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Purification and Some Properties of Carbon Monoxide Dehydrogenase from *Acinetobacter* sp. Strain JC1 DSM 3803

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A brown carbon monoxide dehydrogenase from CO-autotrophically grown cells of *Acinetobacter* sp. strain JC1, which is unstable outside the cells, was purified 80-fold in seven steps to better than 95% homogeneity, with a yield of 44% in the presence of the stabilizing agents iodoacetamide (1 mM) and ammonium sulfate (100 mM). The final specific activity was 474 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, NAD(P), flavin mononucleotide, flavin adenine dinucleotide, and ferricyanide were not reduced by the enzyme, but methylene blue, thionin, and dichlorophenolindophenol were reduced. The molecular weight of the native enzyme was determined to be 380,000. Sodium dodecyl sulfate-gel electrophoresis revealed at least three nonidentical subunits of molecular weights 16,000 (α), 34,000 (β), and 85,000 (γ). The purified enzyme contained particulate hydrogenase-like activity. Selenium did not stimulate carbon monoxide dehydrogenase activity. The isoelectric point of the native enzyme was found to be 5.8; the K_m of CO was 150 μM. The enzyme was rapidly inactivated by methanol. One mole of native enzyme was found to contain 2 mol of each of flavin adenine dinucleotide and molybdenum and 8 mol each of nonheme iron and labile sulfide, which indicated that the enzyme was a molybdenum-containing iron-sulfur flavoprotein. The ratio of densities of each subunit after electrophoresis (α:β:γ = 1:2:6) and the number of each cofactor in the native enzyme suggest a $\alpha_2\beta_2\gamma_2$ structure of the enzyme. The carbon monoxide dehydrogenase of *Acinetobacter* sp. strain JC1 was found to have no immunological relationship with the enzymes of *Pseudomonas carboxydohydrogena* and *Pseudomonas carboxydovorans*.

Carbon monoxide dehydrogenase (CO-DH) is an enzyme responsible for the oxidation of CO in several gram-positive and -negative carboxydobacteria which are able to grow aerobically with CO as the sole source of carbon and energy (7, 14, 22, 31, 34–36, 39, 43, 58). Studies on the CO-DHs in the purified state, in partially purified preparations, and in extracts revealed that the enzymes from several carboxydobacteria are, with few exceptions, similar in biochemical and immunological properties (2, 6, 14, 22, 23, 25, 34–36, 39, 43).

Acinetobacter sp. strain JC1 DSM 3803 is a new carboxydobacterium isolated from soil in Seoul, Korea (7). Preliminary studies with cell extracts showed that the CO-DH of this bacterium is an inducible enzyme which is loosely bound to the inner face of the cytoplasmic membrane (6, 18). The enzyme was found to be unstable outside the cell and to have no immunological relationship with that of *Pseudomonas carboxydohydrogena* (6), which implies the presence of a novel CO-DH in *Acinetobacter* sp. strain JC1.

In this study, we examined purified CO-DH of *Acinetobacter* sp. strain JC1 in some detail to determine whether the basis for CO oxidation is diverse in the carboxydobacteria and to learn more about the process. Before the purification, we established experimental conditions to stabilize the enzyme in vitro.

MATERIALS AND METHODS

Bacterial strain and cultivation. *Acinetobacter* sp. strain JC1 DSM 3803 was cultivated under CO autotrophy with a gas mixture of 30% CO–70% air in standard mineral medium as described previously (20). CO (99.5%, vol/vol) was purchased from Dongjin Gas Co., Seoul, Korea. Growth was measured with a spectrophotometer by turbidity determined at 650 nm. Cells were harvested in mid-exponential growth phase and washed once in 0.05 M Tris hydrochloride buffer (pH 7.5; standard buffer) and stored at −20°C. After 2 weeks of cultivation of cells from a small inoculum, a yield of 6 g (wet weight) of cells per liter was obtained.

Protein determination. Protein was determined by modified biuret reactions (12). Proteins in crude cell extracts were estimated by the same method after the extracts were boiled in 20% NaOH for 10 min (40).

Enzyme assay. CO-DH activity was assayed photometrically at 30°C by measuring the CO-dependent reduction of thionin dye ($\varepsilon_{595} = 4.2 \times 10^4\ M^{-1}\ cm^{-1}$) in standard buffer at 595 nm, using anaerobic cuvettes as described previously (20). The reaction was started by addition of the enzyme preparations after stabilization of the base line of the reaction mixture.

