Eley, M. H., Namihira, G., Hamilton, L., Munk, P., & Reed, L. J. (1972) *Achiv. Biochem. Biophys. 152*, 655–669.
Engel, A. (1983) *Micron 13*, 425–436.
Fuller, C. C., Reed, L. J., Oliver, R. M., & Hackert, M. L. (1979) *Biochem. Biophys. Res. Commun. 90*, 431–438.
Griffin, T. A., Lau, K. S., & Chuang, D. T. (1988) *J. Biol. Chem. 263*, 14008–14014.
Guest, J. R., Darlison, M. G., Spencer, M. E., & Stephens, P. E. (1984) *Biochem. Soc. Trans. 12*, 220–223.
Hainfeld, J. F., Wall, J. S., & Desmond, E. J. (1982) *Ultramicroscopy 8*, 263–270.
Hansen, B. A., Lane, R. S., & Dekker, E. E. (1974) *J. Biol. Chem. 249*, 4891–4896.
Hu, C.-W. C., Griffin, T. A., Lau, K. S., Cox, R. P., & Chuang, D. T. (1986) *J. Biol. Chem. 261*, 343–349.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Lamvik, M. K. (1978) *J. Mol. Biol. 122*, 55–68.
Mosesson, M. W., Hainfeld, J., Haschemeyer, R. H., & Wall, J. (1982) *J. Mol. Biol. 153*, 695–718.
Oliver, R. M., & Reed, L. J. (1982) in *Electron Microscopy of Proteins* (Harris, J. R., Ed.) Vol. 2, pp 1–48, Academic Press, London.
Perham, R. N., & Roberts, G. C. K. (1981) *Biochem. J. 199*, 733–740.
Perham, R. N., Duckworth, H. W., & Roberts, G. C. K. (1981) *Nature 292*, 474–477.
Pettit, F. H., Hamilton, L., Munk, P., Namihira, G., Eley, M. H., Willms, C. R., & Reed, L. J. (1973) *J. Biol. Chem. 248*, 5282–5290.
Pettit, F. H., Yeaman, S. J., & Reed, L. J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4881–4885.
Radford, S. E., Laue, E. D., Perham, R. N., Miles, J. S., & Guest, J. R. (1987) *Biochem. J. 247*, 641–649.
Reed, L. J. (1974) *Acc. Chem. Res. 7*, 40–46.
Reed, L. J., & Oliver, R. M. (1968) *Brookhaven Symp. Biol. 21*, 397–412.
Reed, L. J., Damuni, Z., & Merryfield, M. L. (1985) *Curr. Top. Cell. Regul. 27*, 41–49.
Stephens, P. E., Darlison, M. G., Lewis, H. M., & Guest, J. R. (1983) *Eur. J. Biochem. 133*, 481–489.
Steven, A. C., Hainfeld, J. F., Trus, B. L., Steinert, P. M., & Wall, J. S. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6363–6367.
Wall, J. (1978) in *Introduction to Analytical Electron Microscopy* (Hren, J. J., Goldstein, J. I., & Joy, D. C., Eds.) pp 333–342, Plenum, New York.
Watanabe, H., Green, G. D. J., & Shaw, E. (1979) *Biochem. Biophys. Res. Commun. 89*, 1354–1360.
Yang, H., Hainfeld, J. F., Wall, J. S., & Frey, P. A. (1985) *J. Biol. Chem. 260*, 16049–16051.
Yang, H., Frey, P. A., Hainfeld, J. F., & Wall, J. S. (1986) *Biophys. J. 49*, 56–58.
Zhang, B., Kuntz, M. J., Goodwin, G. W., Harris, R. A., & Crabb, D. W. (1987) *J. Biol. Chem. 262*, 15220–15224.

Carbonyl Sulfide Inhibition of CO Dehydrogenase from *Rhodospirillum rubrum*†

Michael R. Hyman,‡ Scott A. Ensign,§ Daniel J. Arp,‡ and Paul W. Ludden*,§

Department of Biochemistry, University of California, Riverside, California 92521, and Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin—Madison, Madison, Wisconsin 53706

Received February 27, 1989; Revised Manuscript Received May 9, 1989

ABSTRACT: Carbonyl sulfide (COS) has been investigated as a rapid-equilibrium inhibitor of CO oxidation by the CO dehydrogenase purified from *Rhodospirillum rubrum*. The kinetic evidence suggests that the inhibition by COS is largely competitive versus CO (K_i = 2.3 μM) and uncompetitive versus methylviologen as electron acceptor (K_i = 15.8 μM). The data are compatible with a ping-pong mechanism for CO oxidation and COS inhibition. Unlike the substrate CO, COS does not reduce the iron–sulfur centers of dye-oxidized CO dehydrogenase and thus is not an alternative substrate for the enzyme. However, like CO, COS is capable of protecting CO dehydrogenase from slow-binding inhibition by cyanide. A true binding constant (K_D) of 2.2 μM for COS has been derived on the basis of the saturable nature of COS protection against cyanide inhibition. The ability of CO, CO_2, COS, and related CO/CO_2 analogues to reverse cyanide inhibition of CO dehydrogenase is also demonstrated. The kinetic results are interpreted in terms of two binding sites for CO on CO dehydrogenase from *R. rubrum*.

