JOURNAL OF BACTERIOLOGY, Dec. 1981, p. 904-911
0021-9193/81/120904-08$02.00/0

Vol. 148, No. 3

Purification and Some Properties of Carbon Monoxide Dehydrogenase from *Pseudomonas carboxydohydrogena*

Y. M. KIM AND G. D. HEGEMAN*

Microbiology Group, Biology Department, Indiana University, Bloomington, Indiana 47405

Received 22 May 1981/Accepted 31 July 1981

A soluble yellow CO dehydrogenase from CO-autotrophically grown cells of *Pseudomonas carboxydohydrogena* was purified 35-fold in seven steps to better than 95% homogeneity with a yield of 30%. The final specific activity was 180 μmol of acceptor reduced per min per mg of protein as determined by an assay based on the CO-dependent reduction of thionin. Methyl viologen, nicotinamide adenine dinucleotide (phosphate), flavin mononucleotide, and flavin adenine dinucleotide were not reduced by the enzyme, but methylene blue, thionin, and toluylene blue were reduced. The molecular weight of native enzyme was determined to be 4×10^5. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate revealed at least three nonidentical subunits of molecular weights 14,000 (α), 28,000 (β), and 85,000 (γ). The ratio of densities of each subunit after electrophoresis was about 1:2:6 (α/β/γ), suggesting an $\alpha_3\beta_3\gamma_3$ structure for the enzyme. The purified enzyme was free of formate dehydrogenase and nicotinamide adenine dinucleotide-specific hydrogenase activities, but contained particulate hydrogenase-like activity with thionin as electron acceptor. Known metal-chelating agents tested had no effect on CO dehydrogenase activity. No divalent cations tested stimulated enzyme activity. The native enzyme does not contain Ni since cells assimilated little ^{63}Ni during growth, and the specific ^{63}Ni content of the enzyme declined during purification. The isoelectric point of the native enzyme was found to be 4.5 to 4.7. The K_m for CO was found to be 63 μM. The spectrum of the enzyme and its protein-free extract revealed that it contains bound flavin. The cofactor was flavin adenine dinucleotide based on enzyme digestion and thin-layer chromatography. One mole of native enzyme contains at least 3 mol of noncovalently bound flavin adenine dinucleotide.

Pseudomonas (Seliberia) carboxydohydrogena DSM1083, like a variety of other bacteria (3, 20, 24, 38; D. H. Davis, Ph.D. thesis, University of California, Berkeley 1967; S. Kirkconnell, Ph.D. thesis, Indiana University, Bloomington, 1978), is able to grow aerobically at the expense of carbon monoxide (CO) as a sole source of energy and carbon. Other bacteria (e.g., some *Clostridium* spp.; 5, 6, 9, 11, 33) are able to oxidize CO, but apparently gain no benefit from the process.

Other workers have examined some gram-negative bacteria and have partially characterized the CO-oxidizing system (3, 21-23, 26, 38). To learn whether the basis of CO oxidation is the same or similar in other utilitarian CO-oxidizing bacteria and to learn more of the mechanism of the process, we decided to examine the enzyme(s) of *P. carboxydohydrogena* in some detail. Specific questions asked included the following. (i) What kind(s) of enzyme(s) are involved in CO oxidization? (ii) What prosthetic groups do such enzymes have? (iii) Are there intermediates detectable in the process of CO oxidation? (iv) Is nickel necessary for CO oxidation as in the case of the non-utilitarian *Clostridium* enzyme (9)?

To those ends, the purification and characterization of purified CO dehydrogenase (CO:acceptor oxidoreductase, CO-DH) from *P. carboxydohydrogena* were carried out.

MATERIALS AND METHODS

Bacterial strain and conditions of cultivation. A culture of the type strain of *P. carboxydohydrogena* DSM1083 (*Seliberia carboxydohydrogena* Z-1062) was obtained from J. Schmidt, University of Arizona, Tempe. The standard mineral medium used for autotrophic growth was prepared by the method of Doudoroff (8). *P. carboxydohydrogena* was cultivated in a fermentor (16 liters; New Brunswick Scientific Co.) with standard mineral medium and stirred at 200 rpm at 30°C. The gas mixture of 30% CO-70% air for submerged cultures in standard mineral medium was prepared in a 40-liter ballast vessel and circulated by a bellows pump. The gas mixture was periodically analyzed with a Hewlett-Packard model 5710A gas

chromatograph equipped with a Spherocarb (80/100 mesh) column (1.8 m by 2 mm), and the ballast vessel was refilled with an appropriate atmosphere as needed. CO, (chemically purified; 99.5% minimum, vol/vol), was purchased from Matheson. Growth was measured by turbidity determined using a Klett-Summerson colorimeter with a red filter (no. 66). After 2 weeks of cultivation from a small inoculum, a yield of 5 g of wet cells per liter was obtained. Cells were harvested by centrifugation at 10,000 × *g* for 30 min, washed once by resuspension in the standard buffer, and stored at −20°C.