H_2 (99.5%, vol/vol; Dongjin Gas Co.) was substituted for CO as the substrate to test the hydrogenase activity of the purified enzyme. To test NAD-specific hydrogenase activity, β-NAD (5 mM) was used as the electron acceptor. For the formate dehydrogenase activity test, β-NAD (5 mM) and sodium formate (0.5 mM) were used as the electron acceptor and substrate, respectively (20).

Electrophoresis. Nondenaturing polyacrylamide gel electrophoresis (PAGE) of the native enzyme was performed in gels containing 7.5% acrylamide by the method of Laemmli (30) but without sodium dodecyl sulfate (SDS), as described previously (20). SDS-PAGE of the purified enzyme in 12.5% acrylamide gel was conducted by the procedure of Laemmli (30) with several modifications; all electrophoresis was performed in a vertical slab gel apparatus at 100 to 150 V. Proteins were stained with Coomassie brilliant blue R-250 (CBB) by a modification (20) of the method of Weber and Osborn (55). Activity staining of CO-DH, hydrogenase, and formate dehydrogenase was carried out in standard buffer, using a nondenaturing gel strip in the presence of 0.05%

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phenazine methosulfate, 0.25% nitroblue tetrazolium, and appropriate substrate as described by Kim and Hegeman (20).

Enzyme purification. All purification steps were carried out at 4°C except when noted. A 71-g portion of thawed cells was suspended in 284 ml of standard buffer containing 1 mM iodoacetamide and 100 mM ammonium sulfate (stabilizing standard buffer) and disrupted by sonic treatment (10 s/ml) in portions of 20 ml. The suspension was centrifuged at 15,000 × *g* for 30 min. The resulting supernatant (crude extract) was then treated with protamine sulfate to a final concentration of 0.054%, left in ice for 10 min, and then centrifuged at 100,000 × *g* for 90 min. The supernatant fluid (soluble fraction) was next made 35% saturated with respect to ammonium sulfate. After 2 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 2 h, the solution was centrifuged again at 15,000 × *g* for 30 min, and the precipitate was suspended in a small volume of cold stabilizing standard buffer. The brown suspension was then dialyzed against three 3-liter changes of stabilizing buffer for 7 h. The dialysate was then applied to a Sepharose 6B column (1.8 by 91 cm) and eluted with the same buffer at a flow rate of 5.0 ml/cm^2 per h with 60 cm of hydrostatic pressure. Fractions with the highest specific activity were pooled and applied to a DEAE Sephadex A-25 column (1.8 by 18 cm) with a 1-cm layer of Sephadex G-25 (coarse) at the top. The column was prewashed with at least 3 total bed volumes of stabilizing standard buffer before application of the sample. Elution was carried out with 600 ml of a linear 0 to 0.4 M NaCl gradient in stabilizing standard buffer. Fractions were collected at a flow rate of 11.4 ml/cm^2 per h, and the brown-colored fractions with the highest specific activity were pooled and applied to a hydroxylapatite column (1.3 by 9.5 cm) preequilibrated with stabilizing standard buffer. The column was eluted with a linear ammonium sulfate gradient (300 ml, 0 to 0.5 M) at a rate of 52.5 ml/cm^2 per h. The brown fractions containing peak CO-DH activity were pooled and made 60% saturated with ammonium sulfate to yield a concentrated CO-DH preparation. After 3 h, the solution was centrifuged at 15,000 × *g* for 30 min. The sediment was then suspended in a small volume of stabilizing standard buffer and dialyzed against five 300-ml changes of the same buffer. The pure CO-DH was stored at −20°C under air.

Immunological analysis. Antiserum against purified CO-DH was raised in a New Zealand White rabbit as described previously (20). Double-immunodiffusion assays were carried out in 1.2% agarose (type V; Sigma Chemical Co., St. Louis, Mo.) gel by the method of Ouchterlony and Nilsson (45) except that immunoprecipitates were stained with 0.25% CBB (29). Analysis of immunoprecipitated CO-DH (23) was done by a modification of the SDS-PAGE technique of Laemmli (30) as described above.

Cofactor analysis. Cofactors in the purified CO-DH were analyzed by using an enzyme preparation that had been extensively dialyzed against standard buffer to remove iodoacetamide and ammonium sulfate. Purified CO-DH of *Pseudomonas carboxydovorans* (24) was used as the internal standard.

