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Carbon Monoxide:Methylene Blue Oxidoreductase from *Pseudomonas carboxydovorans*

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The enzyme carbon monoxide:methylene blue oxidoreductase from CO autotrophically grown cells of *Pseudomonas carboxydovorans* strain OM5, was purified to homogeneity. The enzyme was obtained in 26% yield and was purified 36-fold. The enzyme was stable for at least 6 days, had a molecular weight of 230,000, gave a single protein and activity band on polyacrylamide gel electrophoresis, and was homogeneous by the criterion of sedimentation equilibrium. Sodium dodecyl sulfate gel electrophoresis revealed a single band of molecular weight 107,000. Carbon monoxide:methylene blue oxidoreductase did not catalyze reduction of pyridine or flavin nucleotides but catalyzed the oxidation of CO to CO_2 in the presence of methylene blue, thionine, toluylene blue, dichlorophenolindophenol, or pyocyanine under strictly anaerobic conditions. The visible spectrum revealed maxima at 405 and 470 nm. The millimolar extinction coefficients were 43.9 (405 nm) and 395.5 (275 nm), respectively. Absorption at 470 nm decreased in the presence of dithionite, and the spectrum was not affected by the substrate CO. Maximum reaction rates were found at pH 7.0 and 63°C; temperature dependence followed the Arrhenius equation, with an activation energy ($\Delta H°$) of 36.8 kJ/mol (8.8 kcal/mol). The apparent K_m was 53 μM for CO. The purified enzyme was incapable of oxidizing methane, methanol, or formaldehyde in the presence of methylene blue as electron acceptor.

Pseudomonas carboxydovorans strain OM5 is capable of growing autotrophically with CO or H_2 + CO_2 as well as heterotrophically on a restricted number of organic substrates with oxygen as an electron acceptor (15). Experiments on the oxidation of carbon monoxide in cell extracts of *P. carboxydovorans* provided indirect evidence that CO is oxidized according to the equation CO + H_2O + methylene blue (ox) $\rightarrow$ CO_2 + methylene blue (red) and that neither free formate nor hydrogen gas are intermediates of the CO oxidation reaction (15, 16).

However, double-label experiments with *Azotobacter* and *Azomonas* strains employing ^{13}CO and either $H_2{}^{18}O$ or $^{18}O_2$ showed that the second oxygen atom in the oxidation of CO to CO_2 by these strains comes neither from O_2 nor water and that, therefore, other possible sources must be sought (12).

In the present work, purification of the carbon monoxide:methylene blue oxidoreductase (CO:MB oxidoreductase) from *P. carboxydovorans* to homogeneity provides direct evidence that neither hydrogenase nor formate dehydrogenase, which are both active in CO-grown cells, are involved in the CO oxidation reaction, that CO is the sole substrate of the enzyme, and that the second oxygen atom in the CO_2 evolved must be derived from water. Data on the molecular, structural, and spectral properties of the pure enzyme are also presented.

MATERIALS AND METHODS

Growth of bacteria. *P. carboxydovorans* strain OM5 (DSM 1227) was cultivated autotrophically on CO in mineral medium under the conditions described (15). Cells grown in a 10-liter fermentor (Braun, Melsungen, Germany) supplied with a gas mixture of 50% CO and 50% air were harvested in the late exponential phase of growth (optical density at 436 nm $\approx$ 6), washed in 50 mM KH_2PO_4-NaOH buffer, pH 7.0, and stored at −20°C under air.

Protein determination. The method of Bradford (3) was used for protein estimation.

Enzyme assays. The reduction of methylene blue with CO, H_2, or formate was measured spectrophotometrically at 30°C in a serum-stoppered cuvette (diameter = 1 cm) flushed with the indicated gases by following the decrease of methylene blue absorption at 615 nm (ϵ_{615} = 37.1 cm^2/μmol) as described previously (16). The assay for CO:MB oxidoreductase contained (1.0 ml final volume): 46 mM potassium phosphate buffer (pH 7.0); glucose (0.1 mM); an electron acceptor of the indicated concentration (usually 50 μM); and 0.01 ml of mix (1 U of glucose oxidase + 1 U of catalase in buffer). Serum-stoppered cuvettes were flushed with the following gases for at least 5 min: CO (CO:MB oxidoreductase), H_2 (hydrogenase, H_2:MB

oxidoreductase), or N_2 in the presence of 100 μM formate (formate oxidase, formate:MB oxidoreductase). The reactions were initiated with 10 μl of enzyme solution (approximately 0.2 to 8 mg of protein per ml).

