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., Hyman, M. R., & Ludden, P. W. (1989) *Biochemistry* (following paper in this issue).
Hausinger, R. P. (1987) *Microbiol. Rev. 51*, 22–42.
Krzycki, J. A., & Zeikus, J. G. (1984) *J. Bacteriol. 158*, 231–237.
Peterson, G. L. (1977) *Anal. Biochem. 83*, 346–356.
Pezacka, E., & Wood, H. G. (1988) *J. Biol. Chem. 263*, 16000–16006.
Ragsdale, S. W., & Wood, H. G. (1985) *J. Biol. Chem. 260*, 3970–3977.
Ragsdale, S. W., Ljungdahl, L. G., & DerVartanian, D. V. (1982) *Biochem. Biophys. Res. Commun. 108*, 658–663.
Ragsdale, S. W., Clark, J. E., Ljungdahl, L. G., Lundie, L. L., & Drake, H. L. (1983a) *J. Biol. Chem. 258*, 2364–2369.
Ragsdale, S. W., Ljungdahl, L. G., & DerVartanian, D. V. (1983b) *Biochem. Biophys. Res. Commun. 115*, 658–665.
Ragsdale, S. W., Ljungdahl, L. G., & DerVartanian, D. V. (1983c) *J. Bacteriol. 155*, 1224–1237.
Ragsdale, S. W., Wood, H. G., & Antholine, W. E. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6811–6814.
Raybuck, S. A., Bastian, N. R., Orme-Johnson, W. H., & Walsh, C. T. (1988) *Biochemistry 27*, 7698–7702.
Shanmugasundarum, T., Kumar, G. K., & Wood, H. G. (1988) *Biochemistry 27*, 6499–6503.
Terlesky, K. C., Nelson, M. J. K., & Ferry, J. G. (1986) *J. Bacteriol. 168*, 1053–1058.
Terlesky, K. C., Barber, M. J., Aceti, D. J., & Ferry, J. G. (1987) *J. Biol. Chem. 262*, 15392–15395.
Walsh, C. T., & Orme-Johnson, W. H. (1987) *Biochemistry 26*, 4901–4906.
Wood, H. G., Ragsdale, S. W., & Pezacka, E. (1986) *Biochem. Int. 12*, 421–440.

Nickel-Specific, Slow-Binding Inhibition of Carbon Monoxide Dehydrogenase from *Rhodospirillum rubrum* by Cyanide†

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ABSTRACT: The inhibition of purified carbon monoxide dehydrogenase from *Rhodospirillum rubrum* by cyanide was investigated in both the presence and absence of CO and electron acceptor. The inhibition was a time-dependent process exhibiting pseudo-first-order kinetics under both sets of conditions. The true second-order rate constants for inhibition were 72.2 M^{-1} s^{-1} with both substrates present and 48.9 and 79.5 M^{-1} s^{-1}, respectively, for the reduced and oxidized enzymes incubated with cyanide. CO partially protected the enzyme against inhibition after 25-min incubation with 100 μM KCN. Dissociation constants of 8.46 μM (KCN) and 4.70 μM (CO) were calculated for the binding of cyanide and CO to the enzyme. Cyanide inhibition was fully reversible under an atmosphere of CO after removal of unbound cyanide. N_2 was unable to reverse the inhibition. The competence of nickel-deficient (apo) CO dehydrogenase to undergo activation by $NiCl_2$ was unaffected by prior incubation with cyanide. Cyanide inhibition of holo-CO dehydrogenase was not reversed by addition of $NiCl_2$. $^{14}CN^-$ remained associated with holoenzyme but not with apoenzyme through gel filtration chromatography. These findings suggest that cyanide is a slow-binding, active-site-directed, nickel-specific, reversible inhibitor of CO dehydrogenase. We propose that cyanide inhibits CO dehydrogenase by being an analogue of CO and by binding through enzyme-bound nickel.