Enzyme assay. CO-DH activity was assayed by measuring the CO-dependent decrease of absorbancy of thionin dye ($\epsilon_{595} = 4.2 \times 10^4\ M^{-1} \cdot cm^{-1}$) at 30°C. Anaerobic silica cuvettes (1-cm light path, 4.2-ml total volume; Precision Cells) with Teflon caps with drilled holes (0.75 mm in diameter) were filled with CO-saturated 0.05 M Tris-hydrochloride (pH 7.5, standard buffer) containing 20 μM thionin and with 10 μl of 3- or 4-day-old 25 mM sodium hydrosulfite in 10 mM NaOH solution as a reducing agent to remove molecular oxygen (14). The reaction was started by the addition of the enzyme preparations with a syringe, and the reduction of absorbance at 595 nm with standard buffer as a reference was measured with a spectrophotometer coupled to a digital converter and to a strip chart recorder.

To test the hydrogenase activity of purified enzyme, H_2 was substituted for CO as the substrate. In addition, β-NAD (5 mM) was introduced as the electron acceptor for testing NAD-specific hydrogenase activity. For the formate dehydrogenase activity measurement, 5 mM β-NAD was used as the electron acceptor, and 0.5 mM sodium formate was used as the substrate. N_2 was used to flush the standard buffer containing both the acceptor and the substrate.

Protein determination. Protein was measured by modified biuret reagent (Sigma Chemical Co.), using a standard curve prepared previously with bovine serum albumin as a standard (12). The protein contents of crude extracts were determined by the same method after boiling the samples in 20% NaOH for 10 min (20).

Electrophoresis. Analysis of native enzyme was carried at 0 to 2°C by nondenaturing polyacrylamide gel electrophoresis (PAGE) by the method of Laemmli (18), but without sodium dodecyl sulfate (SDS). Denaturation analysis of native enzyme was done by the SDS-PAGE technique of Laemmli (18) at the temperature of circulating tap water. Gels containing 7.5 and 11.5% acrylamide were used for nondenaturing and denaturing PAGE, respectively. All electrophoresis was performed in a vertical slab gel apparatus (Bio-Rad Laboratories) and was conducted with pulsed power by using a constant power supply (Ortec, EC & Co.; model 4100) at 320 V. The pulse rate was adjusted throughout the electrophoresis by the method of Beremand and Blumenthal (1). Gels were stained with 0.25% Coomassie brilliant blue R-250 in 9% acetic acid–45% methanol with the modification of Weber and Osborn (34). Activity staining was carried out by the method of Dietz and Lubrano (7), with some modification. The nondenaturing gel strip was equilibrated with cold standard buffer for 10 min, transferred to a Hungate tube containing 9 ml of standard buffer, and flushed with CO for at least 6 min. A 1.0-ml portion of phenazine methosulfate-nitro blue tetrazolium stock solution (phenazine methosulfate, 0.5 mg/ml; nitro blue tetrazolium, 2.5 mg/ml) was injected with a syringe into the tube, which was wrapped with aluminum foil. The reaction mixture was flushed with CO for 2 min and was left for 1 to 2 min at room temperature. The gel was then washed several times with water and kept in a 7.5% acetic acid solution. H_2 gas was used as the substrate for hydrogenase, and 0.01 M sodium formate and 0.5 mM β-NAD were added to test for formate dehydrogenase activity in an otherwise identical system.

Enzyme purification. All purification steps were carried out at 4°C, except when noted. A 60-g (wet weight) portion of thawed cells was resuspended in 240 ml of cold standard buffer, homogenized with a tissue homogenizer, and disrupted by sonic treatment (10 s/ml) in portions of 30 ml at 0°C. The suspension was centrifuged at 10,000 × *g* for 30 min. The supernatant fluid was referred to as crude extract. The crude extract was then treated with protamine sulfate to a final concentration of 0.054%, left in ice for 10 min, then sedimented at 100,000 × *g* for 90 min. The resulting supernatant fluid (soluble fraction) was next made 40% saturated with respect to ammonium sulfate. After 5 h, this fraction was centrifuged at 15,000 × *g* for 30 min. The resulting supernatant was further treated with ammonium sulfate to achieve a final concentration of 55% of saturation. After 5 h the solution was centrifuged again at 15,000 × *g* for 30 min, and the sediment was resuspended in a small volume of cold standard buffer. The deep yellow suspension was then dialyzed against three 2-liter changes of standard buffer for 20 h. The dialysate was then applied to a Sepharose 6B column (2.6 by 90 cm). Elution was performed with the same buffer at a flow rate of 7.1 ml/cm² per h with 200 cm of hydrostatic pressure. Fractions with the highest specific activity were pooled and applied to a DEAE-Sephadex A-50 column (2.6 by 31.5 cm) which had a 1-cm layer of Sephadex G-25 (coarse) at the top. The column was pre-equilibrated with at least 2 total bed volumes of standard buffer before sample application. Elution was first carried out with 200 ml of 0.15 M NaCl in standard buffer followed by a shallow linear NaCl gradient (400 ml, 0.15 to 0.3 M). Fractions were collected at a flow rate of 1 ml/cm² per h, and the yellow-colored fractions with the highest specific activity were pooled and applied to a hydroxylapatite column (1.5 by 6 cm) pre-equilibrated with the standard buffer. The column was eluted with a linear ammonium sulfate gradient (100 ml, 0 to 0.5 M) at a rate of 2.0 ml/cm² per h. A yellow active fraction that contained all of the CO-DH activity was eluted at about 0.15 M ammonium sulfate and was dialyzed against three 500-ml changes of cold standard buffer, each for 2 h.

Salt concentration in each fraction after DEAE-Sephadex A-50 and hydroxylapatite chromatography was determined by measuring the conductivity of each fraction with a conductivity bridge by using the conductivity of known NaCl and ammonium sulfate solutions, respectively, as standards.