Alcohol dehydrogenase was assayed according to *Boehringer Informationen* (2), and catalase was assayed by the method of Bergmeyer (1).

Enzyme purification. With the exception of the chromatography on ECTEOLA cellulose and gel filtration on Sephadex G-25, which were performed at room temperature (22°C), all purification steps were carried out at 0 to 4°C.

A 22-g amount of thawed cells was suspended in 20 ml of 50 mM KH_2PO_4-NaOH buffer, pH 7.0, and was disrupted by sonication (60 s/ml) in portions of 5 ml (Braun-Sonic 300 disintegrator; Quigley-Rochester, Rochester, N.Y.). Whole cells and large-cell debris were removed by centrifugation at 10,000 × g for 20 min. The supernatant fluid is referred to as crude extract.

The crude extract was centrifuged at 100,000 × g for 1 h. The resulting supernatant was layered on top of a discontinuous sucrose gradient (100,000 × g supernatant, 9.66 ml; 5% sucrose in buffer, 12 ml; 10, 15, 20, and 30% sucrose in buffer, 10 ml each) and centrifuged at 20,000 rpm for 40 h (Vacufuge with swing-out rotor [3 by 60 ml]; Christ, Osterode, Germany).

After centrifugation, 1.5-ml fractions were collected. Sucrose concentration was determined by means of a refractometer (Carl Zeiss, Oberkochen, Germany). The protein from the fractions containing highest CO:MB oxidoreductase activity was dialyzed in the absence of air (desiccator filled with N_2 in the presence of alkaline pyrogallol) against 4.5 liters of 50 mM KH_2PO_4-NaOH buffer, pH 7.0, for 20 h. The dialysate was concentrated with the aid of dry Sephadex G-25 coarse, and the protein solution was then layered on top of a ECTEOLA cellulose column (2.6 by 12 cm) equilibrated with 50 mM KH_2PO_4-NaOH buffer, pH 7.0. Elution was performed with a linear KCl gradient (100 ml, 0 to 1 M KCl). Fractions (1.8 ml each) were collected, and those with the highest activity were pooled and dialyzed anaerobically as before. In a final step, CO:MB oxidoreductase was subjected to sedimentation into a linear sucrose density gradient (10 to 30% sucrose, wt/vol) of 60-ml volume (20,000 rpm for 40 h with a swing-out rotor [3 by 60 ml]). Fractions 1.5 ml each were collected, and sucrose was removed from the combined active fractions by means of gel filtration on a small (5-ml) Sephadex G-25 column. The pure enzyme was stored at −20°C under air.

Solubility of carbon monoxide and hydrogen. At 30°C and atmospheric pressure, 19.4 μl of CO or 16.7 μl of H_2 is dissolved in 1 ml of water (13).

Preparation of gas mixtures. Gas mixtures of carbon monoxide, hydrogen, and nitrogen were prepared by filling the required quantities of gas into a syringe (100 ml). CO (99.997%, vol/vol), H_2 (99.9%, vol/vol), O_2 (99.995%, vol/vol), N_2 (99.99, vol/vol), and CO_2 (99.995%, vol/vol) were obtained from Messer Griesheim, Düsseldorf, Germany.

Electrophoresis. Polyacrylamide gel electrophoresis (250 mM Tris-borate buffer, pH 7.9) was performed in a flat gel apparatus for vertical slab electrophoresis (Panto-Phor, Müller, Hann.-Münden, Germany) as described by Stegemann (20), using a current of 100 mA and a voltage of 200 to 300 V. Protein was stained with Coomassie brilliant blue R 250 (10). Activity staining was performed in 50 mM KH_2PO_4-NaOH buffer, pH 7.0, in the presence of 0.05 mM Nitro Blue Tetrazolium and 0.1 mM phenazine methosulfate under an atmosphere of 100% CO.

Sodium dodecyl sulfate electrophoresis was performed in 10% gels by the method of Weber and Osborn (22).

