Reactivity of Carbon Monoxide Dehydrogenase from *Rhodospirillum rubrum* with Carbon Dioxide, Carbonyl Sulfide, and Carbon Disulfide

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ABSTRACT: The reactivities of CO_2 and the related compounds COS and CS_2 with the nickel- and iron−sulfur-containing carbon monoxide dehydrogenase (CODH) from *Rhodospirillum rubrum* have been investigated. Both CO_2 and COS were substrates for CODH in a reductant-dependent reaction resulting in the formation of CO. CO_2 was reduced to CO and H_2O, while COS was reduced to CO and H_2S. CO was a potent inhibitor of CO_2 reduction at dissolved concentrations as low as 1 μM, but this inhibition could be prevented by quantitatively trapping CO as it was formed by including reduced hemoglobin in the assays. The addition of hemoglobin to the assays also allowed the formation of CO to be monitored in real time by following the decrease in absorbance at 433 nm resulting from carboxyhemoglobin formation. A variety of low-potential reductants, including dithionite, titanium(III) citrate, and dithionite-reduced viologens (methyl and benzyl), were suitable electron donors for the reduction of CO_2 and COS. Dithionite-reduced methyl viologen supported the highest rates of CO_2 and COS reduction, and the stimulation of CO_2 reduction (170-fold increased rate over dithionite alone) was much more dramatic than the stimulation of COS reduction (2.6-fold increased rate over dithionite alone). CO_2 was reduced to CO with a K_m for CO_2 of 190 μM and a V_{max} of 44 μmol of CO formed min^{-1} (mg of protein)$^{-1}$, while COS was reduced with a K_m for COS of 2.2 μM and a V_{max} of 0.51 μmol of CO formed min^{-1} (mg of protein)$^{-1}$. The CO_2/COS analog CS_2 interacted with CODH as a reversible, rapid-equilibrium inhibitor of CO_2 reduction, and the pattern of inhibition observed for CS_2 was competitive vs CO_2 (K_i = 43 μM).

A variety of diverse bacteria possess CO-oxidizing enzymes termed CO dehydrogenases (CODH)[1] which allow the bacteria to assimilate CO as a source of carbon and energy. While all CODH enzymes studied to date are able to catalyze the two-electron oxidation of CO to CO_2, the physiological functions and biochemical properties of these enzymes differ greatly among different organisms. In acetogenic, methanogenic, and sulfate-reducing bacteria, CODHs are complex, O_2-labile, nickel- and iron−sulfur-containing enzymes (Schauder et al., 1986; Ragsdale, 1991; Ferry, 1992). In addition to catalyzing the oxidation of CO to CO_2, these enzymes reduce CO_2 to CO and catalyze the synthesis or breakdown of acetyl-CoA (Spormann & Thauer, 1989; Ragsdale, 1991; Ferry, 1992). The purple, non-sulfur photosynthetic bacteria *Rhodospirillum rubrum* and *Rhodocyclus gelatinosa* contain inducible CO oxidation systems allowing the organisms to grow anaerobically with CO as their sole carbon and energy source (Uffen, 1976; Dashekvicz & Uffen, 1979; Bonam et al., 1989). Finally, a number of aerobic bacteria collectively referred to as carboxydobacteria contain inducible CODHs which allow the organisms to grow autotrophically with CO (Meyer et al., 1986). These O_2-stable enzymes differ from the CODHs of anaerobes in containing flavin and a molybdopterin cofactor instead of nickel (Meyer, 1982).

Several features of the CODH from *R. rubrum* make it an attractive model enzyme for studying the roles of nickel and iron−sulfur centers in catalyzing CO oxidation. *R. rubrum* CODH is a simpler enzyme than the nickel-containing CODHs from other anaerobes: It is a monomeric enzyme containing a single nickel atom and 7−8 iron atoms apparently arranged in 4Fe-4S clusters, and it lacks the ability to catalyze the synthesis or degradation of acetyl-CoA (Bonam & Ludden, 1987; Ensign et al., 1989a). The enzyme is induced by CO, and its induction is not repressed by the presence of other carbon sources (Bonam et al., 1989). When *R. rubrum* is cultured on growth medium depleted of nickel, the enzyme is still induced by CO and accumulates in a nickel-deficient state that can be subsequently activated by the addition of nickel chloride both *in vivo* and *in vitro* (Bonam et al., 1988). Through comparative biochemical and spectroscopic studies of nickel-deficient CODH and holo-CODH, nickel has been shown to play an essential role in the binding and activation of CO and the transfer of electrons from CO to the iron−sulfur centers of the enzyme during catalysis (Stephens et al., 1989; Ensign et al., 1989a,b).

Although *R. rubrum* CODH has been studied in some detail with respect to the mechanism of CO oxidation, the reverse reaction, the reduction of CO_2 to CO, has not been investigated for this enzyme. In a previous study the presence of 10 mM $NaHCO_3$ had no effect on the rate of CO oxidation catalyzed by CODH, indicating that CO_2 does not have a high affinity for binding the enzyme relative to CO (Hyman et al., 1989). However, an interaction between CO_2 and CODH has been established by demonstrating that the presence of CO_2 leads to a reversal of inhibition exerted by cyanide, which is a slow-binding inhibitor of CODH

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[1] Abbreviations: BSA, bovine serum albumin; CODH, carbon monoxide dehydrogenase; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; DMSO, dimethyl sulfoxide; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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(Hyman et al., 1989). Cyanide appears to inhibit CODH by binding at the CO oxidation site of the enzyme, and the reversal of cyanide inhibition effected by CO_2 is analogous to a similar reversal effected by CO (Ensign et al., 1989b). In one additional study that impacts the potential interaction of CO_2 with CODH, the CO_2 analog carbonyl sulfide (COS) was found to be a potent rapid-equilibrium competitive inhibitor of CO oxidation catalyzed by CODH (Hyman et al., 1989). In addition, COS was able to both protect CODH against slow-binding inhibition by cyanide and effect the reversal of cyanide inhibition in a manner analogous to CO and CO_2 (Hyman et al., 1989).

In order to provide further information concerning the catalytic mechanism of CODH and to clarify the previous observations regarding the interactions of CO_2 and COS with the enzyme, these latter two compounds have been investigated for their abilities to serve as substrates for CODH in a reversal of the CO oxidation reaction catalyzed by CODH. In this paper both CO_2 and COS are shown to be substrates for *R. rubrum* CODH, and the respective reactions are biochemically and kinetically characterized. The related compound, CS_2, is shown to be a rapid-equilibrium competitive inhibitor of CO_2 reduction. These studies are compared and discussed with relation to recently published studies concerning the reactivities of CO_2, COS, and CS_2 with the multifunctional CODH from the acetogenic bacterium *Clostridium thermoaceticum* (Anderson & Lindahl, 1994; Kumar et al., 1994).

EXPERIMENTAL PROCEDURES

Materials

CO (99.99+%) and COS (97.5% minimum) were purchased from Matheson (Chicago, IL).

Argon (99.9% minimum) was purchased from AL Compressed Gases (Salt Lake City, UT). Traces of O_2 were removed from argon by passage over a heated, copper-based catalyst. Traces of CO_2 were removed from COS and argon by passage over soda lime followed by bubbling through 12 M KOH. CS_2 (99.99%) was obtained from EM Science (Gibbstown, NJ). All other chemicals were of research grade.