Carbon monoxide dehydrogenase [carbon monoxide:(acceptor) oxidoreductase; EC 1.2.99.2] from the photosynthetic bacterium *Rhodospirillum rubrum* catalyzes the oxidation of CO to CO_2 in the presence of suitable electron acceptors (Bonam et al., 1984; Bonam & Ludden, 1987). CO dehydrogenase from *R. rubrum* is similar to the CO dehydrogenases purified from methanogenic and acetogenic bacteria in that it is an iron–sulfur- and nickel-containing, oxygen-labile protein (Bonam & Ludden, 1987). However, there are several significant differences between the enzyme from *R. rubrum* and the other CO dehydrogenases studied to date. The *R. rubrum* enzyme is a monomer of 61 800 molecular weight which apparently functions solely to oxidize CO to CO_2. In contrast, the acetogenic and methanogenic CO dehydrogenases are much larger (200 000–400 000 molecular weight) multimeric enzymes which, in addition to oxidizing CO to CO_2, function physiologically and in vitro to catalyze the biosynthesis and/or degradation of acetate and acetyl-CoA (Ragsdale & Wood, 1985; Wood et al., 1986; Terlesky et al., 1986, 1987). A further distinction of particular significance is that CO dehydrogenase from *R. rubrum* may be purified as an inactive, nickel-deficient protein from cells exposed to CO and grown in nickel-deficient media. This nickel-deficient enzyme, or "apo-CO dehydrogenase", contains less than 0.1 mol of Ni/mol of protein and is activated upon addition of $NiCl_2$ (Bonam et al., 1988). Purified apo-CO dehydrogenase differs from holo-CO dehydrogenase only in the nearly com-

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plete absence of nickel; the iron contents of the two enzymes are identical. No other nickel-deficient CO dehydrogenases have been reported (Hausinger, 1987; Walsh & Orme-Johnson, 1987). The monomeric nature of CO dehydrogenase from *R. rubrum*, its high catalytic efficiency, and the existence of a nickel-deficient form of the enzyme make it an ideal model enzyme for studying properties of the nickel- and iron–sulfur-containing CO dehydrogenases in general.

Cyanide has been found to be a potent inhibitor of all CO dehydrogenases studied to date. Cyanide has been shown to inhibit CO dehydrogenase activity in a time-dependent fashion when incubated with cell extracts of the anaerobic, fermentative bacterium *Clostridium pasteurianum* (Thauer et al., 1974), acetogenic bacteria (Diekert & Thauer, 1978), and methanogenic bacteria (Daniels et al., 1977; Krzycki & Zeikus, 1984). A similar inhibition is observed with purified enzymes from the acetogens *Acetobacterium woodii* (Ragsdale et al., 1983a,b) and *Clostridium thermoaceticum* (Drake et al., 1980) and the methanogens *Methanosarcina thermophila* (Terlesky et al., 1986) and *Methanosarcina barkeri* (Grahame & Stadtman, 1987). Incubation of enzyme and cyanide in the presence of CO generally decreases the extent and rate of inhibition (Thauer et al., 1974; Diekert & Thauer, 1978; Ragsdale et al., 1983b; Grahame & Stadtman, 1987). Cyanide-inactivated CO dehydrogenase can generally be reactivated by addition of CO (Thauer et al., 1974; Diekert & Thauer, 1978; Ragsdale et al., 1983a,b; Drake et al., 1980; Terlesky et al., 1986). These results suggest that cyanide is competitive with CO, binding at or near the CO binding site on the enzyme (Terlesky et al., 1986). Despite the widespread observations of cyanide inhibition, a detailed kinetic analysis has not been previously presented.

