II (Fig. 1D), and IgG antibodies against CODH II were specific for CODH II and did not react with CODH I (Fig. 1D). Ouchterlony double immunodiffusion employing CODH I, CODH II, and IgG antibodies directed against CODH I showed that the enzymes share no common antigenic determinants (data not shown).

The presence of Ni, Fe, and acid-labile sulfur in both CODHs is apparent from analysis by inductively coupled plasma mass spectroscopy (values in moles of element per mole of enzyme [mean ± standard deviation]: Ni, 1.41 ± 0.01 for CODH I and 1.65 ± 0.03 for CODH II; Fe, 19.83 ± 0.22 for CODH I and 19.25 ± 0.27 for CODH II); neutron activation (Ni, 1.28 ± 0.03 for CODH I and 1.67 ± 0.11 for CODH II; Fe, 22.37 ± 0.27 for CODH I and 24.19 ± 0.98 for CODH II); and colorimetric determination (Fe, 20.7 ± 0.5 for CODH I and 19.6 ± 0.3 for CODH II; acid-labile sulfur, 21.9 ± 1.9 for CODH I and 18.8 ± 1.2 for CODH II). Both enzymes contained less than 0.1 mol of Co, Cr, Cu, Mo, V, or Zn per mol.

**Catalytic properties.** The two CODHs catalyze the CO-dependent reduction of various oxidized electron acceptors and show similar electron acceptor specificities (CO oxidation activities at 70°C and pH 8.0, in units per milligram) for CODH I (BV, 5,200 [set at 100%]; MV, 1,400 [27%]; methylene blue, 1,248 [24%]; PMS, 572 [11%]; FAD, 364 [7%]; FMN, 104 [2%]; DCPIP, 52 [1%] and for CODH II (BV, 3,100 [set at 100%]; MV, 1,000 [32%]; PMS, 341 [11%]; methylene blue, 155 [5%]; FAD, 31 [1%]; FMN, 31 [1%]; DCPIP, 31 [1%]; NADP$, NAD$, INT-MPMS, ubiquinone Q$_10$, and horse heart cytochrome c were not reduced by the two enzymes. At saturating electron acceptor concentrations (20 mM), the following specific CO oxidation activities (in units per milligram) were obtained for CODH I: 29,400 (BV) and 15,800 (MV). For CODH II the activities were 27,000 (BV) and 13,800 (MV).

Both CODHs catalyze the formation of a total of two electrons from CO and water (CO + H$_2$O → CO$_2$ + 2H$^+$ + 2e$^-$). Examination of the amounts of MV reduced in the presence of limiting CO concentrations indicated molar ratios of 2.15 (±0.21):1 (CODH I) and 2.08 (±0.08):1 (CODH II), which are consistent with the functioning of MV as a one-electron acceptor.
CO oxidation followed first-order kinetics. The apparent $K_m$ was 4 mM MV for both CODHs, 30 $\mu$M CO for CODH I, and 18 $\mu$M CO for CODH II (assayed at 70°C and pH 8.0). The apparent $V_{\text{max}}$, $k_{\text{cat}}$, and $k_{\text{cat}}/K_m$ of CODH I were 18,900 $\mu$mol min$^{-1}$ mg$^{-1}$, 39,000 s$^{-1}$, and 1.3 · 10$^9$ M$^{-1}$ s$^{-1}$, respectively, and of CODH II were 14,200 $\mu$mol min$^{-1}$ mg$^{-1}$, 31,000 s$^{-1}$, and 1.7 · 10$^9$ M$^{-1}$ s$^{-1}$, respectively.

CODH I (CO oxidation activity of 14,400 U mg$^{-1}$) could oxidize H$_2$ with MV as an electron acceptor (0.81 U mg$^{-1}$). The reverse reaction, or the formation of H$_2$ from CO, was not catalyzed. CODH II (CO oxidation activity of 10,900 U mg$^{-1}$) could neither oxidize nor produce H$_2$ from reduced MV, but it could produce H$_2$ from CO (0.14 U mg$^{-1}$).