Methods

Cell Growth and Purification of CODH. *R. rubrum* (ATCC 11170) cells were grown in 15-L polypropylene carboys with illumination from overhead projector bulbs contained in submerged water-jacketed tubes. The growth medium and induction conditions were identical to those described previously (Ensign et al., 1989b), except that the MOPS buffer concentration of the medium was decreased from 4 to 0.5 g/L and the pH of the growth medium was maintained at pH 7.0 by the addition of concentrated malic acid with a pH control system (Cole Parmer Instrument Co., Chicago, IL). CODH was purified anaerobically essentially as described previously (Bonam & Ludden, 1987) with the following changes. Cells were disrupted at 16 000 psi in a French pressure cell equipped with a rapid-fill kit (Aminco). After sedimentation and resuspension of the chromatophore membranes, CODH was solubilized by heat treatment as described (Bonam & Ludden, 1987), except that the duration of heating was increased by 25%. Lesser durations of heat treatment result in two copurifying forms of CODH which are referred to as peak 1 and peak 2 CODH due to their different migrations during preparative scale native gel electrophoresis (Bonam & Ludden, 1987; Ensign & Ludden, 1991). Both peak 1 and peak 2 CODH contain the catalytic 62-kDa protein, but peak 2 CODH contains an additional 22-kDa polypeptide which apparently serves as the physiological electron acceptor and membrane anchor protein for CODH (Ensign & Ludden, 1991). The additional heating time results in the quantitative denaturation and precipitation of the 22-kDa protein, leaving only monomeric and fully active peak 1 CODH, which is the form that has been used for previous biochemical and spectroscopic studies of CODH (Ensign & Ludden, 1991). After solubilization, CODH was further purified by DEAE-cellulose and hydroxylapatite chromatography as described (Bonam & Ludden, 1987). For the final purification step, preparative native gel electrophoresis was replaced by chromatography on a Hi-Load Q Sepharose FPLC column (Pharmacia). CODH was eluted from the column with a linear gradient of 50 to 400 mM NaCl in 100 mM MOPS buffer, pH 7.5, concentrated by ultrafiltration, and stored in liquid nitrogen. Purified CODH was homogenous as determined by SDS−PAGE and had a specific activity of 5200 μmol of CO oxidized min^{-1} (mg of protein)$^{-1}$. No hydrogenase activity (H_2 evolving) could be detected in the purified CODH preparation.

Assay of CO Oxidation. CODH activity was measured spectrophotometrically at pH 7.5 and 25 °C using the CO-dependent methyl viologen reduction assay described previously (Ensign et al., 1989b). All spectrophotometric measurements were performed using a Shimadzu UV-160 spectrophotometer containing a water-jacketed cell holder maintained at 25 °C.

Gas Chromatographic Assay of CO_2 and COS Reduction. Carbon monoxide formed from the reduction of CO_2 or COS was quantified using a Shimadzu GC-8A gas chromatograph equipped with a molecular sieve 5A column (0.3 × 100 cm) and a thermal conductivity detector using argon as the carrier gas. The gas chromatograph was operated isothermally with an injector/detector temperature of 100 °C, a column temperature of 50 °C, and a carrier gas flow rate of 15 mL/min. The gas chromatograph was interfaced to a Shimadzu Model CR601 integrator. CO_2 reduction assays were performed under an atmosphere of argon in serum vials (25 mL) sealed with butyl rubber stoppers in a shaking water bath maintained at 25 °C. The vials contained, in a total volume of 2 mL, 100 mM MOPS buffer, pH 7.5, 2 mM dithionite, 0.05 mM methyl viologen, 0.1 mM EDTA, and 1 mg/mL BSA. Substrates were added to the assay vials as a combination of $NaHCO_3$ (10 μmol) and CO_2 (4.5 μmol). Assays were initiated by the addition of CODH (1.07 μg). At the desired time points assays were terminated by the addition of 0.1 mL of acetic acid, and a sample of the gas phase was removed using a gastight syringe and analyzed for CO. CO formed from the reduction of COS was determined similarly except that the components of the assay mixture were altered as described below to allow the simultaneous quantification of H_2S formed from COS.

Quantitation of H_2S and CO Formed from COS Reduction. H_2S was quantified spectrophotometrically after chemical conversion to methylene blue using a modification of the protocol described by Brumby et al. (Brumby et al., 1965). Assays were performed under argon in stoppered serum vials

(25 mL) containing 2 mL of assay buffer and a glass test tube (10 × 40 mm) to which 0.8 mL of 1% zinc acetate was added. The assay buffer contained 100 mM MOPS buffer, pH 7.5, 2 mM titanium citrate [added from an 80 mM stock solution prepared as described previously (Seefeldt & Ensign, 1994)], 0.1 mM EDTA, and 1 mg/mL BSA. Fifty microliters of COS was added as the substrate, and the assays were initiated by the addition of CODH (35.6 μg). At the desired time points the assays were terminated by the addition of 0.1 mL of acetic acid to the assay buffer. The vials were incubated an additional 2 h to allow sufficient time for all the H_2S generated in the course of the assays to be trapped in the zinc acetate solution. Samples of the gas phase were then removed using gastight syringes and were analyzed for CO by gas chromatography as described above. The vials were then opened, and the glass test tubes containing the zinc acetate and zinc sulfide precipitate were removed and transferred to serum vials (13 mL) which contained 5 mL of H_2O. The vials were sealed with butyl rubber stoppers, 1 mL of 0.5% *N,N*-dimethylphenylenediamine hydrochloride in 5 M HCl and 0.2 mL of 0.023 mM $FeCl_3$ in 1.2 M HCl were added, and the vials were vortexed vigorously. After 30 min, aliquots of each vial were diluted 5-fold by the addition of water, and the absorbance of the samples was measured at 670 nm. Reference standards were prepared using sodium sulfide prepared anaerobically in H_2O and which had been standardized by iodometric titration. The reference standards, as well as standards of CO for analysis by gas chromatography, were subjected to the same incubation conditions and manipulations as described above.

Spectrophotometric Assay of CO_2 and COS Reduction. The formation of CO was measured spectrophotometrically at 25 °C by monitoring the formation of carboxyhemoglobin using a modification of the CO quantification assay described previously (Bonam et al., 1984). Assays were performed in 2-mL (1-cm path length) anaerobic quartz cuvettes filled completely with assay buffer components such that there was no gaseous headspace present. The cuvettes had been modified by fusing to each a serum bottle-style quartz top (7 × 13 mm at mouth), allowing the cuvettes to be sealed either with red rubber serum vial stoppers or gray butyl stoppers and aluminum crimp seals. The sealed cuvettes were evacuated and flushed with argon on a vacuum manifold. Anaerobic buffer (1.9−2 mL) containing 100 mM MOPS, pH 7.5, 0.2 mg/mL hemoglobin, 2 mM dithionite, and 0.1 mM EDTA was transferred to cuvettes using a gastight syringe. Where indicated, methyl viologen or other accessory reductants were added to the cuvettes from stock solutions prepared in MOPS buffer. Dithionite-reduced solutions of methyl viologen used in these experiments were stable for the duration of the assays, and the addition of CODH, $NaHCO_3$, and hemoglobin did not lead to changes in the spectrum of, or the steady concentration of, the reduced methyl viologen. $NaHCO_3$ was prepared as either a 50, 500, or 1000 mM solution in anaerobic H_2O, while COS was prepared as a partially saturated aqueous solution under 3% COS and 97% argon. Samples of $NaHCO_3$ or COS solutions were transferred to the assay cuvettes, and the assay components were allowed to equilibrate for 2 min at 25 °C. Assays were initiated by the addition of CODH. The progress of assays was monitored by following the decrease in absorbance at 433 nm vs time. Standard curves were generated by titrating samples of a saturated solution of CO prepared in water into assay cuvettes and measuring the absorbance at 433 nm after each addition. All buffers and reagents added to assay cuvettes were made anaerobic either by evacuating and flushing with CO_2-free argon on a vacuum manifold or by sparging vigorously with CO_2-free argon.