The purified CO-DH was stored at −70°C.

Subunits of CO-DH. The numbers of subunits in CO-DH were determined by nondenaturing-denaturing two-dimensional PAGE.

The purified enzyme was first subjected to nondenaturing PAGE (7.5% acrylamide). After electrophoresis was completed, a 0.5- by 1.0-cm block which contained the yellow CO-DH band was cut from the slab, placed in test tube, and equilibrated with two changes of 5 ml of Laemmli sample buffer with SDS (18) at 55°C for 2 h. The gel piece was then placed onto the top of a denaturing gel (11.5% acrylamide) and was electrophoresed for subunit analysis. To estimate the numbers of each subunit in CO-DH, the density of each subunit after staining with Coomassie brilliant blue was measured with a densitometer.

Preparation of antiserum against CO-DH. A New Zealand white rabbit was immunized with 200 μg of the purified CO-DH homogenized 1:1 in Freund adjuvant (Difco Laboratories). Injections (0.2 ml) were made intradermally at multiple points along the flanks, and the remainder was injected intramuscularly into the rear quadrants. The rabbit was injected with an additional 100 μg of protein at 2 weeks. After 4 weeks the rabbit was bled; 35 ml was removed from the ear. Blood was stored overnight at 4°C, and erythrocytes were removed after clotting by centrifugation at 2,000 × *g* for 10 min at 4°C before freezing. The final recovery was 15 to 20 ml of serum per bleeding.

Test for nickel in CO-DH. It had been reported that the CO-DH from *Clostridium thermoaceticum* contains Ni (9). To see whether the CO-DH from *P. carboxydohydrogena* also contains Ni, *P. carboxydohydrogena* was cultivated under standard culture conditions with 100 μCi of $^{63}NiCl_2$ (ICN Chemical & Radioisotope Division; specific activity, 11.1 mCi/mg of Ni) per liter, and CO-DH from dialyzed $(NH_4)_2SO_4$ precipitates was precipitated with rabbit antiserum. Precipitation was allowed to occur for 5 min at room temperature, and the mixture was centrifuged at 5,000 × *g* for 20 min at 4°C.

The precipitate was collected on glass fiber filter paper (Whatman Ltd.; GF/A, 2.4 cm), dried for 5 min under a 250-W infrared lamp, and then immersed in toluene-based scintillator. The amount of ^{63}Ni in CO-DH on the filter was measured by using a Beckman LS-230 liquid scintillation counter.

Cofactor analysis. The spectrum of the purified native enzyme solution was measured in a Cary 14 spectrophotometer in the visible range (300 to 600 nm).

Extraction of the cofactor from purified enzyme was carried out with trichloroacetic acid by the method of Swoboda and Massey (32). The visible light spectrum of the protein-free flavin derivative in aqueous solution was determined after removal of the trichloroacetic acid by repeated extraction with diethyl ether.

To confirm the chemical identity of the cofactor, the extracted flavin derivative was subjected to thin-layer chromatography along with some reference compounds: riboflavin, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD). Phosphodiesterase (*Crotalus adamanteus;* Sigma) and alkaline phosphatase (Bethesda Research Laboratories) were used to treat FMN, FAD, and the flavin cofactor for 30 min at 37°C. Enzyme-treated and untreated references and samples were then applied on silica gel plates (0.25 mm thick, 20 by 20 cm; EM Laboratories, Inc.) for ascending chromatography in a rectangular glass chamber which was saturated with 200 ml of one of several solvents tested. Running times ranged from 55 min to 3.5 h, depending on the solvent used. Each separated spot after thin-layer chromatography was then visualized by native fluorescence under longwave UV light.

The number of moles of FAD per mole of CO-DH was estimated by photometric analysis (32). $\epsilon_{450} = 11.3 \times 10^3\ M^{-1} \cdot cm^{-1}$ (37) was used as the molar absorbancy coefficient to calculate the amount of FAD.

RESULTS

Purification. The CO-DH was purified 35-fold in seven steps with a yield of 30% and a final specific activity of about 180 μmol of thionin reduced per min per mg of protein (Table 1). The purified enzyme had a yellow color and was found to be completely stable for 5 months at −70°C under air. The enzyme activity in whole cells was stable for over 1 year at −20°C under air.

Purified enzyme from the hydroxylapatite column was judged better than 95% homogeneous (Fig. 1A) after densitometric scanning of the gel stained with Coomassie brilliant blue after nondenaturing PAGE. It was possible to see a yellow CO-DH band with the unaided eye on nondenaturing polyacrylamide gel after PAGE with about 40 μg enzyme.

Activity staining with CO or with H_2 as the substrate revealed that the purified enzyme had both CO-DH and hydrogenase activities. Activity staining with sodium formate did not show any formate dehydrogenase activity in the purified enzyme. Hydrogenase and formate dehydrogenase activities of the purified enzyme were rechecked by enzyme assay. The purified enzyme was free of measurable formate dehydrogenase and NAD-specific hydrogenase activities, but with thionin as acceptor had hydrogenase activity amounting to 8.5% of the CO-oxidizing activity.