Neither CODH catalyzed the exchange of $^{14}$C from the carbonyl group of [1-14C]acetil-CoA with $^{12}$C from $^{12}$CO. The amount of radioactivity in the aqueous phase containing [1-14C]acetil-CoA under an atmosphere of pure CO remained constant in the presence of CODH I or CODH II (0.5 mg of CODH ml$^{-1}$) when assayed for up to 2 h at 30, 50, and 70°C. The same results were obtained when purified CODH was replaced by cell-free extracts, irrespective of whether the extracts were prepared in the presence or absence of dithionite. Cell-free extracts of C. hydrogenoforans (CODH activity of 1,438 U mg$^{-1}$) were assayed for ACS activity by examination of acetate production from 5-methyltetrahydrofolate, CO, and CoA as detailed in Materials and Methods. Acetate was below the detection limit (0.05 nmol min$^{-1}$ mg$^{-1}$ of protein$^{-1}$) after 2, 4, and 6 h of incubation, reflecting the absence of ACS activity in C. hydrogenoforans. Control experiments with cell-free extracts of C. thermoaacetitum (CODH activity of 13.1 U mg$^{-1}$) showed an ACS activity of 2.8 nmol of acetil-CoA synthesized min$^{-1}$ mg$^{-1}$ of protein$^{-1}$. An EPR signal with a $g_{\text{av}}$ of 2.06 has been attributed to the reduced Ni-X-[4Fe-4S] cluster A, which is the site of acetil-CoA synthesis in the bifunctional CODH-ACS from acetogens and methanogens (16, 47). In accordance with the absence of ACS activity in both CODHs from C. hydrogenoforans, we were not able to demonstrate this EPR signal in CO-reduced CODH I or CODH II at temperatures between 10 and 130 K. Ribulose-1,5-bisphosphate carboxylase activity, assayed according to published procedures (9), could also not be demonstrated in C. hydrogenoforans.

Both CODHs showed an unusual dependence of CO oxidation activity on temperature, with maxima at 95°C (CODH I, 37,949 kU mg$^{-1}$) and 105°C (CODH II, 40,666 kU mg$^{-1}$) and half-lives at 90°C under N$_2$ of 7 h (CODH I) and 2 h (CODH II). The half-lives at 100°C were 7 min for CODH I and 10 min for CODH II. At 114°C, CODH II retained 90% of its maximum activity seen at 105°C, and CODH I retained 28% of its maximum activity seen at 95°C. The pH optima at 70°C were 9.0 (CODH I) and 8.0 (CODH II). The two enzymes were inactivated upon exposure to air at 23°C with half-lives of 20 min (CODH I) and 60 min (CODH II).

**Spectral characterization.** The UV-visible absorption spectra of purified CODH I (Fig. 2A) and CODH II (Fig. 2B) were similar under the various conditions examined. Treatment of either enzyme with CO or dithionite resulted in bleaching of the FeS-like shoulder extending from 350 to 550 nm and centered around 419 nm, referring to a reduced type of spectrum (Fig. 2A and B, traces c and d). The shoulder reappeared when the enzymes treated with CO (Fig. 2A and B, trace c) were exposed to air (Fig. 2A and B, trace b). When dithionite was removed from the as-isolated enzymes kept under N$_2$, the spectra shown in Fig. 2A and B (trace a) were obtained. The spectra are similar to those of the air-oxidized enzymes (Fig. 2A and B, trace b) and therefore are believed to represent an oxidized state. Corresponding oxidized-minus-reduced-difference spectra are shown in the insets. The following extinction coefficients ($\varepsilon$, per millimolar per centimeter) were calculated for the two CODHs in the CO-reduced (Fig. 2A and B, trace c) or oxidized (Fig. 2A and B, trace a) state: CODH I, $\varepsilon_{320} = 191.9$ (oxidized) or 172.8 (reduced), $\varepsilon_{419} = 74.0$ (oxidized) or 35.5 (reduced); CODH II, $\varepsilon_{320} = 200.4$ (oxidized) or 203.7 (reduced), $\varepsilon_{419} = 83.8$ (oxidized) or 42.8 (reduced). Assuming an extinction coefficient of about 4 per mM per Fe in a 4Fe cluster, the calculated approximate number of 4Fe clusters is 4.6 in CODH I and 5.2 in CODH II.