Absorption spectra of the purified enzyme and protein-free flavin cofactor in aqueous solution were measured in cells of 1-cm light path at room temperature with a Hitachi 200-20 spectrophotometer in the visible range (300 to 550 nm). Protein-free flavin cofactor solution was prepared from the purified enzyme by the trichloroacetic acid extraction technique of Swoboda and Massey (54), followed by removal of the trichloroacetic acid by repeated extraction with diethyl ether (20). To determine the chemical identity of the flavin, the extracted brown-colored solution was subjected to thin-layer chromatography after concentration of the solution with a Mini-Vapour vacuum evaporation system (Tokyo Scientific Instruments, Inc.). Ascending thin-layer chromatography of the concentrated extracts along with some reference compounds (riboflavin, flavin mononucleotide, and flavin adenine dinucleotide [FAD]) was performed on silica gel plates (0.25-mm thick, 20 by 20 cm; Sigma) for 100 min in a rectangular glass chamber which had been presaturated with the developing solvent *n*-butanol–acetone–acetic acid–water (5:2:1:3, by volume) (15). Separated spots after thin-layer chromatography were then visualized by native fluorescence under long-wave UV light. The number of moles of FAD per mole of CO-DH was estimated by photometric analysis (54), and $\varepsilon_{450} = 11.3 \times 10^3$ M^{-1} cm^{-1} (57) was used as the molar absorbancy coefficient to calculate the amount of FAD.

Molybdenum of the purified CO-DH was determined by the improved dithiol method of Hart et al. (13), which involves the selective formation and extraction of the complex of molybdenum with toluene-3,4-dithiol (Tokyo Chemical Industry, Inc.) under specific experimental condition. The method was standardized with molybdic acid (Sigma) solution (13).

Iron was determined by the method of Miller and Massey (44), in which the colored ferrous iron-*o*-phenanthrolinate was separated from the trichloroacetic acid-treated protein solution by extraction into *n*-amyl alcohol before determination of A_{510}. The amount of iron was calculated from the molar extinction coefficient of ferrous iron-*o*-phenanthroline, $\varepsilon_{510} = 1.11 \times 10^4$ M^{-1} cm^{-1} (32).

Labile sulfide of the purified enzyme was estimated by the modified methylene blue method of Brumby et al. (5), using 0.3 ml of the purified enzyme solution, *N*,*N*-dimethyl-*p*-phenylenediamine (E. Merck, Darmstadt, Federal Republic of Germany), and ferric chloride under acidic condition. Under the experimental conditions, 1 μmol of sulfide was considered to give an A_{670} of 17.3 cm^{-1} (5).

RESULTS

Stabilization of CO-DH. Iodoacetamide, ammonium sulfate, dithiothreitol, cysteine, EDTA, and phenylmethylsulfonyl fluoride were tested for ability to stabilize the labile CO-DH of *Acinetobacter* sp. strain JC1. The chemicals at appropriate concentrations were added to the cell suspension before sonic treatment, and the enzyme activity in cell extracts was measured at several time intervals by the standard assay method.

Among the chemicals tested, a mixture of 1 mM iodoacetamide and 100 mM ammonium sulfate was found to be most effective; the enzyme was completely stable for 4 months at −20°C under air in the presence of both stabilizing agents. However, addition of the two agents into the completely inactivated enzyme preparation did not reactivate the enzyme. Iodoacetamide or ammonium sulfate alone also exhibited a stabilizing effect, but only for 3 or 4 days. EDTA and phenylmethylsulfonyl fluoride were found to be totally ineffective, and dithiothreitol and cysteine were determined inappropriate for this test since these reducing agents themselves were found to reduce thionin dye nonspecifically in the absence of CO.

Purification. The CO-DH was purified 80-fold in seven

TABLE 1. Purification of CO-DH from CO-autotrophically grown cells of *Acinetobacter* sp. strain JC1

Purification step	Total protein[a] (mg)	Sp act[b]	Purification (fold)	Total activity[c]	Recovery (%)
Crude extract	2,755.0	5.9	1.0	16,255	100
Soluble fraction	1,606.4	13.8	2.3	22,168	136
Ammonium sulfate (35-55%)	430.0	62.0	10.5	26,660	164
Dialysis	425.6	60.7	10.3	25,834	159
Sepharose 6B	80.1	195.4	33.1	15,652	96
DEAE A-25	23.5	400.5	67.9	9,412	58
Hydroxylapatite	15.0	474.0	80.3	7,110	44

[a] Determined by the biuret method.
[b] Micromoles of thionin reduced per milligram of protein per minute.
[c] Micromoles of thionin reduced per minute.

steps, with a yield of 44% and a specific activity of about 474 μmol of thionin reduced per min per mg of protein (Table 1).