Carbon monoxide is oxidized by at least four different classes of enzyme. These are (1) ammonia- and methane-oxidizing monooxygenases found in autotrophic nitrifying bacteria (Tsang & Suzuki, 1982) and methanotrophic bacteria (Colby et al., 1977), respectively; (2) the CO oxidases that are O_2 insensitive, contain molybdenum, Fe-S clusters, and FAD, and are found in aerobic carboxydotrophic bacteria (Meyer, 1985); (3) the CO dehydrogenases that are O_2 labile and multimeric, contain nickel, zinc, and Fe-S clusters, and are found in strictly anaerobic acetogenic and methanogenic bacteria (Drake et al., 1980; Grahame & Stadtman, 1987); and (4) the CO dehydrogenase from *Rhodospirillum rubrum*, which is O_2 labile, monomeric, and contains Fe-S clusters, 1 mol of nickel/mol of enzyme, and substoichiometric amounts of zinc (Bonam et al., 1984, 1988; Ensign et al., 1989a).

Despite superficial similarities, the two classes of nickel-containing CO dehydrogenases are sufficiently different to warrant separate classification. The physiological role of the

† This research was supported by the College of Agricultural and Life Sciences at the University of Wisconsin—Madison and by Grant DE-FG02-87ER13691 to P.W.L. from the U.S. Department of Energy. S.A.E. was supported by Training Grant GM07215 from the National Institutes of Health, and M.R.H. was supported by Grant DE-FG03-84ER13257 to D.J.A. from the U.S. Department of Energy during this study.

* Address correspondence to this author.
‡ University of California.
§ University of Wisconsin—Madison.

0006-2960/89/0428-6821$01.50/0

CO dehydrogenases in acetogens and methanogens is the reduction of CO_2 to CO and the insertion of CO into the carboxyl position of acetyl-CoA (Diekert et al., 1985). While these complex enzymes can be conveniently assayed by following a partial reaction of electron-acceptor-dependent CO oxidation, it would be more appropriate to regard them as acetyl-CoA synthases. In contrast, the CO dehydrogenase from *R. rubrum* is specifically induced by CO and apparently catalyzes solely the oxidation of CO to CO_2, according to the reaction

$$CO + H_2O \rightarrow CO_2 + 2H^+ + 2e^-$$

Although two classes of CO dehydrogenase are recognized, the nickel present in both classes of enzyme is thought to serve a similar role. An interaction between CO and nickel has been demonstrated for CO dehydrogenase from *Clostridium thermoaceticum* by using magnetic spectroscopic techniques (Ragsdale et al., 1983a). Likewise, a nickel-free form of CO dehydrogenase from *R. rubrum* is catalytically inactive and rapidly activated upon addition of $NiCl_2$ (Bonam et al., 1988). These observations are consistent with the notion that nickel is the site of CO binding and activation in both classes of enzyme. Specific inhibitors of CO dehydrogenase may provide a means to further characterize the CO-binding site and the putative catalytic role of nickel. However, few inhibitors of CO dehydrogenases are known. To date, only O_2, cyanide, and alkyl halides have been described as inhibitory compounds (Krzycki & Zeikus, 1984; Grahame & Stadtman, 1987; Bonam et al., 1984). Oxygen inactivates CO dehydrogenases by an unknown mechanism. That these enzymes are sensitive to O_2 is not surprising given the strictly anaerobic growth requirements of many CO-dehydrogenase-expressing bacteria. Cyanide inhibits all CO dehydrogenases and has recently been shown to be a nickel selective, competitive, active-site-directed, slow-binding inhibitor of CO dehydrogenase from *R. rubrum* (Ensign et al., 1989b). This quantitative approach has rationalized many of the previous qualitative descriptions of cyanide inhibition of CO dehydrogenases. Reports of inhibition by methyl iodide and related alkyl halides are more diverse, although the inhibition in vivo is generally described as a time-dependent process that is accelerated by the presence of CO and can be reversed by treatment with light (Diekert & Thauer, 1978). A recent report indicates that methyl iodide acts as a methyl donor for the synthesis of acetyl-CoA using purified CO dehydrogenase from *C. thermoaceticum* (Pezacka & Wood, 1988). Similarly, coenzyme A analogues have recently been shown to be competitive inhibitors of the carbonyl exchange reaction catalyzed by CO dehydrogenase from *C. thermoaceticum* (Raybuck et al., 1988). However, the partial, reversible reaction of methylviologen-dependent CO oxidation is unaffected by these compounds. It is evident that none of the compounds described above act as conventional, rapid-equilibrium inhibitors of CO oxidation. In this work, we demonstrate that COS is such an inhibitor of CO dehydrogenase from *R. rubrum*.

Materials and Methods

Materials

CO (99.99+%), N_2 (99.998+%), CO_2 (99.5%), and carbonyl sulfide (COS) (97.5% min) were purchased from Matheson Gas Products Inc. (Chicago, IL). Traces of O_2 were stripped from CO and N_2 by passage over a heated, copper-based catalyst (R3-11, Chemical Dynamics Corp., South Plainsfield, NJ). COS was used without further purification [principal contaminants (mol %) CS_2 (0.19), CO_2 (1.8), N_2/CO (1.0), O_2 (0.1), H_2S (0.01)] (Matheson Technical Brief TB-202). All other chemicals were of research grade.