Between 10 and 75 K, the two CODHs in their as-isolated state revealed only weak paramagnetic signals (Fig. 3A and B, traces a and e). The spectra did not change upon exposure of the enzymes to air, with the exception of a slight increase of the
signal at $g = 4.29$ (Fig. 3A and B, traces b and f). Reduction of the as-isolated enzymes (Fig. 3A and B, traces a and e) with CO (Fig. 3A and B, traces c and g) or dithionite (Fig. 3A and B, traces d and h) exhibited complex EPR signals. The reduced CODH signals originated from two different paramagnetic components (Fig. 3A and B, traces c, d, g and h). One paramagnetic component shows a rhombic signal with $g$ values ($g_z$, $g_y$, $g_x$) of 2.04, 1.94, and 1.90 ($g_{av} = 1.94$), which is the same for both CODHs. The other paramagnetic component shows a different rhombic signal with $g$ values ($g_z$, $g_y$, $g_x$) of 1.97, 1.87, and 1.77 ($g_{av} = 1.86$) for CODH I, and $g$ values ($g_z$, $g_y$, $g_x$) of 1.99, 1.80, and 1.74 ($g_{av} = 1.84$) for CODH II. The two paramagnetic components can be differentiated by their temperature dependence. The rhombic signal at $g_{av} = 1.94$ (CODH I and II) appeared between 10 and 40 K with maximum intensity at 10 to 25 K, whereas the signals at $g_{av} = 1.86$ (CODH I) or 1.84 (CODH II) were apparent at 10 K and absent at 25 K. The $g$ values and temperature dependence of the reduced signal at $g_{av} = 1.94$ (CODH I and II) suggests that it originates from an $S = 1/2$ [4Fe-4S]$^{2+}$ cluster (32). The reduced signals at $g_{av} = 1.86$ (CODH I) or 1.84 (CODH II) can be assigned to a second but faster relaxing $S = 1/2$ [4Fe-4S]$^{2+}$ cluster (32). Integration of the spectra and comparison with a spin standard of copper EDTA give a spin concentration for CO-reduced CODH I of 2.74 mol of spin/mol of CODH I for the $g_{av} = 1.94$ EPR signal and 2.18 mol of spin/mol of CODH I for the $g_{av} = 1.86$ EPR signal. The spin concentration for CO-reduced CODH II was 2.87 mol of spin/mol of CODH II for the $g_{av} = 1.94$ EPR signal and 2.17 mol of spin/mol of CODH II for the $g_{av} = 1.84$ EPR signal. The air-oxidized CODHs at 10 K (Fig. 3A and B, trace b) and 25 K (Fig. 3A and B, trace f) exhibited an axial-type EPR signal near $g_{av}$ of 2.01, which was not detectable above 75 K. We suggest that this signal emerges from an oxidized [3Fe-4S]$^{2+}$ cluster produced by the oxidative damage of one of the [4Fe-4S] clusters in analogy to previous reports (3, 18, 40).