Cyanide is of interest as a potential CO analogue and inhibitor of CO dehydrogenase due to its isoelectronic structure with CO and similar metal-ligating properties. Nickel has been proposed as the CO binding site of CO dehydrogenase (Ragsdale et al., 1982, 1983c; Walsh & Orme-Johnson, 1987). The existence of an apo form of CO dehydrogenase from *R. rubrum* allows the study of the nickel specificity of cyanide inhibition. In this study, a kinetic analysis of cyanide as an inhibitor of the nickel-containing (holo) CO dehydrogenase from *R. rubrum* is presented, and the nickel dependency of cyanide inhibition is established by comparing the effects of cyanide on holo- vs apo-CO dehydrogenase.

Materials and Methods

Materials

CO (99.99+%) and N_2 (99.998+%) were purchased from Matheson (Chicago, IL). Gases were stripped of trace O_2 by passage over a heated copper-based catalyst (R3-11, Chemical Dynamics Corp., South Plainsfield, NJ). $K^{14}CN$ was obtained from Amersham (Arlington Heights, IL). Ultrapure $NiCl_2$, $Fe(NO_3)_3$, $CaCl_2$, and $MgSO_4$ were obtained from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were of analytical grade.

Methods

Cell Growth and Protein Purification. R. rubrum (ATCC 11170) cells for holo-CO dehydrogenase purification were grown under N_2 as described (Bonam & Ludden, 1987). Fifty micromolar $NiCl_2$ was added to the cell culture at an optical density of 3.5, 3 h prior to inducing CO dehydrogenase by sparging with 100% CO for 30 min. The cell culture was left under CO and intermittently sparged over an additional 36 h prior to harvesting. Cells for apo-CO dehydrogenase purification were grown under N_2 in a 50-L polypropylene carboy illuminated by two overhead projector bulbs in a water-jacketed tube submerged in the culture and were induced for CO dehydrogenase as described above. The growth medium was depleted of trace metals by passage over a 3 × 100 cm column of Bio-Rad Chelex 100 chelexing resin. Ultrapure metal salts were added to complete the medium. Holo-CO dehydrogenase was purified as described (Bonam & Ludden, 1987). Apo-CO dehydrogenase was purified as described (Bonam et al., 1988) under stringent metal-free conditions. Purified holo-CO dehydrogenase had a specific activity of 5200 units/mg (1 unit = 1 μmol of CO oxidized/min) when assayed with methylviologen as electron acceptor (see below). Purified apo-CO dehydrogenase had a specific activity of 57.9 units/mg, which was activated 35-fold to 2054 units/mg upon addition of 10 mM $NiCl_2$. Protein was determined by the method of Peterson (1977) using Sigma Grade A bovine serum albumin dried over P_2O_5 as standard.

Assay of CO Dehydrogenase Activity. CO dehydrogenase activity was determined according to the CO-dependent methylviologen ($\epsilon_{578} = 9.7\ mM^{-1}\ cm^{-1}$) reduction assay at 25 °C as previously described (Bonam et al., 1984) with the following modifications. Assays were performed at pH 7.5 in 100 mM MOPS buffer containing 10 mM methylviologen and 1 mM EDTA. Potassium cyanide was added to assay cuvettes prior to addition of enzyme for inhibition studies performed under catalytic (turnover) conditions. Spectrophotometric assays were performed on a Shimadzu UV-160 UV–visible recording spectrophotometer.

Protein Oxidation. Dithionite-reduced CO dehydrogenase was oxidized by addition of the oxidized redox dye indigo carmine (indigodisulfonate, Sigma Chemical Co.). The oxidized protein was then stripped of dye by anaerobic Sephadex G-25 gel filtration. Due to the extreme oxygen lability of oxidized enzyme, all manipulations were performed in an anaerobic box containing less than 1 ppm oxygen (Vacuum/Atmospheres Dri-Lab glovebox Model HE-493). The oxygen concentration in the box was monitored continuously by a Teledyne oxygen meter. All column and dilution buffers were preevacuated/flushed with N_2 and allowed to equilibrate in the anaerobic box for several days.