Reduction of artificial electron acceptors. CO-DH has been shown to reduce several artificial electron acceptors in the presence of CO. The standard assay method was used with various acceptors (methyl viologen, NAD, NADP, FAD, FMN, methylene blue, thionin, toluylene blue, 2,6-dichlorophenol indophenol, and ferricyanide) to determine the range of electron acceptors and to select the best acceptor for measuring CO oxidation. Among those electron acceptors tested, only three (methylene blue, thionin, and toluylene blue) were reduced by the enzyme when CO was used as substrate. The reduction rate was the highest when thionin was used as acceptor. Methylene blue and toluylene blue were reduced at 83 and 20% of this rate, respectively.

TABLE 1. *Purification of CO-DH from CO-autotrophically grown cells of P. carboxydohydrogena*

Purification step	Total protein (mg)[a]	Sp act[b]	Purification (-fold)	Total activity[c]	Recovery (%)
Crude extract	1,736	5.2	1	9,027	100
Soluble fraction	1,108	16	3.1	17,728	196
Ammonium sulfate (40 to 55%)	329	41	7.9	13,489	149
Dialysis	327	42	8.1	13,734	152
Sepharose 6B	105	82	15.8	8,610	95
DEAE A-50	40	140	27.0	5,600	62
Hydroxylapatite	15	180	35.0	2,700	30

[a] Biuret method.
[b] Micromoles of thionin reduced per milligram of protein per minute.
[c] Micromoles of thionin reduced per minute.

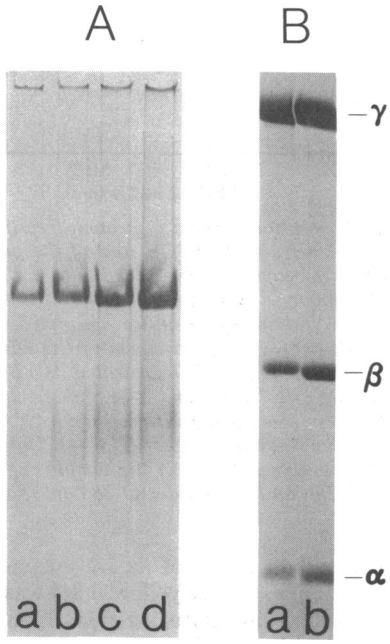

FIG. 1. *A, CO-DH after nondenaturing PAGE of the purified enzyme stained with Coomassie brilliant blue. Gels containing 7.5% acrylamide were run by the method of Laemmli (18) without SDS; 5 μg (a), 10 μg (b), 20 μg (c), and 40 μg (d) of enzyme were applied. B, Dissimilar subunits in the purified CO-DH. Denaturing PAGE (11.5% acrylamide, 0.1% SDS) was carried out with the active CO-DH band after nondenaturing PAGE (7.5% acrylamide) of the purified enzyme. Original enzyme concentrations for non-denaturing PAGE were 20 μg (a) and 40 μg (b).*

Molecular weight and structure. The molecular weight of the purified enzyme was determined to be 4×10^5 by using Sepharose 6B column (2.6 by 88 cm) chromatography by the method of Whitaker (36) with reference proteins of known molecular weight. Two-dimensional nondenaturing-denaturing PAGE revealed the presence of three nonidentical subunits (Fig. 1B) in the native enzyme; the molecular weights of the subunits were found to be 14,000 (α), 28,000 (β), and 85,000 (γ) by using SDS-gel electrophoresis with several molecular weight references. The ratio of integrated densities of each Coomassie brilliant blue-stained subunit after electrophoresis was about 1:2:6 (α:β:γ), suggesting an unusual $\alpha_3\beta_3\gamma_3$ structure for CO-DH based on the assumption that all subunits were stained to the same extent with Coomassie brilliant blue.

Isoelectric point (pI). The pI of the purified enzyme was determined by isoelectric focusing in thin slabs of polyacrylamide gel according to LKB application note 250 using LKB 2117 Multiphor (LKB Instruments, Inc.), with a slight modification: acrylamide concentration, 4.2%; final concentration of ampholine (LKB), 2.4% (pH range of 3.0 to 9.5); and the addition of 50 μl of *N,N,N',N'*-tetramethyl-ethylenediamine to the gel mixture.

The pI of the enzyme was found to be 4.5 to 4.7. This result is in good agreement with the observation that CO-DH activity was not detected in supernatants of DEAE A-50- or QAE A-50-treated soluble fraction in 0.1 M Tris-hydrochloride buffer (pH 8).

Enzyme kinetics. The rate of reduction of thionin by the purified enzyme with CO as substrate was completely proportional to the amount of enzyme added.

The standard assay method was used to measure the dependence of the rate of thionin reduction on CO concentration to determine the K_m and V_{max} of the purified CO-DH, except that volumetric gas mixtures containing various concentrations of CO in argon (Matheson; prepurified, 99.998% minimum) were used to flush the standard buffer before the addition of enzyme. Amounts of CO in the mixture were adjusted by using a Rotameter (Matheson; model 7401 T). Thionin reduction followed Michaelis-Menten kinetics. Assuming that 1 mol of thionin can be reduced by two electrons which come from the oxidation of 1 mol of CO, the K_m and V_{max} for

CO were found to be 63 μM and 183 μmol of CO oxidized per mg of protein per min, respectively, following Lineweaver-Burk (19) and Hofstee (13) plots. The solubility of CO at 30°C under atmospheric pressure was taken to be 19.15 ml in 1 liter of water (30).