Intracellular location of the two CODHs. Osmotic lysis, which is a gentle method of cell breakage, revealed 71% of the total CODH activity was in the cytoplasmic fraction and 29% was in the membrane fraction of C. hydrogenoformans (Table I). Western blotting indicated that all fractions contained...
CODH I as well as CODH II. It was, therefore, of interest to examine the intracellular location of the two CODHs in intact cells of _C. hydrogenoformans_. For this purpose, immunogold labeling of ultrathin sections of CO-grown bacteria was employed (Fig. 4). Low-temperature embedding in Lowicryl K4M resin resulted in good preservation of the bacterial ultrastructure (Fig. 4 A). The distribution of the gold label was the same for the specific IgG antibodies directed against CODH I or CODH II and was independent of the growth phase (Table 2; Fig. 4C to H). Longitudinal and cross-sections showed the gold label nearly exclusively close to the cytoplasmic membrane (Fig. 4C, D, F, and G; Table 2). Tangential sections, which leave a major part of the inner aspect of the cytoplasmic membrane exposed, revealed significant labeling (Fig. 4E and H), which is also indicative of a location of the two enzymes at the membrane. The preference of the gold label for the cytoplasmic side of the membrane (Fig. 4C, D, F, and G; Table 2) suggests a close association of CODHs with the entire H2-evolving hydrogenase activity. The experiments reported in Table 3 examined the conditions of the CO-driven formation of H2 by cytoplasmic membranes of _C. hydrogenoformans_. H2 evolution increased considerably when both CODH I and protein B were present. Under the same conditions, CODH II or protein B alone had no effect. Apparently, protein B functions as a specific electron acceptor of CODH I, transferring the electrons to the hydrogenase. Although oxidized flavins are able to accept electrons from both CODHs, they could not functionally replace protein B. Protein B refers to a greenish-brown-colored protein fraction which eluted at 0.67 M NaCl from the Macro-Prep High Q anion exchanger in step 3 of the CODH purification scheme (Table 1). The fraction presumably contains one or several ferredoxins, according to the negative charge, and a broad shoulder centered around 390 nm and extending from 360 to 500 nm in the oxidized UV-visible absorption spectrum. We have not been able to identify a fraction from the Macro-Prep High Q anion exchanger that would couple the electron transfer from CODH II to the hydrogenase.

**CO-dependent production of NADPH.** Other than the purified CODHs, cytoplasmic fractions of _C. hydrogenoformans_ catalyzed the CO-dependent reduction of NADP+ (2 mM) with a rate of 0.31 μmol of NADP+ reduced min⁻¹ mg⁻¹, NADPH formation increased 1.5-fold upon addition of CODH II (0.5 mg of CODH II per mg of cytoplasmic protein). CODH I or NAD+ had no effect.

**DISCUSSION**

*C. hydrogenoformans* contains two distinct CODHs. CODH I and CODH II of *C. hydrogenoformans* are distinct proteins according to the results which we have obtained. CODH I (subunit mass, 62.5 kDa; holoenzyme mass, 125 kDa) and CODH II (subunit mass, 64.5 kDa; holoenzyme mass, 129 kDa) differ slightly in their subunit and holoenzyme masses (Fig. 1A and B) and are immunologically unrelated to each other (Fig. 1D). Their N-terminal sequences and proteolytic peptide patterns (Fig. 1C) indicate different primary structures. The enzymes show different temperature optima and catalytic properties. CODH I and CODH II from *C. hydrogenoformans*, as well as the CODH from *R. rubrum*, are very similar in sharing high CO-oxidizing activity, high affinity for CO, and comparable holoenzyme and subunit masses (6, 7).

Searches in the National Center for Biotechnology Information, SwissProt, Protein Information Resource, and The Institute for Genomic Research (TIGR) databases revealed that the N terminus of the CODH II subunit (Fig. 1B), as well as the N terminus of the 49-kDa chymotryptic peptide (Fig. 1C), is located on a DNA stretch from *C. hydrogenoformans*. This piece of DNA shows highest homology to cooS, which is the CODH subunit of *R. rubrum* (23). The experimentally determined subunit mass of CODH II (64.5 kDa; Fig. 1B) differs by less than 5% from the mass (67.3 kDa) which can be calculated from the *C. hydrogenoformans* CooS sequence (23). The mass of the 49-kDa chymotryptic fragment (Fig. 1C) of the CODH II subunit (Fig. 1B) compares favorably to the 49.6 kDa calculated from the CooS sequence. These considerations can be taken as evidence for the relatedness of the CODH II subunit with CooS. The amino acid sequences of *C. hydrogenoformans* CooS, the CODH from *R. rubrum* (55% identity), and the CO-oxidizing β-subunit of CODH-ACS from *C. thermoaceticum* (48% identity) are closely related (23).