Numeric Constants and Data Analysis. The solubilities of COS (23.2 mM) and CO (1.04 mM) in aqueous solutions at 25 °C were calculated using published Bunsen coefficients for the gases (Wilhelm et al., 1977). The concentration of CO_2 in equilibrium with HCO_3^- at pH 7.5 was calculated from the following equation, which applies to aqueous solutions with no gaseous headspace present (Butler, 1982):

$$[CO_2] = C_T \frac{[H^+]^2}{K_{a1}K_{a2} + K_{a1}[H^+] + [H^+]^2} \quad (1)$$

C_T represents the total concentration of carbonate species and CO_2 present, while K_{a1} and K_{a2} are the ionization constants for dissolved CO_2 and HCO_3^-, respectively. Values for pK_{a1} and pK_{a2} of 6.3 and 10.3 at 25 °C were used (Butler, 1982). The absorption coefficient of methyl viologen at 578 nm was taken as 9.7 mM^{-1} cm^{-1}. Kinetic constants (K_m, K_i, and V_{max}) were calculated by fitting initial rates to the appropriate equations for rectangular hyperbolas as described by Cleland (Cleland, 1979).

RESULTS

Gas Chromatographic and Spectrophotometric Measurement of CO_2 Reduction by CODH. CODHs from *R. rubrum* and other organisms catalyze the two-electron oxidation of CO to CO_2 according to eq 2:

$$CO + H_2O \rightleftharpoons CO_2 + 2e^- + 2H^+ \quad (2)$$

In spite of the apparent physiological importance of CO_2 reduction to CO in some microorganisms, this reaction has not been demonstrated previously for *R. rubrum* CODH. In order to determine whether purified CODH was capable of catalyzing the reductive formation of CO, the enzyme was incubated in sealed serum vials for various times in the presence of $NaHCO_3$ and CO_2 and with dithionite and reduced methyl viologen added as electron donors. Samples of the gas phase were then removed and analyzed for CO by gas chromatography. As shown in Figure 1, CODH catalyzed the formation of CO from CO_2 in a time-dependent manner. When $NaHCO_3$ and CO_2 were omitted from the reaction vials, no CO formation was observed. No CO formation from CO_2 was observed in the presence of cyanide, a slow-binding inhibitor of CO oxidation, or if CODH was O_2-inactivated by exposure to air prior to performing the assays.

A quantitative analysis of CO formation from CO_2 using the gas chromatographic assay employed in Figure 1 is complicated by several factors. First, the assay is dependent on fixed time point measurements of CO formation, making kinetic analyses difficult. Second, the gas chromatographic analysis is rather insensitive; under the conditions used to generate Figure 1, the lower limit of CO detection was approximately 25 nmol of CO formed per assay vial. Since the reduction of CO_2 to CO is catalyzed at a much slower rate than the oxidation of CO to CO_2, relatively high concentrations of CODH were required to observe CO

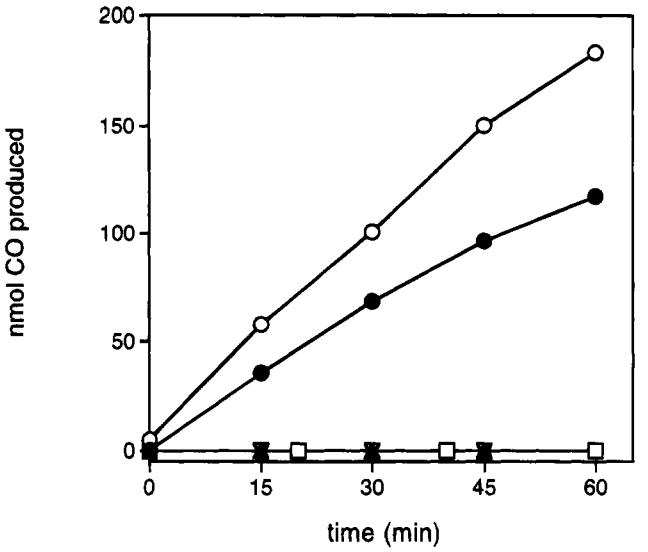

FIGURE 1: Gas chromatographic analysis of CO_2 reduction catalyzed by *R. rubrum* CO dehydrogenase. Assays were performed and CO was quantified by gas chromatography as described under Materials and Methods. (○) 1.07 μg of CODH with CO_2/$NaHCO_3$, (●) 0.535 μg of CODH with CO_2/$NaHCO_3$, (□), 1.07 μg CODH without CO_2/$NaHCO_3$, (▲) 1.07 μg of CODH with CO_2/$NaHCO_3$ and 0.5 mM sodium cyanide, and (▽), 1.07 μg of O_2-inactivated CODH with CO_2/$NaHCO_3$.

formation within a reasonable time frame. At the concentrations used, doubling the amount of CODH added to the assay did not result in a doubling of the rate of CO formation. A further complication arose if assays were performed in vials with a low ratio of gas to liquid phase, or if assays were performed with low concentrations of $NaHCO_3$ (or CO_2 gas) added as substrate. In these cases the rates of CO formation decreased over time until no further CO formation was observed. If the vials were then evacuated and flushed on a vacuum manifold and additional CO_2 was added, CO formation proceeded at the same initial rate as was observed previously. These observations suggest that the accumulation of CO leads to inhibition of further CO_2 reduction, a phenomenon that is addressed in more detail below.

These considerations have been addressed by adapting an extremely sensitive, spectrophotometric-based hemoglobin assay to trap CO formed from CO_2 by CODH in real time during the course of the reaction. This assay takes advantage of the large spectral changes which occur upon the binding of CO to hemoglobin to produce carboxyhemoglobin (Van Assendelft, 1970). The binding of CO leads to a shift in the absorption maximum of hemoglobin from 433 to 419 nm, and the resultant decrease in absorbance at 433 nm or increase in absorbance at 419 nm is linear with respect to CO binding (Bonam et al., 1984). These differences in the spectral features of hemoglobin and carboxyhemoglobin have been exploited in a previous study of *R. rubrum* CODH to prove that CO-dependent methyl viologen reduction is coupled to the consumption of CO (Bonam et al., 1984). In two recent studies, Lindahl, Ragsdale, and co-workers have exploited carboxyhemoglobin formation to quantify CO formation from CO_2 by *C. thermoaceticum* CODH in an electrochemical cell (Lindahl et al., 1990) and to characterize the inhibition of CO_2 reduction by CS_2 (Kumar et al., 1994).

In Figure 2, the changes in the absorption spectrum of dithionite- and methyl viologen-reduced hemoglobin observed upon successive additions (2.08 nmol each) of CO