Methods

Protein Purification. Holo CO dehydrogenase was purified from *R. rubrum* as described previously (Ensign et al., 1989b). All the studies described in this paper made use of enzyme preparations with specific activities of 4200 μmol of CO oxidized min^{-1} (mg of protein)$^{-1}$ when assayed as described below.

Assay of CO Dehydrogenase Activity. The activity of CO dehydrogenase was determined spectrophotometrically by following CO-dependent methylviologen reduction at 578 nm, as described previously (Ensign et al., 1989b). Unless otherwise stated, all assays were performed at room temperature (23 °C) in anaerobic, CO-saturated buffer (100 mM MOPS, pH 7.5) containing 10 mM methylviologen and 1 mM EDTA and were monitored on a Shimadzu UV-160 UV–visible recording spectrophotometer. All assays were initiated by the addition of purified CO dehydrogenase; initial rates were taken from the linear portion of the reaction progress curve (within the first 100 s).

For inhibition studies under assay conditions, COS was added to assay cuvettes in an aqueous solution taken from a 30-mL stoppered serum vial containing 101 kPa COS and 2 mL of buffer (as above) at room temperature. In aqueous solution COS is very slowly hydrolyzed to CO_2 and H_2S (Ferm, 1957). COS solutions were therefore used within 2 h of preparation. Although COS was contaminated with CO (<1%), the amount of CO carried to assays or incubations with COS was found to be insignificant (data not shown).

Incubation Procedures for Experiments with Cyanide. Incubations of CO dehydrogenase with KCN were carried out at room temperature (23 °C) under anaerobic conditions in 10-mL, stoppered, serum vials. The vials were prepared by repeated evacuation and flushing with N_2 followed by addition of deoxygenated buffer (100 mM MOPS, pH 7.5) containing 1 mM sodium dithionite, enzyme solution, and an appropriate volume of anaerobic, COS-saturated buffer. Before the addition of cyanide, a sample of the incubation mixture was removed and assayed for CO-oxidizing activity. To determine the level of inhibition caused by the transfer of COS from the incubation vial to the assay cuvette, this rate was compared to that of CO dehydrogenase not exposed to COS. Cyanide (as freshly prepared KCN in aqueous 1 N NaOH) was then added to each incubation mixture (time of addition designated as $t = 0$) to give a final reaction mixture volume of 1 mL. Samples (less than 5 μL) were withdrawn at the indicated times and assayed for CO-oxidizing activity. The observed rates were corrected for the level of inhibition caused by the carry-over of COS.

Oxidation of CO Dehydrogenase. Oxidation of CO dehydrogenase was carried out in an anaerobic glovebox containing less than 1 ppm of O_2 (Vacuum Atmospheres Dri-Lab glovebox, Model HE-493) following the procedure of Eady et al. (1987) with several modifications that have been described previously (Ensign et al., 1989a).

Determination of CO Concentrations in Solution. CO concentrations in solution were determined by using the CO/hemoglobin binding assay described by Bonam et al. (1984).

Numerical Constants. All kinetic constants were derived by nonlinear, least-squares fit to the appropriate models described in the text (Elsevier Biosoft, Cambridge, England). The solubility of CO (0.88 mM) and COS (20.3 mM) in buffer at room temperature (23 °C) was calculated by using an α value for CO in water of 0.02208 (Dean, 1985) and a

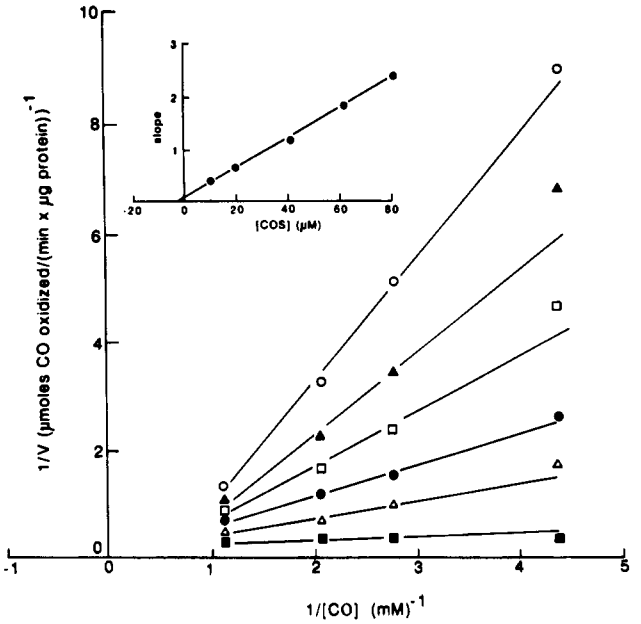

FIGURE 1: Competitive inhibition of CO oxidation by *R. rubrum* CO dehydrogenase in the presence of COS. All assays of CO-oxidizing activity were conducted in 1.5-mL, anaerobic, stoppered cuvettes that contained 1 mL of 0.1 M MOPS buffer (pH 7.5), 1 mM EDTA, and 10 mM methylviologen. The CO concentration in the assay cuvettes was varied by sparging the cuvettes with CO/N_2 mixtures of varying compositions. COS was added to the CO/N_2 sparged cuvettes from a saturated solution in buffer (as above). The reaction was initiated by the addition of 2 μL of stock enzyme solution (0.014 mg/mL). COS concentrations (in solution) were (■) 0, (△) 10.2, (●) 20.3, (□) 40.6, (▲) 60.9, and (○) 81.2 μM. The inset shows a plot of the slopes of the main panel versus inhibitor concentration. Slope units are (μM·min·μg)/μmol.