Searches in the TIGR database revealed that the N terminus of the CODH I subunit (Fig. 1B) matches the amino acid sequence of an open reading frame identified in the *C. hydrogenoformans* genome. The deduced protein shows 74% homology to CooS (CODH II). The experimentally determined subunit mass of CODH I of 62.5 kDa (Fig. 1B) differs by only 8% from the mass (67.5 kDa) deduced from the sequence of this protein. These considerations identify the predicted *C. hydrogenoformans* protein as the CODH I subunit.

**Both CODHs are membrane-associated homodimeric NiFeS proteins.** Intact CO-grown cells of *C. hydrogenoformans* contain more than 92% of the total CO dehydrogenase population in association with the inner aspect of the cytoplasmic membrane (Fig. 4 and Table 2). The intracellular location of both CODHs was independent of the growth phase. Upon cell disintegration, a high proportion (~70%) of both CODHs became solubilized (Table 1), reflecting rather weak noncovalent interactions of the enzymes with the membrane.

Both CODHs of *C. hydrogenoformans* are homodimers with an α2 subunit structure (Fig. 1A and B) which is similar to the CODH from *R. rubrum* but different from acetogenic or methanogenic CODHs-ACSs, which are αβ2 tetramers or αγβε pentamers (15, 16, 47). The two *C. hydrogenoformans* CODHs contain Ni, Fe, and acid-labile sulfur. The mean Ni content values obtained with different methods (CODH I, 1.28 to 1.41 atoms of Ni/mol of dimer; CODH II, 1.65 to 1.67 atoms of Ni/mol of dimer) suggest the presence of 1 atom of Ni per mol of subunit of catalytically fully competent CODH and indicates an ~67% (CODH I) or ~83% (CODH II) occupancy of the Ni site. In *R. rubrum*, the occupancy of the CODH Ni site of ~65% (6) results from the biosynthesis of a mixture of an enzyme containing the full complement of 2 atoms of Ni/
FIG. 4. Localization of CODH I and CODH II on immunogold-labeled ultrathin sections of CO-grown *C. hydrogenoformans*. (A) Ultrastructure of the cell envelope after low-temperature embedding. CM, cytoplasmic membrane; S, surface layer; WL1, first wall layer; WL2, second wall layer; WL3, third wall layer. (B) Control labeling was with preimmune serum and gold-labeled secondary IgG antibodies. IgG antibodies directed against CODH I or CODH II were absent. (C and F) Longitudinal and cross-sections after labeling with IgG antibodies directed against CODH I and the gold-labeled secondary IgG antibodies. (D and G) Treated as for panels C and F, except that IgG antibodies directed against CODH II were used. (E) Tangential section parallel to the long cell axis; all other conditions were as for panels C and F. (H) Tangential section through a cell pole; all other conditions were as for panels C and F. Bar, 0.1 μm.
mol of dimer and a Ni-deficient apo-CODH (8, 31). The absence of EPR signals attributable to Ni(III) or Ni(I) in both C. hydrogenoformans CODHs under all conditions examined is interpreted as a divalent Ni (Fig. 3).