Determination of molecular weight. Analytical ultracentrifugation experiments were performed in a Beckman-Spinco model E ultracentrifuge equipped with monochromator, photoelectric scanner, and multiplexer. Double-sector cells with a 12-mm light path were used. The molecular weight was determined by a low-speed sedimentation equilibrium method (11). The sedimentation equilibrium experiments were performed at 5,200 rpm and 5°C for 19 h. The molecular weight of CO:MB oxidoreductase was also determined by sucrose gradient centrifugation (14).

Spectroscopy. Absorption spectra were recorded on a Zeiss DMR 21 spectrophotometer with cuvettes of 1-cm light path. Fluorescence spectra were measured on a Hitachi model 204 spectrofluorometer.

Enzymes and chemicals. Protein standards for molecular weight determinations as well as NAD(P), flavin mononucleotide, catalase, and alcohol dehydrogenase were obtained from Boehringer Mannheim GmbH, Mannheim, Germany. Acrylamide, *N′,N′*-methylenebisacrylamide, *N,N,N′,N′*-tetramethylenediamine, sodium dodecyl sulfate, and viologen dyes were obtained from Serva, Heidelberg, Germany. Glucose oxidase was from Fluka AG, Buchs, Switzerland, and all other chemicals from E. Merck, AG, Darmstadt, Germany.

RESULTS

Purification. Preliminary experiments aimed at the purification of CO:MB oxidoreductase from crude extracts showed that among ion-exchanging resins only anion-exchanging celluloses (DEAE, triethylaminoethyl, and ECTEOLA) were effective for separation purposes, whereas carboxymethylcellulose was ineffective. Precipitation with ammonium sulfate as well as concentration with Amicon ultrafilters (XM 50; Amicon, Witten, Germany) or the spinal fluid concentrator (Minicon CS 15; Amicon, Witten, Germany) caused a decrease in recovery of about 50% in activity; therefore, these techniques were avoided. The final purification procedure is summarized in Table 1. The fractions of the ECTEOLA cellulose eluate containing enzyme were dialyzed in the absence of air oxygen and afterwards subjected to sucrose density gradient centrifugation. CO:MB oxidoreductase was found in fractions 23 through 30 (Fig. 1); fractions of high activity (25 through 28) were pooled.

CO:MB oxidoreductase from CO autotrophically grown cells of *P. carboxydovorans* was purified 35.5-fold with a yield of 26% and a

TABLE 1. *Purification of CO:MB oxidoreductase from CO autotrophically grown cells of P. carboxydovorans*

Purification step	Vol (ml)	Total protein (mg)	Total activity[a]	Sp act[b]	Fold purification	Recovery (%)
Crude extract	38.0	988	54	55	1	100
100,000 × g supernatant	29.0	464	60.7	131	2.4	112
Sucrose density gradient (5 to 30%)	35.0	127	64.5	508	9.3	119
Dialysis	50.0	101.5	48.8	481	8.8	90
Concn with sephadex G-25	8.5	78.4	43.2	551	10.1	80
ECTEOLA eluate	8.5	27.6	31.1	1,130	20.6	58
Dialysis	8.6	25.4	27.4	1,080	19.7	51
Sucrose density gradient (10 to 30%)	5.0	7.1	13.8	1,940	35.5	26

[a] Micromoles of CO oxidized $\cdot$ min^{-1}.
[b] Nanomoles of CO oxidized $\cdot$ min^{-1} $\cdot$ mg of protein^{-1}.

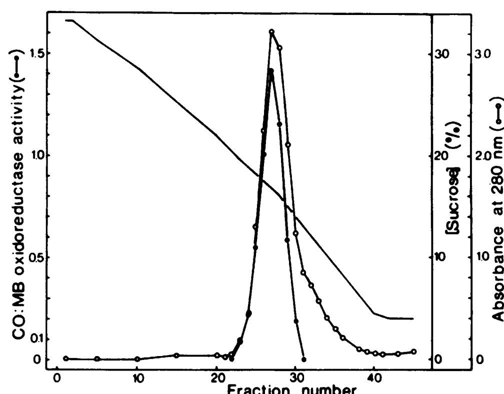

FIG. 1. *Linear sucrose density gradient centrifugation (final step in Table 1). The dialyzed ECTEOLA cellulose eluate was subjected to sedimentation in a linear sucrose density gradient (10 to 30% sucrose in buffer) of 60-ml volume as described in the text. Symbols: CO:MB oxidoreductase activity (ΔE_{615} per minute) (●); absorbance at 280 nm (O); sucrose concentration as measured refractometrically (——).*