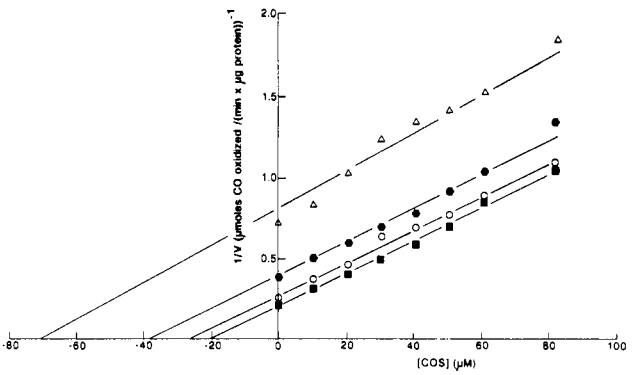

FIGURE 2: Effect of methylviologen concentration on COS inhibition of CO oxidation by CO dehydrogenase. CO oxidation assays were conducted as described for Figure 1 except that CO-saturated buffer (0.88 mM CO) was used in all assays and the stock CO dehydrogenase solution was 0.018 mg/mL. COS was added to the assay cuvettes to the concentrations shown in the figure. The assays were conducted in the presence of (△) 1, (●) 3, (○) 6, and (■) 10 mM methylviologen as electron acceptor.

q value (g of gas/100 mL at 101 kPa) for COS in water of 0.122 (Winkler, 1906). The extinction coefficient (ϵ) for methylviologen at 578 nm was taken as 9.7 mM^{-1} cm^{-1}.

Results and Discussion

Inhibition Patterns of COS versus CO and Methylviologen for CO Dehydrogenase. Carbonyl sulfide (COS) is a colorless, odorless, and tasteless gas with properties similar to those of CO_2 and CS_2. COS is regarded a linear molecule with three resonance structures (Ferm, 1957). Because of the reasonable structural analogy of COS to the substrate (CO), and likely product (CO_2), of CO dehydrogenase, initial experiments with COS tested the effects of this compound when added directly to CO-dependent methylviologen reduction assays. Addition of COS to an assay resulted in an inhibition that reached completion within the 5-s mixing time before the initiation of data collection; therefore, no time dependency to the inhibition was apparent. The inhibition was characterized as a function of [COS] at various fixed CO concentrations (Figure 1). The double-reciprocal plot of velocity versus [CO] reveals a pattern characteristic of competitive inhibition. However, the pattern deviates from classic competitive inhibition at high concentrations of CO where less than expected inhibition was observed. A replot of the slopes of this plot versus [COS] (inset of Figure 1) provided an upper limit estimate of the K_i for COS of 2.3 μM.

The effects of COS on methylviologen reduction assays were also analyzed as a function of methylviologen concentration. A plot of reciprocal velocity versus [COS] for assays run in the presence of various fixed methylviologen concentrations is shown in Figure 2. The parallel, nonintersecting lines are indicative of uncompetitive inhibition. An estimate of the K_i for COS versus methylviologen of 15.8 ± 2.7 μM was obtained directly from the plot in Figure 2 by modifying the values of apparent K_i (X intercept) by the factor $1 + K_m/[S]$, using an experimentally derived value of the K_m for methylviologen of 3.5 mM (at pH 7.5).

Taken together, the results presented in Figures 1 and 2 are compatible with a ping-pong kinetic mechanism in which methylviologen first oxidizes reduced CO dehydrogenase in a step that necessarily precedes the competitive and mutually exclusive binding of CO and COS. The apparent deviation from classical competitive inhibition caused by COS is not inconsistent with the fundamental framework of such a mechanism, although it does imply a more complex process. Notably, a kinetic analysis of methylviologen-dependent CO oxidation by CO dehydrogenase from *C. thermoaceticum* was also interpreted in terms of a ping-pong mechanism (Diekert & Thauer, 1978).

Reversibility of COS Inhibition. While the kinetic analyses above imply a rapid-equilibrium inhibition of CO dehydrogenase by COS, the results presented provide no direct evidence for a reversibility of the inhibition. To test this, samples of CO dehydrogenase were incubated under anaerobic conditions with 101 kPa of COS. Aliquots (10 μL) of COS-treated enzyme gave residual activities of less than 10% when removed directly from the incubation and assayed for CO-oxidizing activity. However, the residual activity reached 94% when the COS was first removed from the incubation by repeated evacuation and reequilibration with 101 kPa of CO. This experiment demonstrates two important points. First, COS appears to be a fully reversible inhibitor of CO oxidation. Second, appropriate controls must be conducted to take account of the effect of COS which is concomitantly transferred to an assay cuvette when COS-treated CO dehydrogenase is assayed for CO-oxidizing activity. This is necessary given the potent effect of COS on CO-oxidizing activity and the high solubility of COS in water.