Effect of divalent cations and chelating agents. The known metal chelating agents tested (1 mM NaCN, NaN_3, EDTA, and *o*-phenanthroline) did not show any significant effect on CO-DH activity under standard assay conditions. EDTA was the most effective agent, reducing CO-DH activity by 7%, suggesting that no easily removed divalent metal is necessary for enzyme activity.

Some divalent cations were tested for their effects on the CO-DH activity under standard assay conditions. None of the metals tested (Mn^{2+}, Co^{2+}, Mg^{2+}, Cu^{2+}, Ca^{2+}, Ba^{2+}, Ni^{2+}, Zn^{2+}) at 1 mM concentration stimulated the enzyme activity. Cu^{2+} eliminated the enzyme activity completely, suggesting that a necessary sulfhydryl group was inactivated by this ion. However, incubation in the presence of 1 mM *N*-ethylmaleimide or iodoacetamide did not significantly reduce enzyme activity (data not shown).

Ni is not present in *P. carboxydohydrogena* CO-DH. When the dialyzed fraction was treated with antiserum there remained less than 0.001% of the enzyme activity in the supernatant, indicating that all the CO-DH activity was precipitated. The precipitated CO-DH from ^{63}Ni-grown cells did not contain a significant amount of Ni (4.67×10^{-4} mol of Ni per mol of CO-DH), suggesting that Ni is not present in the CO-DH of *P. carboxydohydrogena*. The amount of CO-DH was calculated from the specific activity of the dialyzed fraction and of the purified enzyme. This result agrees with the observation that the CO-DH activity was not affected by metal chelating agents and was not activated by nickel ion. During almost threefold purification of ^{63}Ni-CO-DH, the specific activity of ^{63}Ni decreased from 4,750 cpm/mg of protein (soluble fraction) to 2,260 cpm/mg of protein (dialyzed fraction), and the total radioactivity decreased almost 74% although only 23% of the total enzyme activity was lost.

FAD is present. The yellow color of purified CO-DH suggested that it might contain a certain cofactor(s). The visible light spectrum of the native enzyme first showed that it might contain bound flavin, and that of the trichloroacetic acid-treated, protein-free extract of this enzyme revealed that the cofactor was likely a flavin derivative (Fig. 2). The extracted flavin was found to be FAD after enzymatic digestion and thin-layer chromatography in various solvents (Table 2), and it appeared that at least 3 mol of

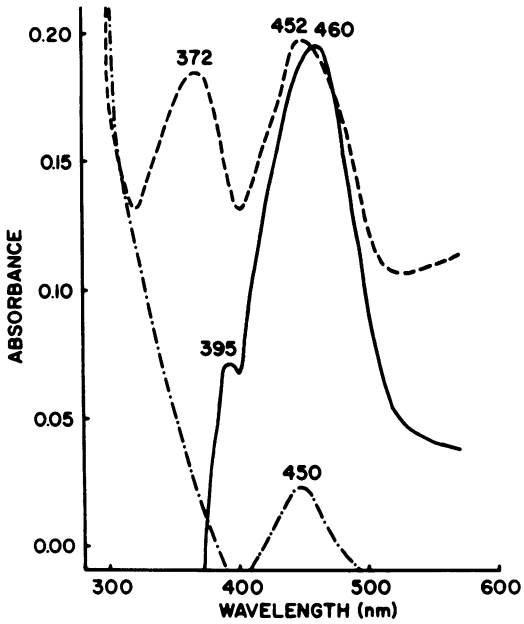

FIG. 2. *Absorption spectra of protein-free trichloroacetic acid extract of CO-DH and of the purified holoenzyme. Spectra were measured with a Cary 14 spectrophotometer. Symbols: spectrum of reduced CO-DH with sodium hydrosulfite against oxidized CO-DH with H_2O_2 (——); spectrum of CO-DH after flushing with CO against CO-saturated 0.05 M Tris-hydrochloride (pH 7.5) buffer (-·-); and spectrum of reduced protein-free extract with sodium hydrosulfite against oxidized extract with H_2O_2 (---). Untreated intact enzyme shows peaks in the visible region at 450 and 333 nm and a shoulder at 380 nm (data not shown).*

FAD is bound to 1 mol of native enzyme. As mentioned above, a clear yellow band was seen on the gel after nondenaturing PAGE, but this disappeared with some loss of activity staining function when the gel was stored in standard buffer for 40 h at 4°C. Exogenously added FAD was found to accelerate activity staining of this gel. Furthermore, there was no yellow band on the gel after electrophoresis with 0.1% SDS. All of the above results indicate that FAD is noncovalently bound to the apoprotein. However, enzyme activity was not increased when the purified enzyme was pretreated with exogenously added FAD (6.36 nM) for 20 min at room temperature, indicating that this cofactor is tightly bound to the protein.

DISCUSSION

Among several known utilitarian and non-utilitarian CO-oxidizing bacteria, only one of each type, *Clostridium thermoaceticum* (9) and *Pseudomonas carboxydovorans* (22), have been ex-

TABLE 2. *Relative migration distances (R_f values) of marker flavins and protein-free extract from purified CO-DH*[a]

Sample[b]	R_f		
	Solvent 1[c]	Solvent 2[d]	Solvent 3[e]
Riboflavin	0.5	0.4	0.14
FMN	0.3	0.24	0.01
FMN + phosphatase	0.5/0.3	0.4/0.24	0.14/0.01
FAD	0.16	0.2	0.0
FAD + diesterase	0.3	0.25	0.01
FAD + diesterase + phosphatase	0.5/0.3	0.4/0.24	0.14/0.01
Extract	0.16	0.2	0.0
Extract + diesterase	0.3	0.24	0.01
Extract + diesterase + phosphatase	0.48	0.4	0.14

[a] R_f values were calculated by dividing distances of spot centers from the starting point with distance of solvent front from the starting point.