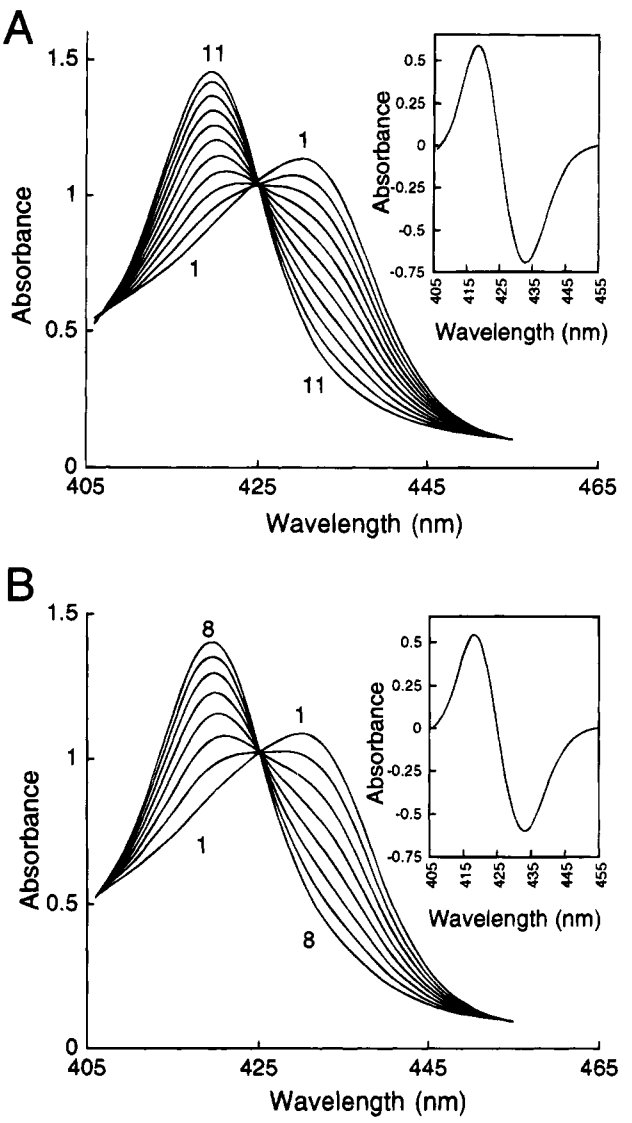

FIGURE 2: Comparison of CO-dependent and CODH- and CO_2-dependent changes in the absorption spectrum of hemoglobin. Hemoglobin-binding assays were performed as described under Materials and Methods with 0.05 mM methyl viologen as an accessory reductant. Spectra were recorded vs a reference blank containing all assay components except hemoglobin. (A) CO-dependent conversion of hemoglobin to carboxyhemoglobin. Spectrum 1 was recorded in the absence of CO. Each subsequent spectrum was recorded after the addition of 2.08 nmol of CO, culminating in spectrum 11, which had a total of 20.8 nmol of CO present. The inset shows the difference spectrum obtained by subtracting spectrum 1 from spectrum 11. (B) Time-dependent conversion of hemoglobin to carboxyhemoglobin in the presence of CODH and CO_2. Assay mixtures contained 2.5 mM $NaHCO_3$ and 0.285 μg of CODH. Immediately after CODH was added to the cuvette, spectrum 1 was recorded. Subsequent spectra were recorded at 1-min intervals, culminating with spectrum 8, which was recorded 7 min after initiation of the assay. The inset shows the difference spectrum obtained by subtracting spectrum 1 from spectrum 8. Spectra were recorded vs a reference blank containing all assay components except hemoglobin.

are compared to the time-dependent spectral changes observed in a cuvette prepared identically, but containing CODH and $NaHCO_3$. The spectral changes observed with $NaHCO_3$ and CODH present are identical to the changes seen upon addition of CO. The time course of CODH-catalyzed CO_2 reduction was monitored by continuously following the decrease in absorbance at 433 nm due to carboxyhemoglobin formation. The progress curves were linear for at least 5

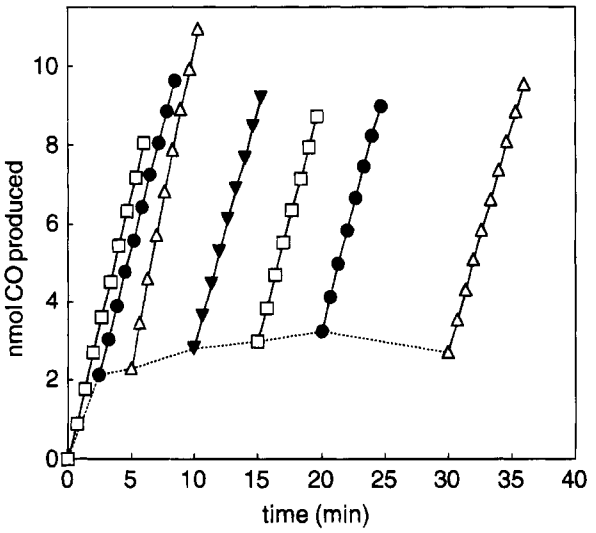

FIGURE 3: Inhibition of CO_2 reduction by CO. Assays were performed in anaerobic cuvettes as described under Materials and Methods in the presence of 0.05 mM methyl viologen and 2.5 mM $NaHCO_3$. Assays were initiated by the addition of CODH (0.196 μg). For these assays hemoglobin was initially omitted from the assay cuvettes. At the times indicated by the plot symbols connected by the dashed line, hemoglobin was added to a final concentration of 0.2 mg/mL from a 20 mg/mL stock solution prepared in MOPS buffer containing 2 mM dithionite; the cuvettes were rapidly mixed and placed in the spectrophotometer, and the formation of carboxyhemoglobin was monitored spectrophotometrically by following absorbance at 433 nm vs time. The amount of CO (nmol) initially observed after the addition of hemoglobin is indicated by the dashed line. The solid lines represent the time-dependent formation of additional CO with hemoglobin present in the assays. The times at which hemoglobin was added to the assays were as follows: (□) 0, (●) 2.5 min, (△) 5, (▼) 10, (□) 15, (●) 20, and (△) 30 min.

min with either 0.071, 0.142, or 0.285 μg of CODH present, and the slopes of the progress curves were proportional to the amount of CODH added. Specific activities of 13.9, 13.8, and 13.4 μmol of CO formed min^{-1} (mg of protein)$^{-1}$, respectively, were calculated from the slopes of these progress curves. No CO formation was observed in the absence of added CO_2 or if CODH was inhibited by cyanide or inactivated by exposure to O_2 prior to initiating the assays.

In addition to providing a convenient real-time assay for monitoring CO_2 reduction, the spectrophotometric hemoglobin binding assay alleviates the problem of inhibition caused by accumulation of CO by removing CO as carboxyhemoglobin immediately after it is produced by CODH. This is illustrated in Figure 3, which shows the effect of hemoglobin addition on the CODH-catalyzed formation of CO from CO_2 over time. For this experiment, assays were initiated by the addition of CODH to cuvettes containing 2.5 mM $NaHCO_3$ and all other assay components, but which initially lacked hemoglobin. At the times indicated in the figure, hemoglobin was added to the cuvettes, the amount of preexisting CO was quantified by measuring the initial absorbance at 433 nm, and additional CO formation was then monitored by following the further decrease in absorbance at 433 nm over time. In the absence of hemoglobin, the accumulation of approximately 2 nmol of CO (1 μM in solution) led to a significant inhibition of further CO_2 reduction. Under the conditions of Figure 3, the time-dependent formation of CO was essentially fully inhibited within 5 min. Upon addition of hemoglobin to the cuvettes, CO_2 reduction proceeded at the same rate as observed when hemoglobin was present in the cuvette from the outset of the reaction, demonstrating that the inhibition is fully reversible once CO is removed.

Reduction of COS by CODH. COS has been characterized previously as a rapid-equilibrium, competitive inhibitor of CO oxidation catalyzed by CODH (Hyman et al., 1989). The spectrophotometric assay described above was used to determine whether COS could serve as a substrate for the reductive formation of CO. The addition of 1 μmol of COS to an assay cuvette containing CODH resulted in the same pattern of time-dependent spectral changes as that observed with CO_2 and CODH. As observed for CO_2-dependent CO formation, the progress curves of absorbance at 433 nm vs time with COS as a substrate were linear for at least 5 min. In order to obtain progress curves with slopes approximately equivalent to those obtained with CO_2 as the substrate, 30-fold higher concentrations of CODH were required. With 1.8, 3.6, and 7.1 μg of CODH present in the assays, the specific activities with COS as the substrate were 0.473, 0.473, and 0.462 μmol of CO formed min^{-1} (mg of protein)$^{-1}$. No COS-dependent CO formation was observed with cyanide-inhibited or O_2-inactivated CODH.

There are two potential complications that need to be considered concerning the characterization of COS as a substrate for CODH. First, commercially obtained COS is contaminated with up to 1.8% CO_2 (Matheson Technical Brief TB-202). Second, in aqueous solutions COS undergoes hydrolysis to CO_2 and H_2S, although this reaction is sufficiently slow that no significant abiotic hydrolysis should occur during the time frame of the 5 min assays (Ferm, 1957). In order to determine whether contaminating CO_2 contributed to the rates of CO formation seen with COS as substrate, unpurified COS and COS that had been scrubbed with potassium hydroxide to remove CO_2 were compared as substrates for CODH. Identical rates of CO formation were obtained, indicating that contaminating CO_2 is not contributing to the rates of CO formation observed with COS.