COS Is Not a Substrate for CO Dehydrogenase. Potentially COS could be acting either as a dead-end inhibitor or as a poor substrate for CO dehydrogenase. An alternative substrate would be expected to exhibit competitive inhibition versus CO and noncompetitive inhibition versus methylviologen within a ping-pong mechanism, while a dead-end inhibitor would exhibit uncompetitive inhibition versus methylviologen (Cleland, 1986). Therefore, dead-end inhibition by COS is suggested by the kinetic analysis. However, a more unequivocal test of the ability of COS to act as a substrate for CO de-

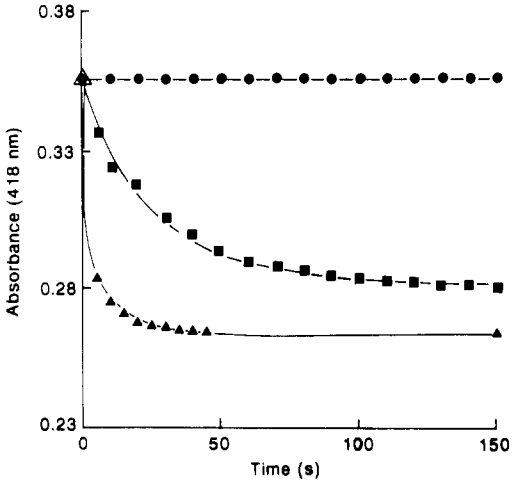

FIGURE 3: Effect of COS on the rate of reduction of iron–sulfur centers of indigo carmine oxidized CO dehydrogenase. CO dehydrogenase was oxidized by indigo carmine as described under Methods. At t = 0, 1 mL of dye-free, indigo carmine-oxidized, CO dehydrogenase (0.7 mg/mL) was added to anaerobic, 1.5-mL stoppered cuvettes that contained (▲) 101 kPa of CO, (●) 101 kPa of COS, or (■) 81 kPa of COS/10 kPa of CO. The figure shows a plot of the absorbance at 418 nm as a function of time for each treatment.

hydrogenase is shown in Figure 3. In this experiment the ability of COS to reduce the iron–sulfur centers of the oxidized form of the enzyme was tested. A spectrophotometric method that follows the decrease in absorbance at 418 nm (due to oxidized iron–sulfur centers) as a function of time was used (Bonam & Ludden, 1987). Exposure of oxidized CO dehydrogenase to 101 kPa of CO led to a complete reduction of the iron–sulfur centers within 20 s (Figure 3). In contrast, enzyme exposed to 101 kPa of COS remained fully oxidized for over 150 s, indicating that no electron transfer occurred between COS and the iron–sulfur centers over the time course of the reaction.[1] The lack of iron–sulfur reduction by COS implies that COS itself is not a substrate for CO dehydrogenase. In the presence of a 91 kPa of CO/10 kPa of COS mixture, a slow rate of iron–sulfur center reduction was observed. This effect is consistent with a decreased rate of CO binding/oxidation arising from the presence of a competitive, rapid-equilibrium inhibitor.

COS Protection of CO Dehydrogenase from Cyanide Inhibition. Cyanide is a slow-binding inhibitor of CO oxidation by CO dehydrogenase from *R. rubrum* (Ensign et al., 1989b). Slow-binding inhibitors exhibit slow rates of association of the enzyme–inhibitor complex (relative to substrate binding) (k_{on}) and very slow rates of enzyme–inhibitor dissociation (k_{off}) (Morrison & Walsh, 1988). In the present study nearly 90% of the CO-oxidizing activity was lost within 5 min ($t_{1/2}$ = 1.4 min) when CO dehydrogenase was incubated with 100 μM KCN at pH 7.5 (Figure 4a). However, in the presence of only 11.5 μM COS (in solution), the rate of inhibition was decreased greater than 5-fold ($t_{1/2}$ = 7.5 min). The semilog plots of percent activity remaining versus time (Figure 4a) indicate that the rate of loss of activity in all incubations was a pseudo-first-order process over the range of COS concentrations tested and the time course of each incubation. From this evidence we suggest that COS, like CO, binds at the active site of the enzyme and therefore prevents cyanide binding.

To quantify this protection by COS against cyanide inhibition, the residual activity was determined after CO dehydrogenase was incubated for 20 min with 100 μM KCN and COS concentrations between 0 and 230 μM (in solution). The results (Figure 4b) indicate that COS protection against cyanide inhibition is an apparently saturable reaction. This saturation curve provides a means of estimating the true binding constant (K_D) for COS. Accordingly, the data from Figure 4b were fitted to a hyperbola (r = 0.986), and an apparent K_D of 27.7 ± 5.5 μM was derived. This value requires modification according to the concentration of KCN present in the incubation according to (Ensign et al., 1989b)

$$K_{D(true)} = K_{D(app)}/(1 + ([KCN]/K_D KCN))$$

Using the previously reported value of the K_D for KCN of 8.46 μM (Ensign et al., 1989b), we can derive a value for $K_{D(true)}$ for COS of 2.18 ± 0.44 μM, a value in close agreement with the maximal K_i value of 2.3 μM derived for COS as a competitive inhibitor of CO oxidation (Figure 1). This K_D value for COS is also comparable to the K_D of 4.68 μM previously determined for CO (Ensign et al., 1989b), indicating that CO dehydrogenase has similar affinities for COS and CO.