[b] Samples were cleaved with alkaline phosphatase or phosphodiesterase as described in the text.

[c] *n*-Butanol-acetone-acetic acid-water (5:2:1:3, vol/vol) (15), 2h.

[d] *n*-Butanol-ethanol-water (50:15:35, vol/vol) (29), 3.5 h.

[e] Acetic acid-acetone-methanol-benzene (5:5:20:70, vol/vol) (28), 55 min.

tensively studied with regard to the mechanism of CO oxidation at the level of the purified enzyme system. The CO-DH from *P. carboxydohydrogena* was purified and characterized in this study to provide more information about aerobic utilitarian oxidation of CO, a process which may have a significant role in the oxidation of CO in the environment (27).

The enzyme was purified by means of conventional methods to better than 95% homogeneity, and the purified enzyme comprised about 3% of the soluble cell protein. The specific activity of this enzyme was almost 100-fold greater than that of *P. carboxydovorans* (1.94 μmol of CO oxidized per min per mg of protein) (22) under the assumption that the reduction of 1 mol of thionin corresponded to the oxidation of 1 mol of CO. The purified enzyme has greater stability than the enzyme of *P. carboxydovorans* which is quite sensitive to storage at −20°C under air. The clostridial CO-DH is very unstable under air (9). The *P. carboxydohydrogena* enzyme had non-NAD-linked hydrogenase activity, but no formate dehydrogenase activity. This result is similar to observations made with the *P. carboxydovorans* enzyme and indicates that free formate is not an intermediate in CO oxidation. However, we still cannot eliminate the possibility of bound formate being an intermediate during CO oxidation.

The occurrence of hydrogenase activity (with thionin but not NAD as acceptor) places this enzyme in an unusual category since other hydrogenases are typically either (i) soluble and act with pyridine nucleotide cofactors, or (ii) insoluble (particulate) and act with dyes, flavin, etc., as acceptors. Since the CO-DH is soluble, but catalyzes the reduction of thionin, it strictly fits neither of these previously established categories. This combination of properties may be explained in two ways. As previously mentioned by Cypionka et al. (3), the CO-oxidizing enzyme may be a loosely attached peripheral membrane protein. Alternatively, the hydrogenase associated with CO-DH is a new kind of hydrogenase which is soluble but cannot reduce NAD.

Previous reports stated that hydrogenase is not involved in CO oxidation even though homogeneous enzyme from *P. carboxydovorans* also showed some hydrogenase activity. In the case of the enzyme from *P. carboxydohydrogena*, the percentage of hydrogenase activity as a fraction of the CO-DH activity is almost 4 times greater than that of *P. carboxydovorans*. To explain the hydrogenase association with CO-DH, we suggest two possibilities. (i) Hydrogenase may be involved in the CO utilization and also in the use of H_2 which may be formed as an intermediate, but not evolved in free form. (ii) Hydrogenase is not involved in CO oxidation, but H_2 is a pseudosubstrate for the enzyme by virtue of its analogy with CO.

The purified enzyme can use several dyes which have redox potentials between +10 mV and +110 mV as electron acceptors. This was also true in preliminary experiments with the crude soluble fraction. This range of artificial electron acceptors is similar to that of the *P. carboxydovorans* enzyme, except that methylene blue was the best acceptor for the *P. carboxydovorans* enzyme (21, 22). This result suggests that a quinone may serve as a physiological electron acceptor during CO oxidation. Several other findings which tend to confirm a quinone in the role of physiological electron acceptor in *P. carboxydohydrogena* are reported in the accompanying paper (16). There is another report about the range of electron acceptors in several carboxydobacteria which supports the notion that quinone is a physiological electron acceptor in aerobic carboxydobacteria when CO is used as an energy source (3). According to those data, thionin is a poor electron acceptor for CO oxidation in *P. carboxydohydrogena*. This disagreement indicates the need for further study. In the case of the clostridial enzyme, electron acceptors of positive potential were not reduced and only dyes of negative potential acted as electron acceptors. Ferredoxin is thought to be

a physiological acceptor for *C. thermoaceticum* (9).

The molecular weight of the purified enzyme was around 4×10^5, which is almost the same as that of the enzyme from *C. thermoaceticum* (4.1×10^5) (9). The subunit structure of the native *P. carboxydohydrogena* enzyme seems to be an unusual $\alpha_3\beta_3\gamma_3$ (α:14k, β:28k, γ:85k), which suggests a complicated mechanism in CO oxidation. When compared with the structure of the enzyme from *P. carboxydovorans* (2.3×10^5, two identical subunits of 1.07×10^5) (22), there are significant differences. A previous report estimates the molecular weight of CO-DH in *P. carboxydohydrogena* to be 2.3×10^5 (3), in disagreement with that (4×10^5) found in the present study. The difference between the two values may arise from the different methods used for molecular size determinations.

Cross-linking experiments with reversible or nonreversible reagents showed that all three kinds of subunit are involved in the cross-linking reaction, but we could not successfully reverse the cross-linking process (data not shown). These experiments, along with transmission electron microscopy and immunological studies, will be continued to better establish the structure of this enzyme.