The Fe and acid-labile sulfide content, along with the EPR spectra (Fig. 3) of CODH I and CODH II, suggests the presence of at least two different redox-active [4Fe-4S] clusters per subunit. The rhombic signal at $g_{av} = 1.94$ (Fig. 3A and B, traces c and g) is assumed to originate from a [4Fe-4S]$^{1+}$ cluster similar to the reduced electron-transferring B cluster of the CODHs from R. rubrum or C. thermoaceticum (32). The second rhombic signal at $g_{av} = 1.86$ of CODH I (Fig. 3A, trace c) and $g_{av} = 1.84$ of CODH II (Fig. 3B, trace c) is believed to come from a faster-relaxing [4Fe-4S]$^{1+}$ cluster similar to the fully reduced CO-oxidizing Ni-X-[4Fe-4S] cluster (C cluster) of the CODHs from R. rubrum and C. thermoaceticum (32). The cluster C of R. rubrum CODH is suggested to be composed of [4Fe-4S], and [FeNi] clusters (30, 31, 53). The presumed B and C clusters in CODH I and CODH II would account for 8 atoms of Fe and 8 atoms of labile sulfur per mol of subunit. Therefore, the extra 3 to 4 atoms of Fe and 1.5 to 3 atoms of labile sulfur per mol of CODH subunit remain to be explained. They could be part of a single [2Fe-2S] cluster in each subunit or part of a single [4Fe-4S] cluster per dimer. The presence of a third [4Fe-4S] cluster bridging the two subunits would agree with the visible spectra of both CODHs (Fig. 2A and B), which show no [2Fe-2S] features. The additional Fe could also be part of a binuclear [NiFe] cluster (22, 31, 60).

**Presumed functions of the two CODHs in the metabolism of C. hydrogenoformans.** According to the scheme presented in Fig. 5, CODH I is involved in the generation of energy, and CODH II is involved in the assimilation of carbon. CODH I generates the electrons from CO which are subsequently channelled via the ferredoxin-like protein B to a hydrogenase, which is the site where intracellular protons are reduced to H$_2$. Ferredoxins couple the electron transport from CODH to hydrogenase in phototrophs (14), acetogens (48), and methanogens (57). The CO-driven proton respiration in C. hydrogenoformans is coupled to the translocation of H$^+$ across the cytoplasmic membrane. This is indicated by experiments where the application of CO pulses to resting cells led to a transient acidification of the bacterial environment from which H$^+$/CO ratios of 0.5 could be extrapolated under conditions where the membrane potential was dissipated by thioanite (KSCN). In R. rubrum (4, 20) or methanogenic Archaea (4, 29, 36, 41), the complex I-related H$_2$-evolving hydrogenases were proposed to be the site of proton translocation. We are, therefore, assuming the same function for the membrane-bound hydrogenase of C. hydrogenoformans, which shows sequence similarities of 80, 95, 72, and 67% (TIGR database) to the large (CooH), small (CooL), proposed H$^+$-translocating (CooK), and TYKY (CooX) subunits of the corresponding [Ni-Fe] hydrogenase of R. rubrum (20).

The anabolic function of CODH II involves the generation of NADPH and part of the CO$_2$ for carbon assimilation. CODH II is able to reduce NADP$^+$ if a cytoplasmic coupling factor X'/XH is present (Fig. 5). The coupling factor X could be a ferredoxin:NADP$^+$ oxidoreductase, in analogy to the CO-dependent reduction of NADP$^+$ in acetogenic bacteria (33, 48), the ability of CODH II to transfer electrons to protons instead to NADP$^+$ might help to regulate the proper ratio of reducing equivalents and CO$_2$ in carbon assimilation.

This study did not intend to resolve the path of the autotrophic fixation of CO or CO$_2$ in C. hydrogenoformans. However, we can conclude from the absence of ribulose-1,5-bisphosphate carboxylase activity in C. hydrogenoformans and the absence of a corresponding sequence on the genome that the Calvin-Benson-Bassham cycle is not operative. Although the genomic sequence of the bacterium (TIGR database) contains an open reading frame which is 86% homologous to the ACS of C. thermoaceticum, we have not been able to demonstrate ACS activity in C. hydrogenoformans. Alternative paths for carbon fixation in C. hydrogenoformans which must be considered in