The possibility that CO_2 reduction is contributing to the COS-dependent formation of CO was further investigated by examining the effect of COS on CO_2-dependent CO formation and, conversely, the effect of CO_2 on COS-dependent CO formation. Figure 4 shows the time courses for the decrease in absorbance at 433 nm vs time with 3.2 μg of CODH present and with either 5 μmol of $NaHCO_3$ or 1 μmol of COS initially added as substrate. Identical rates of carboxyhemoglobin formation were observed with 1 or 2 μmol of COS added as substrate, demonstrating that the assay is saturated with respect to COS (traces 2 and 3). When 5 μmol of $NaHCO_3$ was added to an assay already proceeding with 1 μmol of COS as substrate (see trace 1), there was no change in the rate of carboxyhemoglobin formation. When 1 μmol of COS was added to an assay already proceeding with 5 μmol of $NaHCO_3$ as substrate (see trace 4), a new, much slower rate of carboxyhemoglobin formation was immediately established which was identical to the rate observed with 1 μmol of COS alone. The fact that the same rate of carboxyhemoglobin formation is observed with 1 or 2 μmol of COS, and the fact that this rate does not change upon the addition of CO_2, indicates that the formation of carboxyhemoglobin is due to COS reduction, which occurs at a slower rate than CO_2 reduction under these assay conditions. The results presented in Figure 4 further indicate

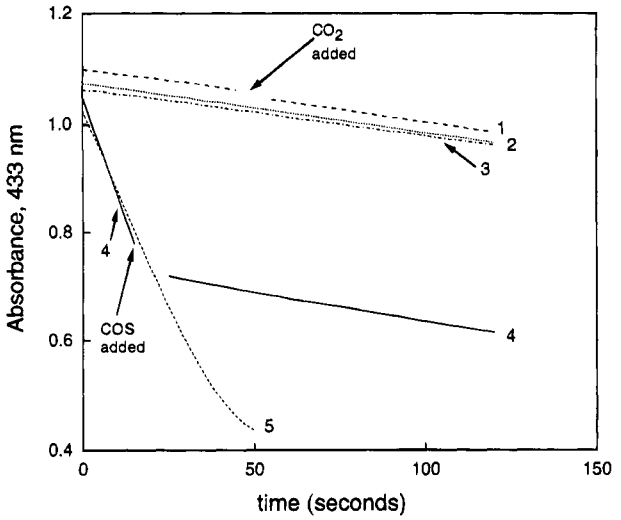

FIGURE 4: Comparison of CO_2 and COS reduction rates, effect of CO_2 on COS reduction, and effect of COS on CO_2 reduction. The decrease in absorbance at 433 nm due to formation of carboxyhemoglobin is plotted vs time. Assay cuvettes were prepared as described under Materials and Methods and contained 0.05 mM methyl viologen and 3.20 μg of CODH. Trace 1 (— —), 1 μmol of COS added initially and 5 μmol of $NaHCO_3$ added at the time indicated by the arrow; trace 2 (•••), 2 μmol of COS added initially; trace 3 (-•-), 1 μmol of COS added initially; trace 4 (—), 5 μmol of $NaHCO_3$ added initially and 1 μmol COS added at the time indicated by the arrow; trace 5 (- - -), 5 μmol of $NaHCO_3$ added initially.

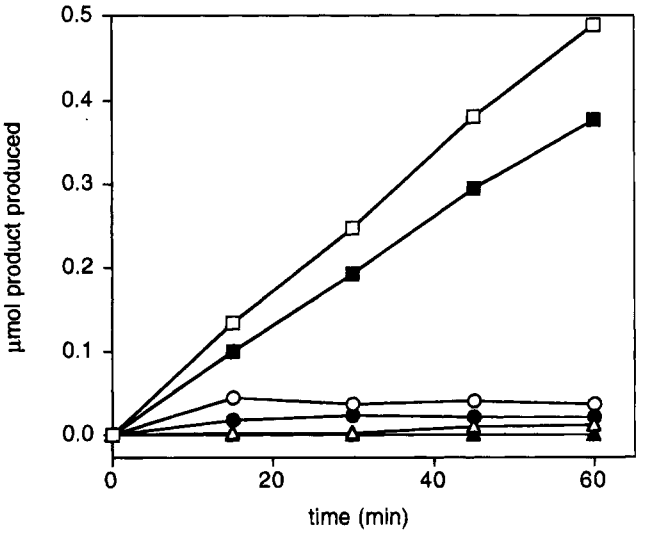

FIGURE 5: Time-dependent formation of CO and H_2S from COS reduction. Assays were performed and CO and H_2S were quantified as described under Materials and Methods. The open symbols represent H_2S produced (μmol), and the filled symbols represent CO produced (μmol). (□, ■) CODH and COS present, (○, ●) cyanide-treated CODH and COS present, (△, ▲) O_2-inactivated CODH and COS present.

that COS is preferentially reduced by CODH at the concentrations of COS and CO_2 added to the assays. The results also demonstrate that the reductions of CO_2 and COS are competing reactions since the simultaneous presence of COS and CO_2 results in a rate of carboxyhemoglobin formation equal to the rate with COS alone, rather than a rate equal to the sum of the two individual reaction rates.

Definitive evidence that COS is serving as a substrate for CODH is provided in Figure 5, which shows that the COS-dependent formation of CO is coupled to the formation of H_2S. H_2S should be formed as a stoichiometric product of COS reduction according to eq 3:

$$COS + 2e^- + 2H^+ \rightleftharpoons CO + H_2S \quad (3)$$

Table 1: Comparison of CO_2 and COS Reduction Rates with Various Reductants[a].

	CO_2 reduction		COS reduction	
reductant	sp act.[b]	rel rate[c]	sp act.[b]	rel rate[c]
2 mM dithionite	0.181	1.0	0.189	1.0
4 mM dithionite	0.222	1.2	0..193	1.0
2 mM dithionite plus				
0.025 mM methyl viologen	8.07	44.6	0..451	2.38
0.050 mM methyl viologen	13.5	74.5	0.480	2.54
0.10 mM methyl viologen	22.6	125	0..476	2.52
0.25 mM methyl viologen	30.1	167	0.500	2.64
0.10 mM benzyl viologen	10.5	58	0.451	2.38
0.025 mM methylene blue	0.193	1.06	0.201	1.06
0.025 mM indigo carmine	0.178	0.984	0.194	1.02
3 mM titanium(III) citrate	0.230	1.27	0.208	1.10

[a] CO_2 and COS reduction assays were performed spectrophotometrically as described under Materials and Methods with 5 mM $NaHCO_3$ or 1 μmol of COS added as substrate. [b] Specific activities are reported as μmol of CO formed min^{-1} mg of $protein^{-1}$. [c] Rates are related to the rates observed with 2 mM dithionite alone as reductant.

For the experiment shown in Figure 5, titanium(III) citrate, a low-potential reductant capable of donating electrons to a number of metalloenzymes (Seefeldt & Ensign, 1994), was used as the reductant for COS reduction in place of dithionite, since dithionite interfered with the subsequent determination of sulfide. Under the conditions used to generate Figure 5, CO, analyzed by gas chromatography, was formed at a rate of 0.18 μmol min^{-1} (mg of protein)$^{-1}$. H_2S, analyzed by the methylene blue assay described in the Methods section, was formed at a rate of 0.23 μmol min^{-1} (mg of protein)$^{-1}$. No CO, and only very low levels of H_2S, was formed when cyanide-inhibited or O_2-inactivated CODH was used in the assays.