Nondenaturing PAGE of the purified enzyme revealed only a single band, indicating the purity of the enzyme preparation (Fig. 1A). The enzyme was judged better than 95% homogeneous after densitometric analysis of the gel stained with CBB. A brown-colored CO-DH band was seen on nondenaturing polyacrylamide gel which was not stained after PAGE.

Activity staining with CO, H_2, or sodium formate as the substrate revealed that the purified enzyme had both CO-DH and hydrogenase activities but no formate dehydrogenase activity. Enzyme assay showed that the purified enzyme was free of measurable NAD-specific hydrogenase and formate dehydrogenase activities, but with thionin as acceptor had particulate hydrogenase-like activity amounting to 10% of the CO-oxidizing activity.

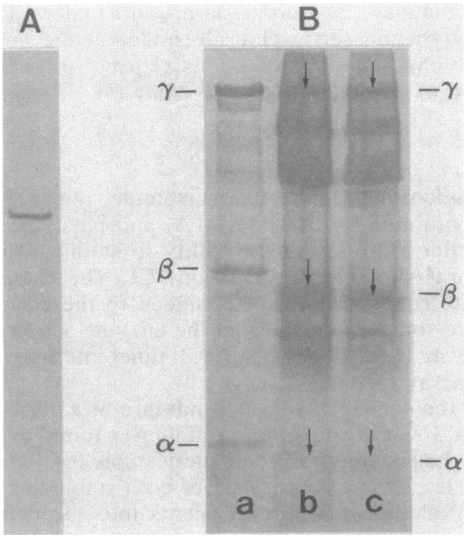

FIG. 1. (A) CO-DH after nondenaturing PAGE of the purified enzyme stained with CBB. The purified enzyme (7 μg) was subjected to PAGE on 7.5% acrylamide gel by the method of Laemmli (30) without SDS as described in the text. (B) Dissimilar subunits in the purified CO-DH. Denaturing PAGE (12.5% acrylamide, 0.1% SDS) was carried out with 20 μg of the purified enzyme (lane a). CO-DHs immunoprecipitated from soluble fractions of *P. carboxydohydrogena* (25 μg) (lane b) and *P. carboxydovorans* (25 μg) (lane c) were run on the gel to compare the sizes of the subunits.

Artificial electron acceptors. The range of artificial electron acceptors and the best acceptor for measuring CO oxidation were determined by the standard enzyme assay method except that thionin was substituted with various acceptors: methyl viologen, NAD^+, $NADP^+$, flavin mononucleotide FAD, methylene blue, thionin, 2,6-dichlorophenolindophenol, and ferricyanide. Among those acceptors, only methylene blue, thionin, and 2,6-dichlorophenolindophenol were reduced by the enzyme when CO was used as the substrate. The reduction rate was the highest when thionin was used as the acceptor. Methylene blue and 2,6-dichlorophenolindophenol were reduced at 67 and 20% of this rate, respectively.

Molecular weight and structure. The molecular weight of the native enzyme was estimated to be 380,000 by using Sepharose 6B column (2.2 by 70 cm) chromatography according to the method of Andrews (1) with reference proteins of known molecular weight. SDS-PAGE revealed the presence of three nonidentical subunits in the native enzyme (Fig. 1B). The molecular weights of the subunits were determined to be 16,000 (α), 34,000 (β), and 85,000 (γ) by using SDS-PAGE with several molecular weight references. SDS-PAGE of immunoprecipitates revealed that the molecular sizes of the α and β subunits of the purified CO-DH were larger than those of *P. carboxydohydrogena* and *P. carboxydovorans* (Fig. 1B). The ratio of densities of each CBB-stained subunit after electrophoresis was about 1:2:6 (α:β:γ), which suggested an $\alpha_2\beta_2\gamma_2$ or $\alpha_3\beta_3\gamma_3$ structure for the purified enzyme.