A comparable experiment to that described in Figure 4b was also conducted with CO_2. Incubation of CO dehydrogenase for 20 min in the presence of 101 kPa of CO_2 only decreased the level of inhibition caused by 100 μM KCN

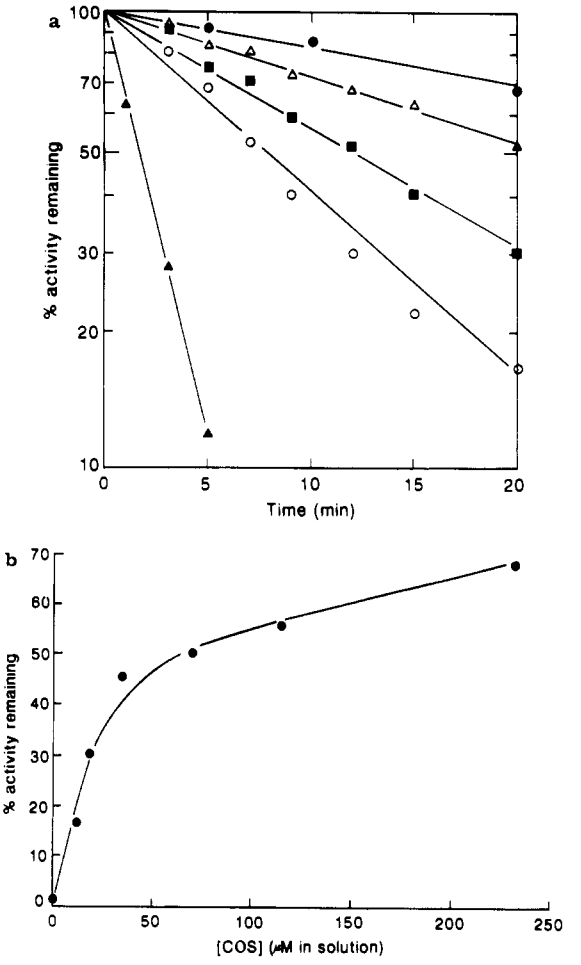

FIGURE 4: Effect of COS on the inhibition of CO dehydrogenase by KCN. Incubations of CO dehydrogenase with KCN and COS were conducted as described under Methods. Each incubation contained CO dehydrogenase at a final concentration of 0.019 mg/mL. (a) Semilog plot of percent activity remaining versus time for CO dehydrogenase incubated with 100 μM KCN and (▲) no, (○) 11.5, (■) 17.25, (△) 69, and (●) 230 μM COS (COS concentrations in solution). (b) Plot of percent activity remaining versus COS concentration (in solution) for CO dehydrogenase incubated for 20 min with 100 μM KCN and COS concentrations from 0 to 230 μM.

[1] Although COS was contaminated by low levels of CO (<1%), this concentration of CO was insufficient to reduce CO dehydrogenase in the presence of at least a 100-fold excess of COS.

by 10% relative to control incubations conducted with KCN and 101 kPa of N_2.

Comparison of the Effects of COS with CO_2 Analogues. A reasonable mechanism for the inhibition of CO dehydrogenase by COS is that the inhibitor is acting as an analogue of the substrate, CO. However, chemically COS is more closely related to CO_2 and CS_2 than CO (Ferm, 1957) and is not known to inhibit other CO-sensitive enzymes such as the nickel-containing hydrogenase from *Azotobacter vinelandii* or nitrogenase from *R. rubrum* (Hyman and Ensign, unpublished results). In contrast, the two other purified enzymes that have been shown to be inhibited by COS are both CO_2-utilizing enzymes. Ribulose bisphosphate carboxylase exhibits a competitive inhibition by COS versus CO_2 (Laing & Christeller, 1980), and COS has recently been shown to be an alternate substrate but not an activator of the enzyme (Lorimer & Pierce, 1989). For carbonic anhydrase, COS is a substrate and competitive inhibitor versus CO_2 (Chengelis & Neal, 1979). The present study showing inhibition of CO dehydrogenase by COS is, therefore, a third example of an interaction of COS with a CO_2-utilizing/generating enzyme.

To compare the effects of CO, COS, and CO_2, the ability of these compounds to reverse cyanide inhibition of CO dehydrogenase was investigated. Previously, we have shown that after the removal of unbound, excess cyanide followed by a 50-fold dilution of cyanide-treated enzyme, cyanide inhibition of CO dehydrogenase is fully reversible (<90 min) in the presence of CO but is only slowly reversed (<10% in 90 min) in the presence of N_2 (Ensign et al., 1989b). The extensive dilution of the cyanide-treated CO dehydrogenase precludes the possibility that the low level of recovery of activity under N_2 is due to, and ultimately limited by, the reestablishment of an equilibrium between enzyme and dissociated cyanide. Therefore, it appears that CO can accelerate the dissociation of cyanide from CO dehydrogenase and that CO can still bind to cyanide-treated CO dehydrogenase, although the catalytic, active site of the enzyme is apparently occupied by cyanide. Similar rate-modifying effects have recently been described for the reversal of slow-binding inhibition of pig liver esterase by substrates and analogues and have been interpreted in terms of two substrate-binding sites on the monomeric enzyme (Allen & Abeles, 1989). Perhaps CO dehydrogenase has two nonequivalent binding sites for CO. Accordingly, the catalytic site cannot bind CO when cyanide is bound (and vice versa) but a second "modulator" site on the cyanide-treated enzyme can bind CO and in so doing alters the binding of cyanide. The modulating effect could be the result of CO binding in close proximity to the catalytic CO-binding (and cyanide-binding) site, resulting in a displacement reaction. Alternatively, this could reflect an allosteric interaction at a distant site. The presence of two CO-binding sites on CO dehydrogenase could also account for the deviations from classical competitive inhibition for COS versus CO that we described in Figure 1.