Effect of Reductants on the Rates of CO_2 and COS Reduction. With the exception of the experiment presented in Figure 5, all of the assays performed to this point used a combination of 2 mM dithionite and 0.05 mM methyl viologen as reductants for substrate reduction. In order to determine whether electron transfer to CODH is rate limiting in the overall reduction of substrate, the rates of CO formation from CO_2 and COS were compared using a number of different reductants as electron donors. The results of this study are presented in Table 1. With dithionite alone as the electron donor, comparable specific rates of CO formation were observed with either CO_2 or COS as the substrate. However, when the low-potential dye methyl viologen was added as an accessory reductant in addition to dithionite, the rate of CO_2 reduction increased dramatically while the rate of COS reduction increased slightly, but to a much lesser extent. With 0.25 mM methyl viologen present in the assay in addition to dithionite, the rate of CO_2 reduction increased 167-fold relative to the rate with dithionite alone, while the rate of COS reduction increased 2.6-fold. The addition of the low-potential dye benzyl viologen as an accessory reductant also led to a dramatic increase in the rate of CO_2 reduction and a lesser increase in the rate of COS reduction. Two higher potential dyes incapable of reducing the iron–sulfur centers of CODH, indigo carmine

and methylene blue, did not increase the rates of CO_2 or COS reduction when added to the assays in addition to dithionite. Titanium(III) citrate, a low-potential reductant already discussed above, served as a reductant, giving rates of CO_2 and COS reduction approximately equal to the rates with dithionite alone.

The stimulation of CO_2 reduction by methyl viologen was further investigated by measuring the rates of CO formation with a wider range of methyl viologen concentrations present. In each case 2 mM dithionite was present in the assays to ensure that all of the viologen was in the reduced state and that an excess of reductant was present. The stimulation of CO_2 reduction was saturable with respect to methyl viologen, and an apparent K_m for reduced methyl viologen of 88 ± 6.2 μM was calculated.

Kinetic Characterization of CO_2 and COS Reduction. The kinetics of CO_2 and COS reduction were investigated using the carboxyhemoglobin assay described above, with 0.1 mg/mL hemoglobin present and with 2 mM dithionite and 0.25 mM methyl viologen present as reductants. The concentration of methyl viologen present was the highest concentration that could be practically used with this assay, since, at higher concentrations, methyl viologen absorption at 433 nm became sufficiently high that it interfered with carboxyhemoglobin quantification. This concentration of methyl viologen was 2.8-fold higher than the K_m and gave rates approximately 80% of the theoretical V_{max} at saturating methyl viologen.

As shown in Figure 6A, the rate of CO production with various concentrations of $NaHCO_3$ followed saturation kinetics. At pH 7.5, a K_m for $NaHCO_3$ (representing all carbonate species in solution) of 3.2 ± 0.21 mM and a V_{max} of 44 ± 1.1 μmol of CO formed min^{-1} (mg of protein)$^{-1}$ (k_{cat} = 45 s^{-1}) were derived for CO_2 reduction. In order to shed light on whether CO_2 or bicarbonate is the actual substrate for CODH, the rate of CO formation was also measured as a function of $NaHCO_3$ concentration at pH 7.8. From eq 1 it can be shown that 5.93% of the total carbonate species in solution at pH 7.5 are present as dissolved CO_2, while at pH 7.8, only 3.06% of the carbonate species are present as dissolved CO_2. At pH 7.8 the rate of CO formation increased in a saturable manner with increasing $NaHCO_3$ concentrations in the same manner as observed at pH 7.5, but higher concentrations of $NaHCO_3$ were required to obtain the same relative rates. The calculated K_m at pH 7.8 was 5.6 mM $NaHCO_3$, a value that is 1.8-fold higher than the K_m at pH 7.5. However, the K_m values, when reported as a function of dissolved CO_2 concentrations, are 0.19 and 0.17 mM at pH 7.5 and 7.8, respectively. These results suggest that the actual substrate for CODH is CO_2 rather than the bicarbonate ion or another dissolved carbonate species.

As shown in Figure 6B, the rate of CO formation from COS also followed saturation kinetics. A K_m of 2.2 ± 0.18 μM COS and a V_{max} of 0.51 ± 0.013 μmol of CO formed min^{-1} (mg of protein)$^{-1}$ (k_{cat} = 0.52 s^{-1}) were derived from the fit of this data. By way of comparison, in the oxidative direction with CO as the substrate and 10 mM methyl viologen as the electron acceptor, CO is oxidized with a K_m for CO of 32 μM and a V_{max} of 7700 μmol of CO oxidized min^{-1} (mg of protein)$^{-1}$ (Ensign & Ludden, 1991). The K_i for the inhibition of CO oxidation by COS was previously determined to be 2.3 μM (Hyman et al., 1989).

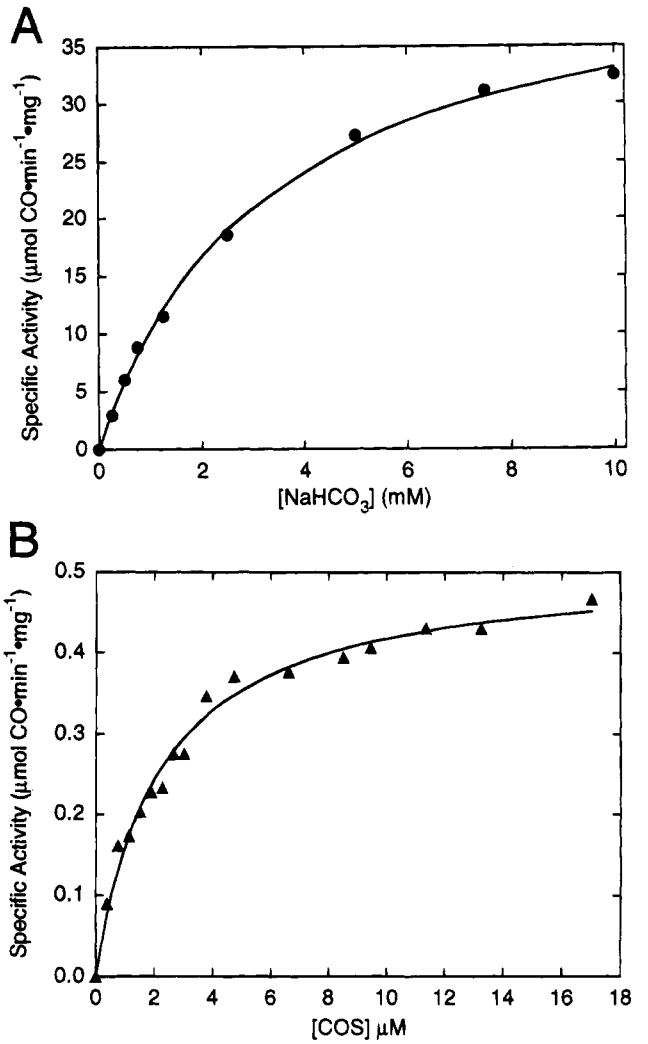

FIGURE 6: Effect of CO_2 and COS concentrations on the rate of CODH-catalyzed CO formation. Assays were performed spectrophotometrically as described under Materials and Methods and with 2 mM dithionite and 0.25 mM methyl viologen added as reductants. Assays of CO_2 reduction were initiated by adding 0.251 μg of CODH, while 3.131 μg of CODH was added to initiate assays of COS reduction. Activities are reported as μmol of CO formed min^{-1} (mg of protein)$^{-1}$. (A) CO_2 reduction. (B) COS reduction.

CS_2 Inhibition of CODH-Catalyzed CO_2 Reduction. CS_2 is a good structural analog of CO_2 and COS and could therefore potentially be an inhibitor of CO oxidation or CO_2 reduction catalyzed by CODH. CS_2 has previously been briefly examined as an inhibitor of CO oxidation by *R. rubrum* CODH and as an effector for the reversal of cyanide inhibition (Hyman et al., 1989). In this previous study, the presence of 10 mM CS_2 resulted in 17% inhibition of CO oxidation when added to an assay saturated with CO, and the presence of 28.9 mM CS_2 resulted in a partial reactivation of cyanide-inhibited CODH (Hyman et al., 1989). Recently, Ragsdale and co-workers have shown that CS_2 interacts with the CODH from *C. thermoaceticum* by competing with CO for binding at the acetyl-CoA synthase site of the enzyme (Kumar et al., 1994). Anderson and Lindahl have recently shown that CS_2, as well as CO and CO_2, serves as an effector for the reversal of cyanide inhibition of CO oxidation by *C. thermoaceticum* CODH in a manner analogous to the reversals described for *R. rubrum* CODH (Anderson & Lindahl, 1994).