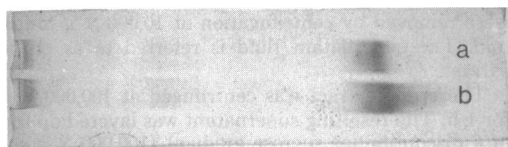

FIG. 2. *CO:MB oxidoreductase examined by polyacrylamide gel electrophoresis of enzyme preparation stained with Coomassie brilliant blue. Gels containing 7.5% acrylamide in 250 mM Tris buffer, pH 7.9, were run at 100 mA and 300 V for 4 h; 53 μg (a) and 106 μg (b) of enzyme were applied.*

specific CO-oxidizing activity of about 1.9 μmol of CO oxidized per min·mg of protein (Table 1).

Stability of purified enzyme. The purified enzyme was found to be stable for 6 days when stored in 50 mM phosphate buffer (pH 7.0) at −20°C under air. Within 18 days only 50% of the initial activity remained; addition of glycerol (50%, vol/vol) was without effect.

Homogeneity. Polyacrylamide gel electrophoresis revealed only one single protein band, indicating the purity of the enzyme preparation (Fig. 2). The purified enzyme was free from formate dehydrogenase activity but contained trace amounts of hydrogenase activity. Hydrogenase activity (36 nmol of H_2 oxidized per min·mg of protein) never exceeded more than 2% of the activity of the purified CO:MB oxidoreductase (1,944 nmol of CO oxidized per min·mg of protein). Attempts to remove the hydrogenase activity completely were not successful. The linearity of log c versus r^2 plots at different protein concentrations indicated that the enzyme was homogeneous by the criterion of ultracentrifugation.

Molecular weight. The molecular weight of the CO:MB oxidoreductase was determined by three different methods: sucrose gradient centrifugation (14), sedimentation equilibrium centrifugation (11), and calculation from subunit structures. A molecular weight of 230,000 ($s^0_{20,w}$ = 10.78 S) was obtained when the purified enzyme was subjected to sucrose gradient centrifugation. From the results of sedimentation equilibrium experiments a molecular weight of 234,000 was extrapolated for the purified enzyme (Fig. 3). This corresponded to the molecular weight as calculated from the number of subunits (214,000) and to the value obtained by sucrose gradient centrifugation (230,000).

Subunit structure. The purified enzyme was treated with denaturating buffer composed of 1% sodium dodecyl sulfate (Serva or Koch-Light)-1% mercaptoethanol in 50 mM phosphate buffer, pH 7.0, and heated for 3 to 8 min (90°C). The enzyme was then subjected to polyacrylamide gel electrophoresis (see Materials and Methods). The dissociated enzyme migrated as one distinct band detectable by staining with Coomassie brilliant blue R 250. The electrophoretic mobility of this band corresponded to a molecular weight of 102,000 to 107,000 when

compared with the mobilities of calibration proteins. Additional treatment of the enzyme with 8 M urea did not influence this result. These measurements support the conclusion that CO: MB oxidoreductase consists of two subunits of identical molecular weight.

Effects of inhibitors. Hypophosphite (NaH_2PO_2), chlorate ($NaClO_3$), cyanide (KCN), azide (NaN_3), and fluoride (NaF) are known as metal-chelating agents and used as inhibitors for metallo-enzymes. Activity of purified CO:MB oxidoreductase was not or only scarcely affected by hypophosphite, chlorate, and fluoride, but the oxidation of CO was completely inhibited by cyanide (Table 2). Formate (50 mM), bicarbonate (10 mM $NaHCO_3$), magnesium (100 mM $MgCl_2$), and molecular hydrogen (enzyme assay as described in Materials and Methods, but in the presence of a gas mixture of 50% CO and 50% H_2 at atmospheric pressure) had no effect on CO:MB oxidoreductase activity.