Isoelectric point. The isoelectric point of the native enzyme was determined to be 5.8 by isoelectric focusing in polyacrylamide gel according to LKB application note 250, using a 2117 Multiphor (LKB Instruments, Inc., Rockville, Md.) with slight modification (20).

Enzyme kinetics. The rate of reduction of thionin with CO catalyzed by the pure enzyme was proportional to the amount of protein added.

The K_m of the purified enzyme was determined by the method of Kim and Hegeman (20), using the standard assay method with various concentrations of CO in the reaction mixture, which were adjusted by a Rotameter (model 7401 T; Matheson Scientific, Inc., Elk Grove Village, Ill.). Variation of thionin reduction rates with different CO concentrations followed Michaelis-Menten kinetics. Assuming that 1 mol of thionin is reduced by two electrons from the oxidation of 1 mol of CO, the apparent K_m for CO was found to be 150 μM. The solubility of CO at 30°C under atmospheric pressure was found to be 19.15 ml in 1 liter of water (53).

Effect of divalent cations and chelating agents. The three known metal-chelating agents tested (10 mM KCN, NaN_3, and EDTA) reduced CO-DH activity by 30 to 40% under standard assay conditions.

Several divalent cations were tested for effects on purified CO-DH activity under standard assay conditions. The enzyme was unaffected or affected very little at 1 mM concentrations of the metals tested (Ni^{2+}, Cu^{2+}, Ba^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+}, Mg^{2+}, and Zn^{2+}); Cu^{2+} eliminated the enzyme activity completely.

Methanol inactivation. The standard assay method was used to test the effect of methanol on CO-DH activity. The enzyme was rapidly inactivated by methanol; complete inactivation occurred at 75 to 100 mM methanol, which suggested that the purified CO-DH may be a molybdoenzyme (33, 34, 36).

Nature and content of flavin. The brown color of air-oxidized CO-DH suggested that the enzyme might contain a cofactor(s). The visible-light spectra of native enzyme and

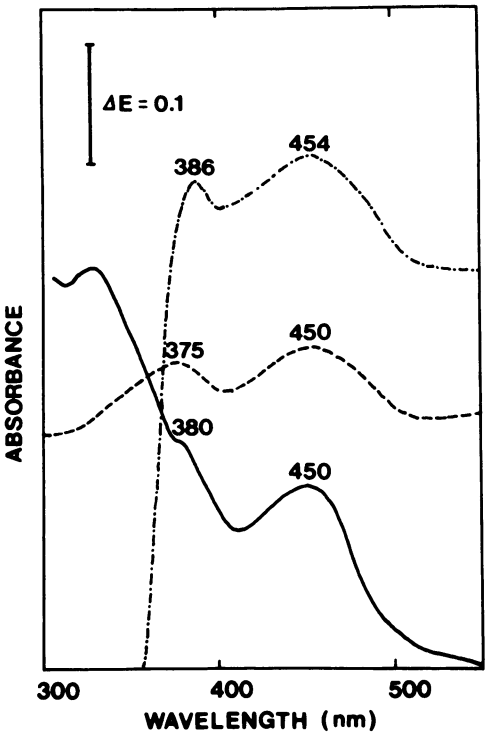

FIG. 2. Absorption spectra of protein-free extracts of CO-DH and of the purified holoenzyme. Protein-free extracts were prepared by the trichloroacetic acid extraction technique (20, 54). Spectra were measured with a Hitachi 200-20 spectrophotometer. Symbols: (——), spectrum of air-oxidized enzyme versus standard buffer; (– – –), spectrum of air-oxidized protein-free extract versus standard buffer; (– · –), difference spectrum of H_2O_2-oxidized minus dithionite-reduced extract.

protein-free extracts revealed that the purified CO-DH might have a bound flavin derivative (Fig. 2). Iodoacetamide and ammonium sulfate did not have a significant effect on the spectra (data not shown). The flavin was found to be FAD, since the R_fs after thin-layer chromatography of the extracts, riboflavin, flavin mononucleotide, and FAD were found to be 0.08, 0.41, 0.22, and 0.07, respectively. Quantitative determination revealed about 2 mol of FAD per mol of enzyme (Table 2). As mentioned above, the CO-DH was seen as a brown band on the gel after nondenaturing PAGE. However, the brown color was not seen on the gel after SDS-PAGE, which indicated that the FAD cofactor was noncovalently bound to the apoprotein.