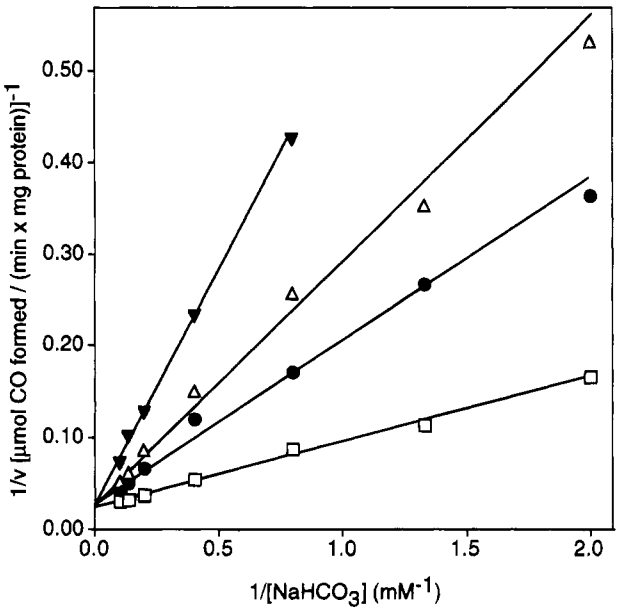

FIGURE 7: Competitive inhibition of CO dehydrogenase-catalyzed CO_2 reduction by CS_2. Assays were performed spectrophotometrically as described under Materials and Methods and the caption to Figure 6. CS_2 was added to the assay cuvettes from a 50 mM solution prepared in DMSO. Cuvettes containing various concentrations of $NaHCO_3$ (0−10 mM) and CS_2 were preincubated for 1 min. The assays were then initiated by the addition of 0.251 µg of CODH. The concentrations of CS_2 present were (□) no CS_2 present; (●) 0.075 mM CS_2, (△) 0.125 mM CS_2, and (▼), 0.25 mM CS_2. The addition of DMSO alone to the assays at the concentrations transferred with CS_2 had no effect on the rates of CO_2 reduction.

On the basis of these observations concerning the interactions of CS_2 with the CODHs from *C. thermoaceticum* and *R. rubrum*, CS_2 was further investigated as an inhibitor of the reduction of CO_2 by *R. rubrum* CODH using the spectrophotometric assay described above. In order to ensure that CS_2 would not interfere with the hemoglobin-binding assay, the absorption spectra of hemoglobin with and without different amounts of CO bound were compared in the absence and presence of 5 mM CS_2. The presence of CS_2 had no effect on either the absorption spectrum of hemoglobin or the magnitude of absorption change seen upon binding of CO to produce carboxyhemoglobin.

In the presence of CS_2, the progress curves of CO formation from CO_2 were linear over time for at least 5 min, but the slopes of the progress curves were reduced relative to those for assays performed in the absence of CS_2. CS_2 thus appears to interact with CODH as a reversible, rapid-equilibrium inhibitor of CO_2 reduction. A kinetic analysis of this inhibition was performed by measuring the rates of CO formation with various concentrations of CO_2 and CS_2 present. As shown in Figure 7, the double-reciprocal plot of velocity vs [$NaHCO_3$] obtained from this study shows a pattern characteristic of competitive inhibition. A K_i for CS_2 of 43 ± 1.9 µM was calculated by fitting the experimental data to the appropriate equation for competitive inhibition (Cleland, 1979).

DISCUSSION

In this paper the reversibility of the CO oxidation reaction catalyzed by *R. rubrum* CODH is established using CO_2 and COS as substrates for the reductant-dependent formation of CO. On the basis of information obtained from previous biochemical and spectroscopic studies of *R. rubrum* CODH (Hyman et al., 1989; Stephens et al., 1989; Ensign et al., 1989a;b Tan et al., 1992) and mechanistic information provided from studies of inorganic metal catalysts (Collman et al., 1987), the oxidation of CO to CO_2 by *R. rubrum* CODH probably proceeds by the following steps: (1) binding of CO to a metal center, possibly nickel, of the fully oxidized enzyme to form an electrophilic metal carbonyl intermediate; (2) nucleophilic attack of water on the metal carbonyl to form a metal−carbohydroxy intermediate with loss of a proton; (3) oxidative decarboxylation of this intermediate, releasing a second proton and CO_2, with donation of two electrons to CODH; (4) transfer of the two electrons released in step 3 to the 4Fe-4S centers of the enzyme; and (5) transfer of the electrons from the 4Fe-4S centers to an electron acceptor to yield the fully oxidized enzyme. The reduction of CO_2 to CO would be expected to proceed by a reversal of the steps outlined above, with CO_2 binding to the active site metal of the fully reduced enzyme, hydroxide release occurring to leave the metal−carbonyl intermediate, and CO release occurring to leave the oxidized form of the enzyme, which then undergoes reduction using electrons donated from a low-potential reductant. COS reduction would be expected to occur in an analogous fashion, except that the leaving group released upon going to the metal−carbonyl intermediate is hydrogen sulfide rather than water (Figure 5).

In a previous attempt to demonstrate the reversibility of the CO oxidation reaction catalyzed by *R. rubrum* CODH, the reduced form of the enzyme, in which the 4Fe-4S centers of the enzyme are in the one-electron-reduced form, was incubated with 0.1 M $NaHCO_3$ in order to determine whether the presence of bicarbonate and CO_2 would lead to the reoxidation of the iron−sulfur centers in the single-turnover reaction shown in eq 4 (Hyman et al., 1989):

$$CO_2 + 2H^+ + 2[4Fe{-}4S]^+ \rightleftharpoons CO + H_2O + 2[4Fe{-}4S]^{2+} \quad (4)$$

No reoxidation of the iron−sulfur centers was observed. This is not surprising, since E° for the reaction shown in eq 4, using values of −550 mV for the reduction potential of the CO_2/CO couple at pH 7.5 and −418 mV for the reduction potential of the 4Fe-4S centers (Smith et al., 1992), is −132 mV. This corresponds to a value for the Gibb's free energy of the reaction in eq 4 of ΔG° = +6.1 kcal/mol and an equilibrium constant of 3.3×10^{-5}. With the concentrations of enzyme (0.7 mg/mL) and CO_2 (5.9 mM; 5.9% of 0.1 M $NaHCO_3$ added is present as dissolved CO_2) present in the single-turnover experiment described previously (Hyman et al., 1989), the enzyme would have undergone only a slight (0.0002%) reoxidation upon attainment of chemical equilibrium. Clearly, the presence of an excess supply of a low-potential reductant (dithionite, titanium(III) citrate, or methyl viologen) is required to drive the reduction of CO_2. In addition, the presence of hemoglobin greatly facilitates the reduction of CO_2, by removing the CO product as carboxyhemoglobin in an exergonic and essentially irreversible reaction. The inhibition of CO_2 reduction by the accumulation of CO which occurs in the absence of hemoglobin (Figure 3) may be due to the attainment of chemical equilibrium in the reaction and/or product inhibition caused by the interaction of CO with CODH.