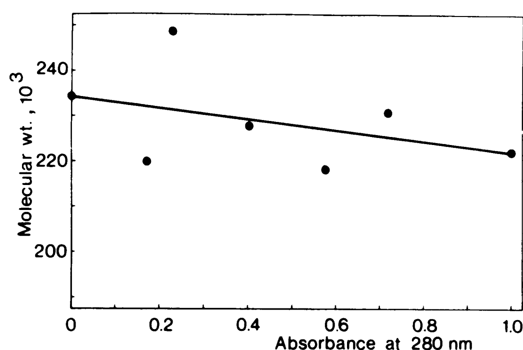

FIG. 3. *Determination of the molecular weight of purified CO:MB oxidoreductase. Equilibrium centrifugation was carried out at different protein concentrations as described in the text; the line was calculated by the method of least squares, and the molecular weight was determined from the apparent molecular weights by extrapolation of the protein concentration to zero.*

Electron acceptors. The reduction of the dyes was impaired by molecular oxygen; therefore, the experiments were conducted under anaerobic conditions as described in Materials and Methods. Purified CO:MB oxidoreductase readily catalyzed the oxidation of carbon monoxide with methylene blue, thionine (Lauth's violet), toluylene blue, dichlorophenolindophenol, or pyocyanine (Table 3). Electron acceptors with a potential lower than −34 mV, such as NAD(P)

TABLE 2. *Effect of metal-chelating agents on CO: MB oxidoreductase activity*[a]

Inhibitor (100 mM)	Inhibition (%)
Hypophosphite (NaH_2PO_2)	8.8
Chlorate ($NaClO_3$)	15.4
Cyanide (KCN)	100
Azide (NaN_3)	64.7
Fluoride (NaF)	16.7

[a] The assays (reduction of methylene blue with CO) were done under standard conditions, but in the presence of 100 mM inhibitor. The enzyme activities refer to values measured in the absence of the inhibitor (1.70 μmol/min·mg of protein), set as 0%.

TABLE 3. *Reduction of electron acceptors with carbon monoxide catalyzed by purified CO:MB oxidoreductase*[a]

Electron acceptor	Concn	λ[b] (nm)	$E^{0'}$ (mV)	CO oxidation rates (% of rate with methylene blue)
Methyl viologen	2.5 mM	605	−440	0
Benzyl viologen	2.5 mM	560	−359	0
NADP, NAD	5 mM	340	−324, −320	0
Neutral red	50 μM	510	−320	0
FAD, FMN[c]	20 μM	450	−219	0
Pyocyanine (perchlorate)	25 μM	620[d]	−34	10
Methylene blue	50 μM	615[e]	+11	100
Thionine (Lauth's violet)	50 μM	595	+70	89
Toluylene blue	50 μM	650[f]	+110	25
Dichlorophenolindophenol	50 μM	610[g]	+217	22
Ferricyanide	40 μM	410	+429	0

[a] Determination of CO oxidation rates photometrically as described in the text; concentrations of electron acceptors as indicated were chosen as high as solubility allowed; activity with methylene blue (1.70 μmol/min·mg of protein) set as 100%.

[b] Wavelength measured.

[c] FAD, FMN, Oxidized flavin adenine dinucleotide or flavin mononucleotide.

[d] Absorption maxima at 375 and 690 nm.

[e] Absorption maximum at 665 nm.

[f] Absorption maximum at 640 to 650 nm.

[g] Absorption maximum at 600 to 620 nm.

and the viologens, were not reduced.

Kinetics. The reduction of methylene blue with CO catalyzed by the pure enzyme proceeded linearly with time (0 to 10 min), and the rate was proportional to the amount of protein added. Variation of methylene blue reduction rates with substrate concentration (CO) followed the Michaelis-Menten equation. The apparent K_m for CO was 53 μM (30°C, 1 mM methylene blue).

Effect of pH and temperature on enzyme activity. Maximum rate of methylene blue reduction with CO occurred at pH 6.8 to 7.0 (50 mM phosphate buffer). Enzyme activities were routinely measured at 30°C, although the activity of CO:MB oxidoreductase increased with increasing temperature (Fig. 4). At 63°C the oxidation of CO proceeded three times faster than at 30°C. The Arrhenius plot was linear and revealed an activation energy (ΔH°) of 36.8 kJ/mol (8.8 kcal/mol).