The K_m for CO of the purified CO-DH from *P. carboxydohydrogena* is 63 μM. This value is comparable to that for the counterpart purified from *P. carboxydovorans* (53 μM) (22), considerably lower than that of *Methanobacterium thermoautotrophicum* extracts (>1 mM) (4), and intermediate between the values determined for the non-utilitarian anaerobes (*Clostridium pasteurianum*, 5 μM in extracts [11, 33]; *Clostridium formicoaceticum* and *C. thermoaceticum*, 2.2 and 150 μM, respectively, in extracts [6]). Since the concentration of CO in the atmosphere is so low (1.3 to 39 nmol/liter depending upon the site of measurement [25]) it seems problematic whether utilitarian CO oxidizers like *P. carboxydohydrogena* can use atmospheric CO for growth. However, it has recently been shown that *P. carboxydovorans* can utilize CO at such low concentrations (2). There may also be microenvironments in which the CO concentration is sufficiently high for CO-DH to afford the bacteria that possess it a selective advantage (27). For instance, it is known that flavonoids and porphyrins are decomposed in the soil with formation of CO (2, 35). Rainwater sometimes contains up to 200 times the concentration of CO expected based upon its partial pressure in the atmosphere (31).

The yellow color of the purified enzyme was found to reflect the presence of noncovalently but tightly bound FAD which is necessary for CO oxidation. One mole of native enzyme appears to have 3 mol of FAD, suggesting that one of the three types of subunits binds the FAD. The spectrum of the *P. carboxydovorans* enzyme showed two peaks at 470 and 405 nm, and it has been suggested on this basis alone that the CO-oxidizing enzyme of this bacterium might be an iron-sulfur protein (22). In the case of the clostridial enzyme, it has been suggested that the CO-DH might be a corrinoid enzyme (9).

It has been reported that the CO-DH of *C. thermoaceticum* is a metallo-nickel enzyme (9) and that active hydrogenase formation in some hydrogen bacteria requires a supply of Ni (10). The CO-DH of *P. carboxydohydrogena* does not have a significant amount of Ni; therefore, Ni is apparently not a necessary factor for CO oxidation in all biological systems. There is also no necessary relationship between hydrogen bacteria and carboxydobacteria with respect to the involvement of this unusual trace metal. This result is also supported by observations that several chelators of divalent metals did not inactivate the enzyme and that exogenously added $NiCl_2$ and other divalent metal salts did not stimulate, but sometimes inactivated, the purified enzyme.

Kirkconnell and Hegeman (17), working with other bacteria, tried to find the source of the second oxygen atom in CO_2 derived from CO by using double-labeling experiments employing ^{13}CO and either $H_2{}^{18}O$ or $^{18}O_2$ and found, paradoxically, that neither water nor O_2 was the immediate source. As mentioned above, we used a completely anaerobic reaction system to measure the CO-DH activity, implying that it was impossible for molecular oxygen to be involved stoichiometrically in the reaction. This indicates that only water can be the source of the second oxygen atom in CO oxidation by *P. carboxydohydrogena* as in the case of *P. carboxydovorans* (21, 22) and the clostridia (9).

From the results mentioned above, we can suggest that oxidation of CO as an energy source by bacteria is mediated by different enzymes of the dehydrogenase type. Taken together with the fact that bacteria from many different biological groups oxidize CO, this suggests either that the ability to use CO evolved independently in different evolutionary lines or at a very early time before divergence occurred, or that genetic exchange, perhaps mediated by plasmids or other wide-ranging mechanisms, has dispersed genes for a common ancestral CO-DH to many different groups of bacteria at some remote time. The striking structural differences between the CO-DHs of *P. carboxydohydrogena* and *P. carboxydovorans* suggest that independent evolution has taken place.

ACKNOWLEDGMENTS

We thank J. Schmidt for the gift of strain DSM1083, S. Kirkconnell for anti-serum preparation, and T. Blumenthal, J. Douglass, and R. Fisher for hospitality, materials, and aid in the use of equipment.

This work was supported by National Science Foundation Research Grant PCM-78-12482.