### Table 2. Distribution of CODH I and CODH II on ultrathin sections of CO-grown C. hydrogenoformans

<table>
<thead>
<tr>
<th>Source (phase) of bacterial cells</th>
<th>CODH I in:</th>
<th>CODH II in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytoplasm, total count/cell (%)</td>
<td>Cytoplasm, total count/cell (%)</td>
</tr>
<tr>
<td>Middle of exponential growth</td>
<td>6.8 ± 1.2 (99.3 ± 2.9)</td>
<td>4.1 ± 1.3 (93.4 ± 9.4)</td>
</tr>
<tr>
<td>End of exponential growth</td>
<td>8.5 ± 1.9 (98.3 ± 3.6)</td>
<td>6.1 ± 1.4 (92.4 ± 7.6)</td>
</tr>
<tr>
<td>Stationary growth</td>
<td>9.0 ± 1.7 (98.8 ± 3.3)</td>
<td>5.0 ± 1.9 (94.2 ± 8.3)</td>
</tr>
</tbody>
</table>

* Gold particles found in attachment to the cytoplasmic membrane of C. hydrogenoformans or present in the cytoplasm after postembedding labeling with the immunogold were counted; for details see Materials and Methods.

**Table 3. Effect of CODH I and CODH II on the CO-dependent formation of H$_2$ by cytoplasmic membranes of CO-grown C. hydrogenoformans**

<table>
<thead>
<tr>
<th>Assay conditions</th>
<th>H$_2$ evolution ($\mu$mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoplasmic membranes plus CODH I plus protein B</td>
<td>30.1</td>
</tr>
<tr>
<td>Cytoplasmic membranes plus CODH II plus protein B</td>
<td>8.8</td>
</tr>
<tr>
<td>Cytoplasmic membranes plus CODH I plus CODH II</td>
<td>8.8</td>
</tr>
<tr>
<td>Cytoplasmic membranes plus CODH II</td>
<td>8.1</td>
</tr>
<tr>
<td>CODH I</td>
<td>0</td>
</tr>
<tr>
<td>CODH II</td>
<td>0.7</td>
</tr>
<tr>
<td>B protein</td>
<td>0</td>
</tr>
<tr>
<td>CODH I plus B protein</td>
<td>0</td>
</tr>
<tr>
<td>CODH II plus B protein</td>
<td>0.8</td>
</tr>
</tbody>
</table>

* Assays contained 5 mg of CODH I or CODH II, 5 mg of protein B, and 1 mg of cytoplasmic membranes as indicated. Cytoplasmic membranes were prepared as detailed in Materials and Methods and washed in buffer A by ultracentrifugation.

H$_2$ formation was measured under an atmosphere of 100% CO.
FIG. 5. Hypothetical scheme showing the function of the two CODHs in *C. hydrogenoformans*. CODH I is involved in energy generation and CODH II serves anabolic functions. For details refer to the text. The scheme is not intended to give correct stoichiometries. Abbreviations: B, ferredoxin-like protein; B$_\text{H_2ase}$, membrane-bound [NiFe] hydrogenase.

future research are the reductive citric acid cycle, the 3-hydroxypropionate cycle, the hydration of CO to formate, or the reduction of CO$_2$ to formate through the action of formate dehydrogenase. Indeed, cytoplasmic fractions of *C. hydrogenoformans* contain formate:oxidized MV oxidoreductase activity (0.7 μmol mg$^{-1}$).

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ADDENDUM IN PROOF

A crystal structure of reduced CODH II has been solved at 1.6-Å resolution (H. Dobbek, V. Svetlichnyi, L. Gremer, R. Huber, and O. Meyer, Science, in press). The structure represents the prototype for Ni-containing CODHs from anaerobic bacteria and archaea. It contains five metal clusters, of which clusters B, B’, and a subunit-bridging, surface-exposed cluster D are cubane-type [4Fe-4S] clusters. The active-site cluster C and C’ are navel, asymmetric [Ni-4Fe-5S] clusters. Their integral Ni ion, which is the likely site of CO oxidation, is coordinated by four sulfur ligands with square planar geometry.

REFERENCES