Spectral properties. Purified and electrophoretically homogeneous CO:MB oxidoreductase was brownish colored and revealed an absorption spectrum with a shoulder at 470 nm and a peak at 405 nm in the visible spectrum (Fig. 5). The millimolar extinction coefficients of the purified enzyme in the oxidized state at 405 and 275 nm were 43.9 and 395.5, respectively. Thus the ratio E_{275}/E_{405} is 9.0 for the oxidized enzyme. The absorption spectrum was not affected by the presence of CO. After reduction with a slight excess of dithionite (50 μM), one would expect an absorption increase in the presence of cytochromes, but (Fig. 5) the shoulder at 470 nm disappeared completely and the peak at 405 nm decreased; this indicates that both

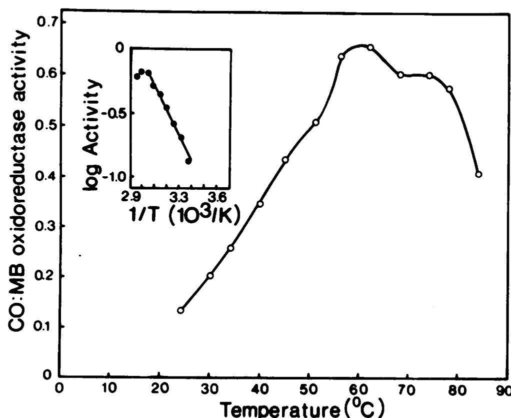

FIG. 4. *Temperature dependence of the CO:MB oxidoreductase. Rate of methylene blue reduction with CO (ΔE_{615} per minute), as described in the text, but in the absence of O_2 trapping system. The inset shows the Arrhenius plot of the same data.*

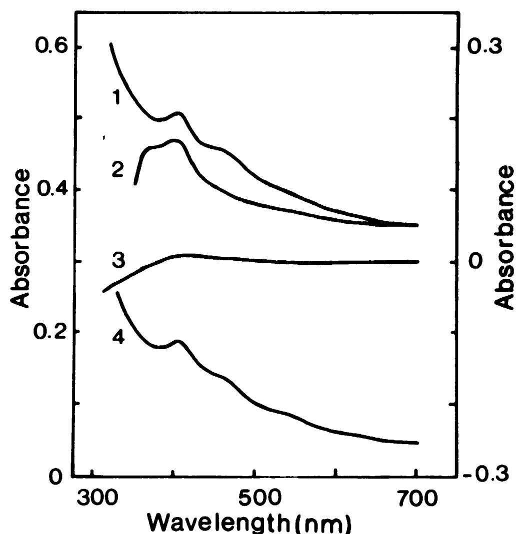

FIG. 5. *Absorption spectra of CO:MB oxidoreductase from P. carboxydovorans. Protein content, 1.05 mg/ml; absorption at 280 nm, 1.740; specific activity, 1, 24 μmol of CO/min·mg of protein. The enzyme was dissolved in 50 mM phosphate buffer, pH 7; spectra were taken against phosphate buffer in the absence (1, 3, 4) or presence (2) of a slight excess of dithionite in both cuvettes. (1) Oxidized spectrum of the enzyme (under air); (2) spectrum after reduction of the enzyme with slight excess of sodium dithionite recorded against buffer with dithionite (50 μM); (3) base line; (4) spectrum in the presence of carbon monoxide (saturated solution) recorded against CO-saturated buffer (atmospheric pressure). Absorbance: left scale for curve 4, right scale for curves 1, 2, and 3.*

absorption maxima are due to the enzyme and not to contaminating cytochromes. Neither the absorption spectrum of the native enzyme nor the fluorescence spectra (excitation and emission spectrum) of the trichloroacetic acid-treated preparation suggested that flavin was a constituent of the enzyme. The absence of flavin from CO:MB oxidoreductase is in agreement with its narrow spectrum of electron acceptors (6, 7).

Other properties of the CO:MB oxidoreductase. Due to the low apparent K_m of 53 μM CO, the clearly exergonic reduction of methylene blue ($E^{o\prime}$ = +11 mV) with carbon monoxide ($E^{o\prime}$ = −540 mV) proceeded to complete oxidation of the substrate. Thus the stoichiometry of the reaction could be determined by adding limiting amounts of CO and by measuring the amount of methylene blue reduced. Methylene blue was reduced by carbon monoxide at a 1:1 molar ratio. This indicates that CO oxidation as mediated by the pure enzyme provides two electrons, and carbon dioxide is the reaction product. The purified enzyme was unable to reduce methylene

blue with methane, methanol, or formaldehyde.