LITERATURE CITED

1. **Beremand, M. N., and T. Blumenthal.** 1979. Overlapping genes in RNA phage: a new protein implicated in lysis. Cell **18**:257–266.
2. **Conrad, R., and W. Seiler.** 1980. Role of microorganisms in the consumption and production of atmospheric carbon monoxide by soil. Appl. Environ. Microbiol. **40**: 437–445.
3. **Cypionka, H., O. Meyer, and H. G. Schlegel.** 1980. Physiological characteristics of various species of strains of carboxydobacteria. Arch. Microbiol. **127**:301–307.
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. **Diekert, G. B., E. G. Graf, and R. K. Thauer.** 1979. Nickel requirement for carbon monoxide dehydrogenase formation in *Clostridium pasteurianum*. Arch. Microbiol. **122**:117–120.
6. **Diekert, G. B., and R. K. Thauer.** 1978. Carbon monoxide oxidation by *Clostridium thermoaceticum* and *Clostridium formicoaceticum*. J. Bacteriol. **136**:597–606.
7. **Dietz, A. A., and T. Lubrano.** 1967. Separation and quantitation of lactic dehydrogenase isoenzymes by disc electrophoresis. Anal. Biochem. **20**:246–257.
8. **Doudoroff, M.** 1940. The oxidative assimilation of sugars and related substance by *Pseudomonas saccharophila* with a contribution to the problem of the direct respiration of di- and polysaccharides. Enzymology **9**:59–72.
9. **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.
10. **Friedrich, B., E. Heine, A. Finck, and C. G. Friedrich.** 1981. Nickel requirement for active hydrogenase formation in *Alcaligenes eutrophus*. J. Bacteriol. **145**: 1144–1149.
11. **Fuchs, G., U. Schnitker, and R. K. Thauer.** 1974. Carbon monoxide oxidation by growing cultures of *Clostridium pasteurianum*. Eur. J. Biochem. **49**:111–115.
12. **Gornall, A. G., C. J. Bardawill, and M. M. David.** 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. **177**:751–766.
13. **Hofstee, B. H. J.** 1959. Non-inverted versus inverted plots in enzyme kinetics. Nature (London) **184**:1296–1298.
14. **Jones, R. W., and P. B. Garland.** 1977. Sites and specificity of the reaction of bipyridylium compounds with anaerobic respiratory enzymes of *Escherichia coli*. Biochem. J. **164**:199–211.
15. **Kato, N., Y. Oman, Y. Tani, and K. Ogata.** 1976. Alcohol oxidases of *Kloeckera* sp. and *Hansenula polymorpha*: catalytic properties and subunit structures. Eur. J. Biochem. **64**:341–350.
16. **Kim, Y. M., and G. D. Hegeman.** 1981. Electron transport system of an aerobic carbon monoxide-oxidizing bacterium. J. Bacteriol. **148**:991–994.
17. **Kirkconnell, S., and G. D. Hegeman.** 1978. Mechanism of oxidation of carbon monoxide by bacteria. Biochem. Biophys. Res. Commun. **83**:1584–1587.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
19. **Lineweaver, H., and D. Burk.** 1934. The determination of enzyme dissociation constants. J. Am. Chem. Soc. **56**:658–666.
20. **Meyer, O., and H. G. Schlegel.** 1978. Reisolation of the carbon monoxide utilizing hydrogen bacterium *Pseudomonas carboxydovorans* (Kistner) comb. nov. Arch. Microbiol. **118**:35–43.
21. **Meyer, O., and H. G. Schlegel.** 1979. Oxidation of carbon monoxide in cell extracts of *Pseudomonas carboxydovorans*. J. Bacteriol. **137**:811–817.
22. **Meyer, O., and H. G. Schlegel.** 1980. Carbon monoxide: methylene blue oxidoreductase from *Pseudomonas carboxydovorans*. J. Bacteriol. **141**:74–80.
23. **Nozhevnikova, A. N., and N. D. Savel'eva.** 1972. Autotrophic assimilation of carbon dioxide by a carbon monoxide oxidizing bacterium. Microbiology **41**:837–843.
24. **Nozhevnikova, A. N., and G. A. Zavarzin.** 1974. On the taxonomy of CO-oxidizing Gram-negative bacteria. Izv. Akad. Nauk SSSR Ser. Biol. **3**:436–440.
25. **Robbins, R. C., K. M. Borg, and E. Robinson.** 1968. Carbon monoxide in the atmosphere. J. Air Pollut. Control Assoc. **18**:106–111.
26. **Romanova, A. K., A. N. Nozhevnikova, I. G. Leonthev, and S. A. Alekseeva.** 1977. Pathways of assimilation of carbon oxides in *Seliberia carboxydohydrogena* and *Achromobacter carboxydus*. Microbiology **46**:719–722.
27. **Spratt, H. G., Jr., and J. S. Hubbard.** 1981. Carbon monoxide metabolism in roadside soils. Appl. Environ. Microbiol. **41**:1192–1201.
28. **Stahl, E.** 1969. Thin-layer chromatography, p. 293. Springer-Verlag, New York.
29. **Stahl, E.** 1969. Thin-layer chromatography, p. 297. Springer-Verlag, New York.
30. **Stephen, H., and T. Stephen.** 1963. *In* Solubilities of inorganic and organic compounds, vol. 1, part 1, p. 364, Pergamon Press, Inc., Elmsford, N.Y.
31. **Swinnerton, J. W., R. A. Lamontagne, and V. J. Linnenbom.** 1971. Carbon monoxide in rainwater. Science **172**:943–945.
32. **Swoboda, B. E. P., and V. Massey.** 1965. Purification and properties of the glucose oxidase from *Aspergillus niger*. J. Biol. Chem. **240**:2209–2215.
33. **Thauer, R. K., G. Fuchs, B. Kaufer, and U. Schnitker.** 1974. Carbon monoxide oxidation in cell-free extracts of *Clostridium pasteurianum*. Eur. J. Biochem. **45**:343–349.
34. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**: 4406–4412.
35. **Westlake, D. W. S., J. M. Roxburgh, and G. Talboat.** 1961. Microbial production of carbon monoxide from flavonoids. Nature (London) **189**:510–511.
36. **Whitaker, J. R.** 1963. Determination of molecular weights of proteins by gel filtration on Sephadex. Anal. Chem. **35**:1950–1953.
37. **Whitby, L. G.** 1953. A new method for preparing flavin-adenine dinucleotide. Biochem. J. **54**:437–442.
38. **Zavarzin, G. A., and A. N. Nozhevnikova.** 1977. Aerobic carboxydobacteria. Microb. Ecol. **3**:305–326.