From the arguments presented above, the ability of a compound to reverse cyanide inhibition can be reasonably equated with the ability of that compound to interact at the putative modulating site of cyanide-treated CO dehydrogenase. As shown in Table I, in addition to CO, CO_2, COS, CS_2, and SO_2 were all capable of reversing the effects of cyanide inhibition to varying degrees. Thus, the putative modulator site will accommodate a number of potential CO and CO_2 analogues. However, not all CO_2 analogues are effective. Thiocyanate (SCN^-), an isoelectronic CO_2 analogue, was not capable of promoting a recovery of activity under the conditions tested.

Table I: Effects of CO/CO_2 Analogues as Inhibitors of CO Oxidation and as Effectors of the Reversal of Cyanide Inhibition of CO Dehydrogenase[a]

		reversal of cyanide inhibition		
	% inhibition of CO-oxidizing activity	concn[b] (mM, in soln)	% activity recovered at t	
			45 min	90 min
N_2	ND[c]	0.56	4	6
CO	ND[c]	0.88	64	87
COS	>99	20.3	38	67
CO_2	~5	32.0	60	73
SO_2	18	20.0	33	47
CS_2	17	28.9	37	30
SCN^-	12	20.0	4	6

[a] The effects of each compound as an inhibitor of CO-oxidizing activity were determined by including each compound in a methylviologen reduction assay cuvette (100 mM MOPS, pH 7.5, with 10 mM methylviologen and 1 mM EDTA) at a concentration of 10 mM. COS was added as a saturated solution in buffer (20.3 mM); CO_2 was added as $NaHCO_3$ (pK_a = 6.3); SO_2 was added as $Na_2S_2O_5$ (pK_a = 7.25); CS_2 was added as a solution in DMSO (10 μL) (10 μL of DMSO in the absence of CS_2 had no effect on CO-oxidizing activity); SCN^- was added as NaSCN. The effects of each compound on the reversal of cyanide inhibition were determined by following the recovery of CO-oxidizing activity of cyanide-treated CO dehydrogenase. CO dehydrogenase was inhibited by cyanide by incubating a stock solution of enzyme with 3 mM KCN for 15 min in an anaerobic serum stoppered incubation vial. After this, the CO-oxidizing activity was inhibited by over 99%. The excess cyanide was removed from the incubation by three cycles of evacuation and reequilibration with O_2-free N_2. Aliquots (5 μL) of the cyanide-treated enzyme were then transferred to 9-mL anaerobic serum vials (0.019 mg of CO dehydrogenase/mL) that contained 1 mL of buffer (100 mM MOPS, pH 7.5) and the additions to the indicated concentrations. N_2, CO, COS, CS_2, and CO_2 were added as gases to a final pressure of 101 kPa. SO_2 and SCN^- were added to a total concentration of 20 mM as $Na_2S_2O_5$ and NaSCN, respectively. At the designated times, aliquots of each incubation were removed and assayed for CO-oxidizing activity. CO dehydrogenase treated similarly, but without KCN, retained 97% of its initial activity. [b] Concentration refers to the total concentration of all species present. [c] ND, not determined.

Converse to the argument above, the ability of a compound to inhibit CO oxidation can reasonably be equated with an interaction at the catalytic site of the enzyme. Under these criteria, the catalytic site appears much more selective than the putative modulator site. The data in Table I show that apart from COS none of the compounds tested were particularly effective as inhibitors of CO oxidation. This includes the final reaction product of CO oxidation, CO_2. This result makes it clear that COS cannot simply be considered as a structural analogue of CO_2. Apparently, the properties of COS are such that it can serve as an analogue of both CO and CO_2.

In the light of the results of the present study on the mode of action of COS on *R. rubrum* CO dehydrogenase, it now becomes of interest to consider the effects of COS on the multimeric CO dehydrogenases of acetogens and methanogens. Unlike *R. rubrum* CO dehydrogenase, these enzymes can operate reversibly in vivo, and both oxidize CO and reduce CO_2. Given the different reactivities of these two classes of CO dehydrogenase with CO_2, a different form of interaction with COS may also occur, especially since COS and CO_2 are so closely chemically related. For example, Ragsdale et al. (1983b) have previously reported that CO_2 can protect against and fully reverse cyanide inhibition of CO dehydrogenase from *Acetobacterium woodii*, and, compatible with a reversible reaction, CO_2 is capable of oxidizing the reduced form of this enzyme. In contrast, we have found that CO_2 could reverse cyanide inhibition of *R. rubrum* CO dehydrogenase as could CO or COS, but CO_2 did not oxidize reduced CO dehydrogenase [after incubation for 1 h at 23 °C (pH 7.5) in

the presence of 0.1 M $NaHCO_3$] or effectively protect against cyanide inhibition.