TABLE 2. Cofactor composition of purified CO-DHs from *Acinetobacter* sp. strain JC1 and *P. carboxydovorans*

Cofactor	Content (mol/mol of enzyme)[a]	
	Acinetobacter sp. strain JC1	*P. carboxydovorans*[b]
FAD	1.7 (2.2)	1.8
Molybdenum	0.8 (1.0)	0.8
Total iron	7.2 (9.1)	7.6
Acid-labile sulfide	7.4 (9.4)	7.4

[a] Calculated on the assumption that the molecular weights of the purified enzymes are 300,000. Values in parentheses were calculated on the assumption that the molecular weight of the purified enzyme is 380,000.

[b] Purified CO-DH from *P. carboxydovorans* was prepared from cells grown under the cultivation conditions used for *Acinetobacter* sp. strain, using conventional techniques as described previously (17, 24).

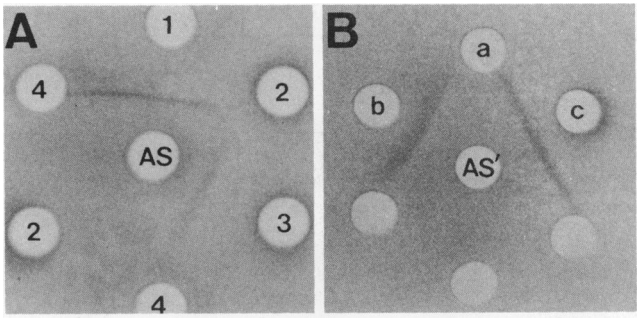

FIG. 3. Double-immunodiffusion patterns for CO-DH from *Acinetobacter* sp. strain JC1. Immunodiffusion assays were performed in 1.2% agarose gel for 24 h at 30°C, followed by staining with CBB. (A) Antiserum prepared against purified CO-DH from *Acinetobacter* sp. strain JC1, 5 μl (AS); purified CO-DH from *Acinetobacter* sp. strain JC1, 4 μg (well 1); soluble fraction from *P. carboxydovorans*, 30 μg (well 2); soluble fraction from *Acinetobacter* sp. strain JC1, 20 μg (well 3); soluble fraction from *P. carboxydohydrogena*, 35 μg (well 4). (B) Antiserum prepared against purified CO-DH from *P. carboxydohydrogena* (20), 5 μl (AS'); purified CO-DH from *Acinetobacter* sp. strain JC1, 7 μg (well a); soluble fraction from *P. carboxydohydrogena*, 20 μg (well b); soluble fraction from *P. carboxydovorans*, 25 μg (well c).

Content of metal and acid-labile sulfide. The purified CO-DH was found to contain 1 mol of molybdenum per mol of enzyme (Table 2). The enzyme was found to have about 8 mol each of total iron and labile sulfide per mol of enzyme. Addition of reducing agent to the reaction mixture had no significant effect on the result. The absorption spectra of the purified CO-DH in the presence of sodium dithionite or CO indicated that the irons were not present in the heme structure (data not shown).

Immunological properties. Double immunodiffusion revealed that the soluble fractions prepared from cells of *P. carboxydohydrogena* and *P. carboxydovorans* grown with CO did not cross-react with antiserum raised against the purified CO-DH (Fig. 3A). The purified enzyme also did not react with antiserum prepared against purified CO-DH of *P. carboxydohydrogena* (Fig. 3B).

DISCUSSION

Unlike CO-DHs in anaerobic CO-oxidizing bacteria (11), all of the CO-DHs of carboxydobacteria other than *Acinetobacter* sp. strain JC1 (6) that have been studied to date are known to be stable at low temperature in the presence of oxygen (2, 20, 25, 41, 42). CO-DH in *Acinetobacter* sp. strain JC1 was very labile, and over 90 and 70% of the enzyme was inactivated in 24 h at 4 and −20°C, respectively, under air (6). We have used several chemicals in attempts to stabilize the CO-DH of *Acinetobacter* sp. strain JC1 to study the mechanism of CO oxidation at the level of the purified enzyme system and found that iodoacetamide and ammonium sulfate together can stabilize enzyme activity for a long time.