DISCUSSION

The procedure for purifying the CO:MB oxidoreductase from *P. carboxydovorans* described here employed two sucrose density gradient centrifugations and chromatography on ECTEOLA cellulose. All the criteria applied (polyacrylamide gel electrophoresis, sodium dodecyl sulfate gel electrophoresis, and sedimentation analysis) indicated that the enzyme is homogeneous. The enzyme was sensitive to storage at −20°C under air. Conditions for stabilizing the pure enzyme still have to be examined. The molecular weight of the purified enzyme was determined by three independent methods, which revealed similar values: 230,000 (sucrose density gradient centrifugation); 234,000 (sedimentation equilibrium); and 214,000 (sodium dodecyl sulfate gel electrophoresis). Gel electrophoresis in the presence of either Serva or Koch-light sodium dodecyl sulfate revealed only one single band corresponding to a molecular weight of 107,000. Therefore CO:MB oxidoreductase of *P. carboxydovorans* is probably composed of two subunits of identical molecular weight. Composition of an enzyme of two subunits of the same molecular weight is not unusual and was also found for *Chromatium* hydrogenase (10) and for trimethylamine dehydrogenase from bacterium W3A1 (19).

The physical data of purified CO:MB oxidoreductase, such as sensitivity to inhibitors, electron acceptor spectrum, pH and temperature dependence of activity, activation energy, K_m for CO, and stoichiometry between CO oxidized and methylene blue reduced were found to be identical to those reported previously for crude cell extracts of *P. carboxydovorans* (16). Maximum rates of methylene blue reduction with CO were found at 63°C. This value is relatively high for an enzyme acting in a mesophilic bacterium.

The brownish color of purified CO:MB oxidoreductase and the visible spectrum of the enzyme with peaks at 405 and 470 nm suggest that CO:MB oxidoreductase may be an iron-sulfur protein. The determination of acid-labile iron and sulfur and measurements of electron paramagnetic resonance spectra will provide an answer. Peaks at 410 nm have also been encountered in purified hydrogenases (see reference 18 for a review).

Recently, extracts of CO autotrophically grown cells of *P. carboxydovorans* were shown to catalyze the oxidation of CO to CO_2, and indirect evidence has been presented that neither hydrogenase nor formate dehydrogenase are involved in the CO oxidation reaction (16). CO:MB oxidoreductase from *P. carboxydovorans* was free from formate dehydrogenase and practically free from hydrogenase activity. This finding directly demonstrates that neither hydrogenase nor formate dehydrogenase is required for the CO oxidation reaction. Purified CO:MB oxidoreductase was assayed under strictly anoxic conditions, indicating that molecular oxygen is not directly involved. Furthermore, the absence of any oxygen-containing compound other than water in the assay, and the liberation of exactly two electrons per molecule of CO clearly indicates that the second oxygen atom in the CO_2 produced must be derived from water; thus, CO oxidation is not due to a monooxygenase reaction, and water is the oxidant for carbon monoxide: $CO + H_2O \rightarrow CO_2 + 2\,H^+ + 2e^-$.

Attempts at reversing the reaction have not yet been made. The soluble methane monooxygenase of *Methylococcus capsulatus* (17), *P. methanica* (8, 9) or of *Methylosinus trichosporium* OB3b (21) catalyzes the oxygenation of CO as well as of *n*-alkanes, ethers, and alicyclic, aromatic, or heterocyclic compounds in a $NADPH_2$-dependent reaction (4, 5). In contrast, CO:MB oxidoreductase of *P. carboxydovorans* oxidized CO as the sole substrate. Oxidation of carbon monoxide by methane bacteria is an energy-consuming process and, therefore, a non-growth oxidation, whereas the reaction of CO-oxidizing hydrogen bacteria provides energy for growth and CO_2 fixation. Different dyes with a potential around 0 mV, such as pyocyanine, methylene blue, or thionine, were shown to act as effective electron acceptors. CO-grown cells of *P. carboxydovorans* contain ubiquinone Q10 as sole quinone in amounts of 4.07 μmol of ubiquinone Q10 per g of the particulate protein (O. Meyer, Ph.D. thesis, Institut für Mikrobiologie, Georg-August-Universität, Göttingen, 1978). Thus, ubiquinone Q10 may be the physiological electron acceptor.

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ADDENDUM IN PROOF

During the preparation of the manuscript, measurements with the more sensitive Perkin-Elmer spectrophotometer model 556 at temperatures of 100°K revealed enzyme-carbon monoxide binding spectra. Treatment of the oxidized enzyme with CO reduced the absorbance at 405 and 470 nm and caused a shift of the 405-nm peak to 410 nm.

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