A further interesting possibility is the use of COS in spectroscopic studies of the *R. rubrum* CO dehydrogenase. EPR signals attributable to nickel can only be observed when the enzyme is in an oxidized state (Ensign et al., unpublished results). Because CO immediately reduces CO dehydrogenase (Figure 3), it has not been possible to demonstrate a direct interaction between CO and nickel. COS, which binds to CO dehydrogenase but does not lead to reduction of Fe-S clusters (Figure 3), may therefore allow for the detection of a nickel–inhibitor interaction. This is particularly attractive since COS is available in numerous isotopic compositions that could aid in the characterization of the direct ligating element.

Summary. The kinetic evidence we have presented indicates that COS is a rapid-equilibrium inhibitor of CO dehydrogenase from *R. rubrum*. COS inhibition is largely competitive with respect to CO and uncompetitive with respect to electron-acceptor concentration. Like the natural substrate CO, COS can protect CO dehydrogenase from inhibition by cyanide. These effects of COS are not achieved through the action of COS as a substrate for CO dehydrogenase. The ability of COS and several closely related CO_2 analogues to reverse cyanide inhibition of CO dehydrogenase is demonstrated.

References

Allen, K. N., & Abeles, R. H. (1989) *Biochemistry 28*, 135–140.

Bonam, D., & Ludden, P. W. (1987) *J. Biol. Chem. 262*, 2980–2987.

Bonam, D., Murrell, S. A., & Ludden, P. W. (1984) *J. Bacteriol. 159*, 693–699.

Bonam, D., McKenna, M. C., Stephens, P. J., & Ludden, P. W. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 31–35.

Chengelis, C. P., & Neal, R. A. (1979) *Biochem. Biophys. Res. Commun. 90*, 993–999.

Cleland, W. W. (1986) in *Investigations of Rates and Mechanisms of Reactions* (Bernascom, C., Ed.) Vol. 6, pp 791–870, Wiley, New York.

Colby, J., Stirling, D., & Dalton, H. (1977) *Biochem. J. 165*, 395–402.

Dean, J. A., Ed. (1985) *Lange's Handbook of Chemistry*, 13th ed., pp 10/3–10/5, McGraw-Hill, New York.

Diekert, G. B., & Thauer, R. K. (1978) *J. Bacteriol. 136*, 597–606.

Diekert, G. B., Fuchs, G., & Thauer, R. K. (1985) in *Microbial Gas Metabolism: Mechanistic, Metabolic and Biotechnological Aspects* (Poole, R. K., & Dow, C. S., Eds.) pp 115–130, Academic Press, London.

Drake, H. L., Hu, S.-I., & Wood, H. G. (1980) *J. Biol. Chem. 255*, 7174–7180.

Eady, R. R., Robson, R. L., Richardson, T. H., Miller, R. W., & Hawkins, M. (1987) *Biochem. J. 244*, 197–207.

Ensign, S. A., Bonam, D., & Ludden, P. W. (1989a) *Biochemistry 28*, 4968–4973.

Ensign, S. A., Hyman, M. R., & Ludden, P. W. (1989b) *Biochemistry 28*, 4973–4979.

Ferm, R. J. (1957) *Chem. Rev. 57*, 621–640.

Grahame, D. A., & Stadtman, T. C. (1987) *J. Biol. Chem. 262*, 3706–3712.

Krzycki, J. A., & Zeikus, J. G. (1984) *J. Bacteriol. 158*, 231–237.

Laing, W. A., & Christeller, J. T. (1980) *Arch. Biochem. Biophys. 202*, 592–600.

Lorimer, G. H., & Pierce, J. (1989) *J. Biol. Chem. 264*, 2764–2772.

Meyer, O. (1985) in *Microbial Gas Metabolism: Mechanistic, Metabolic and Biotechnological Aspects* (Poole, R. K., & Dow, C. S., Eds.) pp 131–152, Academic Press, London.

Morrison, J. F., & Walsh, C. T. (1988) *Adv. Enzymol. Relat. Areas Mol. Biol. 61*, 201–301.

Pezacka, E., & Wood, H. G. (1988) *J. Biol. Chem. 263*, 16000–16006.

Ragsdale, S. W., Ljungdahl, L. G., & DerVartanian, D. V. (1983a) *Biochem. Biophys. Res. Commun. 115*, 658–665.

Ragsdale, S. W., Ljundahl, L. G., & DerVartanian, D. V. (1983b) *J. Bacteriol. 155*, 1224–1237.

Raybuck, S. A., Bastian, N. R., Orme-Johnson, W. H., & Walsh, C. T. (1988) *Biochemistry 27*, 7698–7702.

Tsang, D. C. Y., & Suzuki, I. (1982) *Can. J. Biochem. 60*, 1018–1024.

Winkler, L. W. (1906) *Z. Phys. Chem. 55*, 344–354.