Iodoacetamide is known to bind free sulfhydryl groups irreversibly and prevent them from interacting with other groups. On the basis of this fact, we assume that iodoacetamide may stabilize the enzyme through binding to the free sulfhydryl group(s) in *Acinetobacter* sp. strain JC1 CO-DH when the cells are disrupted, preventing the enzyme from

TABLE 3. Kinetic and molecular properties of CO-DHs from several carboxydobacteria

Species	Mol wt (10^3)	Subunit structure	Subunit size (α:β:γ, 10^3)	Cofactor content (mol/mol of enzyme)				K_m (μM CO)	Reference(s)
				FAD	Mo	Fe[a]	S[b]		
Acinetobacter sp. strain JC1	380 (300)[c]	$\alpha_2\beta_2\gamma_2$	16:34:85	2	2	8	8	150	This study
Pseudomonas carboxydohydrogena	400	$\alpha_3\beta_3\gamma_3$	14:28:85	ND[d]	ND	ND	ND	63	20, 22, 24
	230, 300	ND	ND	2	2	8	8	ND	10, 37
P. carboxydovorans	230–300	$\alpha_2\beta_2\gamma_2$	17:33-34:70–86, 14:25:85	2	2	8	8	53	10, 17, 24, 35–37, 39, 42, 43, this study
P. carboxydoflava	230, 280–300	$\alpha_2\beta_2\gamma_2$	17:33:70	2	2	8	8	ND	10, 36, 37
P. thermocarboxydovorans	230–310	ND	ND	1.8	0.7	6.9	6.9	0.6	2
Bacillus schlegelii	230	ND	ND	ND	ND	ND	ND	ND	25

[a] Nonheme iron.
[b] Acid-labile sulfide.
[c] Values in parentheses indicate alternate assumptions of molecular weight.
[d] ND, not determined.

changing its tertiary structure under an appropriate concentration of salt. In the absence of iodoacetamide and ammonium sulfate, the tertiary structure of the enzyme may change to bring free sulfhydryl groups close to each other. The sulfhydryl groups may then interact to effect further changes in the enzyme structure, resulting in inactivation of the enzyme. The structural changes, however, may not be great and may happen only in a small area around the active center, since the purified enzyme did not differ from the enzyme in crude cell extracts prepared in the absence of iodoacetamide and ammonium sulfate with respect to migration rate on nondenaturing gel or antigenicity (data not shown). The stabilizing effect of iodoacetamide also indicates that a sulfhydryl group is not involved in CO oxidation.

The purified CO-DH of *Acinetobacter* sp. strain JC1 was found to constitute 2.3% of the total soluble cell protein. The 2.5- to 340-fold-higher specific activity of this purified enzyme compared with that of the enzyme isolated from other carboxydobacteria (Table 3) may lend some support to the observation that sodium dithionite can stimulate CO-DH activity (38), since we and Kim and Hegeman (20) added a small amount of sodium dithionite to the reaction mixture to deplete it of molecular oxygen.

The purified CO-DH can reduce several artificial electron acceptors with redox potentials of between 10 and 217 mV. This result suggests that the natural electron acceptor for CO oxidation in *Acinetobacter* sp. strain JC1 may be a quinone or a cytochrome. The nature of the physiological electron acceptor is unknown at present, but a cytochrome such as that in *P. carboxydovorans* (50) may be considered a candidate, since the CO-DH of *Acinetobacter* sp. strain JC1, like that of *P. carboxydovorans* (33, 42, 51), does not reduce UQ_{10} (19).

The molecular weight of the purified enzyme was around 380,000, which is almost the same as that of *P. carboxydohydrogena* (Table 3). It has been reported that the molecular weights of CO-DHs from several carboxydobacteria depend on the method used (2, 10, 25, 36, 39, 42, 43) and that the enzymes from *P. carboxydohydrogena*, *P. carboxydovorans*, and *Pseudomonas carboxydoflava* comigrate with chicken liver xanthine dehydrogenase when applied to a high-performance liquid chromatography sizing column, suggesting an identical molecular weight of 300,000 for the CO-DHs, which may be regarded as the most accurate value obtained so far (37). These findings, together with the fact that Kim and Hegeman (20) used Sepharose 6B column chromatography, as did we, and obtained a higher value for the *P. carboxydohydrogena* enzyme than was determined by other methods, suggest that the actual molecular weight of the purified enzyme may be lower than 380,000; it may be around 300,000, a value similar to those obtained for other carboxydobacterial CO-DHs except the *Alcaligenes carboxydus* enzyme (10), which is smaller than other enzymes. If one assumes that the molecular weight of the native *Acinetobacter* sp. strain JC1 enzyme is 300,000, the subunit structure of the enzyme appears to be $\alpha_2\beta_2\gamma_2$, like the structures of the *P. carboxydovorans* and *P. carboxydoflava* enzymes (Table 3).