The reduction of CO_2 to CO has been investigated for one other purified CODH, the nickel- and iron−sulfur-containing CODH from *C. thermoaceticum* (Lindahl et al., 1990; Kumar et al., 1994). This enzyme differs significantly from *R. rubrum* CODH in several regards: the enzyme is multimeric, highly complex, and multifunctional, catalyzing both the reversible oxidation of CO to CO_2 and the synthesis of acetyl-CoA from CO, coenzyme A, and a methyl group (Ragsdale, 1991). The oxidation of CO and the assembly of acetyl-CoA are proposed to occur at different sites on the enzyme, which have been designated centers C and A, respectively (Shin & Lindahl, 1992; Kumar et al., 1993). Spectroscopic and biochemical evidence suggests that the catalytic center of *R. rubrum* CODH may share properties similar to those of center C of *C. thermoaceticum* CODH (Stephens et al., 1989; Anderson et al., 1993). Since CO and analogs thereof can potentially interact with both center A and C of *C. thermoaceticum* CODH, and since *R. rubrum* CODH contains a single catalytic center capable of catalyzing only the reversible oxidation of CO, the *R. rubrum* enzyme is an excellent model for studying the specific interactions of substrates, products, and inhibitors with the site of CO oxidation and CO_2 reduction.

In the studies of CO_2 reduction by *C. thermoaceticum* CODH, a hemoglobin assay similar to the one described in this paper was used to quantify CO formation (Lindahl et al., 1990; Kumar et al., 1994). In the first study, the reduction of CO_2 to CO was performed electrocatalytically in an electrochemical cell (Lindahl et al., 1990). In the second study, dithionite was used as a source of reducing equivalents to drive the reduction of CO_2 (Kumar et al., 1994). In spite of the similar natures of the CO_2 reduction reactions catalyzed by *C. thermoaceticum* CODH and *R. rubrum* CODH, there are several differences in the kinetics of the respective reactions and the conditions under which activity is observed. For *C. thermoaceticum* CODH, CO_2 reduction was reported to occur only below pH 6.6 (Kumar et al., 1994). At pH 5.9 and 25 °C and with dithionite as the reductant, *C. thermoaceticum* CODH catalyzed the reduction of CO_2 with a K_m for CO_2 of 7.4 mM and a turnover number of 1.3 s^{-1} (Kumar et al., 1994). Our studies of CO_2 reduction were performed at pH 7.5, and under the conditions used in our assay, CO_2 was reduced with a substantially lower K_m for CO_2 (0.19 mM), a higher turnover number (45 s^{-1}), and, accordingly, a 1350-fold higher catalytic efficiency (k_{cat}/K_m).

In their studies of CO_2 reduction by *C. thermoaceticum* CODH, Ragsdale and co-workers also discovered that CS_2 was a rapid-equilibrium inhibitor of both acetyl-CoA synthase (as measured by following an exchange reaction between CO and acetyl-CoA) and CO oxidation/CO_2 reduction activities of the enzyme (Kumar et al., 1994). The inhibition patterns observed for CS_2 were competitive vs CO for the acetyl-CoA exchange assay (K_i = 0.47 mM) and noncompetitive vs CO and CO_2 for CO oxidation (K_i = 12.1 mM) and CO_2 reduction (K_i = 3.2 mM) (Kumar et al., 1994). Treatment of dithionite-reduced *C. thermoaceticum* CODH with CS_2 led to the development of an EPR signal attributed to an interaction between CS_2 and an iron atom of center A (Kumar et al., 1994). No interaction between CS_2 and center C was observed, and it was concluded that CS_2 inhibits reactions at center C by binding at center A. Gorst and Ragsdale, in a separate study, found that COS was a substrate for *C. thermoaceticum* CODH, forming CO and H_2S in a reaction apparently identical to the reduction of COS by *R. rubrum* CODH described in this paper (Gorst, 1991). This result demonstrates that COS interacts with center C as a CO_2 analog and alternate substrate. It is unclear why CS_2 does not interact with the site of CO oxidation/CO_2 reduction in *C. thermoaceticum* CODH in light of our observation that CS_2 is a competitive inhibitor of CO_2 reduction by *R. rubrum* CODH.

COS has recently been shown to be an inhibitor of the acetylene reduction activity of nitrogenase, a complex metalloenzyme which contains a unique molybdenum- and iron−sulfur-containing cofactor which is believed to be the site of substrate reduction (Madden et al., 1990). An interesting feature of nitrogenase is its broad substrate specificity; in addition to the physiologically important reduction of N_2 to ammonia, the enzyme also catalyzes the reduction of a number of alternate substrates, including acetylene, cyanide, protons, cyclopropene, azide, and nitrous oxide (Burris, 1991). In the accompanying paper (Seefeldt et al., 1995), it is shown that COS, as well as CO_2, can serve as a substrate for nitrogenase in the reductive formation of CO in a reaction apparently identical to the reaction catalyzed by CODH. Nitrogenase catalyzed the reduction of COS to CO with a K_m for COS of 3.1 mM and a k_{cat} of 0.15 s^{-1}, while CO_2 was reduced with a K_m for CO_2 of 23 mM and a k_{cat} of 0.0033 s^{-1} (Seefeldt et al., 1995). It is interesting that the k_{cat} for COS reduction by nitrogenase is 45-fold *higher* than the k_{cat} for CO_2 reduction by nitrogenase, while the opposite result was observed with CODH, where the k_{cat} for COS reduction is 88-fold *lower* than the k_{cat} for CO_2 reduction. In the accompanying paper, the CO_2/COS analog CS_2 was also found to be a reversible inhibitor of nitrogenase activity (K_i = 2.9 mM for CS_2 inhibition of acetylene reduction). Although it is unlikely that there is any physiological significance of COS and CO_2 reduction by nitrogenase, it is interesting that the two classes of fundamentally different metalloenzymes are capable of catalyzing these same reactions.

In addition to their reactivities toward CODH and nitrogenase, COS and CS_2 have been characterized as substrates, inhibitors, or inactivators of a limited number of other enzymes. COS is a competitive inhibitor vs CO_2 for the CO_2-utilizing enzymes ribulose bisphosphate carboxylase and carbonic anhydrase (Chengelis & Neal, 1979; Laing & Christeller, 1980). COS will also serve as an alternate substrate for these enzymes in place of CO_2. In the case of ribulose bisphosphate carboxylase, a thiocarboxylation reaction occurs with ribulose bisphosphate to produce 3-phosphoglycerate and 1-thio-3-phosphoglycerate as products (Lorimer & Pierce, 1989). In the case of carbonic anhydrase, COS is hydrolyzed to a monothiocarbonate intermediate, which then spontaneously breaks down to CO_2 and H_2S (Chengelis & Neal, 1979; Miller et al., 1989).

CS_2 is an extremely toxic compound, a feature that can be largely attributed to its reactivity toward metal-containing microsomal cytochrome P-450 enzymes and mixed-function oxidases (Obrebska et al., 1980). CS_2 is oxidized by cytochrome P-450 to form an unstable intermediate, which reacts with water to form monothiocarbonate and reactive sulfur species, which inactivate the enzyme via covalent modification (De Matteis, 1974; Chengelis & Neal, 1987). CS_2 can also react with amino acids in proteins to form

dithiocarbamates, which are potent chelators of metal ions (Reid, 1963). The formation of dithiocarbamates can account for the reactivity of CS_2 toward the copper-containing enzymes cytochrome oxidase, tyrosine oxidase, and ammonia monooxygenase (Hyman et al., 1990). With regard to the interaction of CS_2 with CODH, it seems unlikely that the mode of inhibition involves the formation of a chelating thiocarbamate or the conversion of CS_2 to another reactive species, since there is no time dependency to the inhibition. The reversible and competitive nature of the inhibition suggests that CS_2 mimics CO_2 as a ligand for the metal-containing CO_2/CO binding site of the enzyme.

In summary, in this paper the reversibility of the CO oxidation reaction catalyzed by *R. rubrum* CODH has been demonstrated using the physiological product CO_2 and the related compound COS as substrates for the reductant-dependent formation of CO. The conditions required for obtaining maximal rates of product formation were established, and the respective reactions were kinetically characterized. The ability to assay *R. rubrum* CODH in the reverse direction should prove useful for future mechanistic studies of this nickel- and iron−sulfur-containing enzyme. In addition, the substrate COS and the rapid-equilibrium, competitive inhibitor CS_2 may prove to be useful spectroscopic probes for further studies of the metal centers of CODH.

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