The K_m for CO of purified CO-DH from *Acinetobacter* sp. strain JC1 is considerably higher than values for other bacteria (Table 3). The high K_m of the purified enzyme immediately poses the question as to whether *Acinetobacter* sp. strain JC1 can use atmospheric CO for growth, since the concentration of CO in the atmosphere has been estimated to be 1.3 to 39 μmol/liter of air, depending on the site of measurement (16, 47, 52). However, it has been shown that *Acinetobacter* sp. strain JC1 can grow with 4.3 μmol of CO per liter of air (7), which indicates that it may utilize CO in highly polluted air (2.2 to 4.3 μmol/liter) in urban districts (48). It may also utilize CO in microenvironments in which a high concentration of CO is produced by decomposition of flavonoids and porphyrins (8, 56).

The methylene blue-reducing activity of certain preparations of CO-DH from *P. carboxydovorans* was specifically activated up to eightfold upon incubation aerobically with sodium selenite (36, 38), and the fully activated cytoplasmic CO-DH was found to contain nearly 2 mol of covalently bound selenium per mol of enzyme (36, 38). Since *Acinetobacter* sp. strain JC1 was grown in selenium-free medium throughout the experiments and the CO-oxidizing activity of the purified enzyme was not activated upon aerobic treatment with selenite (data not shown), it may be concluded that selenium is not a constituent of CO-DH and also is not required for CO oxidation in *Acinetobacter* sp. strain JC1.

The CO-DH of *Acinetobacter* sp. strain JC1, like the enzyme of other carboxydobacteria, was found to be a molybdenum-containing iron-sulfur flavoprotein (Table 3). The purified enzyme, however, like that of *Pseudomonas thermocarboxydovorans*, is low in molybdenum content. It has been reported that demolybdo forms of incompletely functional molybdenum hydroxylases are produced as natural products along with active enzymes having 2 mol of molybdenum per mol of enzyme (3, 9) and that the molybdenum content in CO-DH may depend on the amount of molybdenum added to the medium (33, 37, 43). These findings, together with the facts that *Acinetobacter* sp. strain

JC1 was grown with a relatively low concentration of molybdenum (2 mg of $Na_2MoO_4 \cdot 2H_2O$ per liter) and the purified CO-DH from *P. carboxydovorans*, which was grown under the same conditions, was also low in molybdenum (Table 2), suggest that the purified enzyme is a typical molybdenum hydroxylase, as are other carboxydobacterial CO-DHs which contain FAD, molybdenum, iron, and labile sulfide in a 1:1:4:4 ratio (Table 3). The ratio of cofactor further supports the conclusion that the *Acinetobacter* sp. strain JC1 enzyme has a $\alpha_2\beta_2\gamma_2$ structure. The questions of whether bactopterine is a constituent of the molybdenum cofactor (26, 27, 36) and whether the purified enzyme has two different types of iron-sulfur centers, as do other carboxydobacterial CO-DHs (4, 36), need further study.

By using antisera raised against purified CO-DHs from *P. carboxydovorans* and *P. carboxydohydrogena*, CO-DHs from several carboxydobacteria except *Bacillus schlegelii* (25) were shown to share a number of common antigenic groups (23, 24, 39, 46). The purified CO-DH of *Acinetobacter* sp. strain JC1, like that of *B. schlegelii*, has no antigenic sites in common with those of *P. carboxydohydrogena* and *P. carboxydovorans*. This result agrees with the previous report that the enzyme in cell extracts has no immunological relationship with the *P. carboxydohydrogena* enzyme (6).

These results indicate that the CO-DH of *Acinetobacter* sp. strain JC1 is similar in molecular weight, subunit structure, range of artificial electron acceptors, and cofactor composition to other carboxydobacteria. However, it also has two properties, instability and the absence of common antigenic groups, which distinguish it from other carboxydobacterial CO-DHs. Considering the unique CO-DHs in thermophilic bacilli (25) and *Streptomyces* G26 (2), the results presented here indicate that the oxidation of CO as an energy source by aerobic carboxydobacteria is mediated by similar enzymes of the dehydrogenase type which have been evolved in several independent lines from a common ancestral CO-DH.

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