Incubation Procedures. Incubations of enzyme with cyanide were carried out under anaerobic conditions in stoppered glass serum vials at 25 °C. The vials were made anaerobic by repeated evacuation and flushing with N_2. Degassed buffer (100 mM MOPS, pH 7.5) contained 1 mM sodium dithionite for experiments involving reduced CO dehydrogenase. An appropriate volume of anaerobic KCN stock in 0.1 M NaOH was added to the buffer. Reaction of enzyme with inhibitor was initiated by addition of enzyme to the vial. For experiments involving oxidized enzyme, microcentrifuge tubes containing dithionite-free buffer inside glass serum vials were set up in the anaerobic box. One milliliter of 0.1 M sodium dithionite was added to the vial outside the microcentrifuge tube to trap traces of oxygen.

Nickel Activation of Apo-CO Dehydrogenase. Activation of apo-CO dehydrogenase by $NiCl_2$ was carried out as previously described (Bonam et al., 1988), except that activations were performed in the presence of 0.05 mM dithionite and under an atmosphere of 100% N_2 in place of CO.

Metal Analyses. Metal analyses were performed on an Applied Research Laboratories 34000 inductively coupled plasma atomic emission spectrophotometer at the University of Wisconsin Soil & Plant Analysis Laboratory.

Carbon Monoxide Determination. The solubility of CO in water under 100% CO was determined to be 0.88 mM at 25

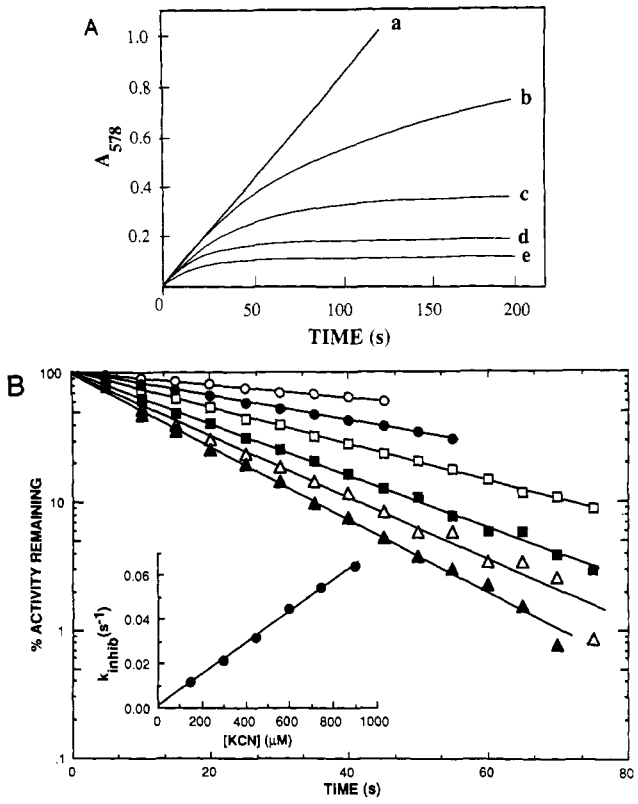

FIGURE 1: (A) Slow-binding inhibition of CO dehydrogenase by cyanide at pH 7.5. Potassium cyanide was added to anaerobic cuvettes containing methylviologen and saturating CO in 100 mM MOPS buffer, pH 7.5. Reaction was initiated by addition of 2 μL of 0.01 mg/mL enzyme solution. Activity was followed spectrophotometrically over a several-minute time course. The levels of potassium cyanide present were (a) 0, (b) 150, (c) 300, (d) 500, and (e) 700 μM. (B) Effect of cyanide concentration on the inhibition of CO dehydrogenase under turnover conditions. Reactions were initiated as described for (A). Activity was determined at 5-s intervals over a 0–80-s time course. The inhibitor concentrations in the cuvettes were (○) 150, (●) 300, (□) 450, (■) 600, (△) 750, and (▲) 900 μM potassium cyanide. Apparent first-order rate constants (k_{inhib}) were derived from the data. The inset shows a plot of k_{inhib} vs [KCN].

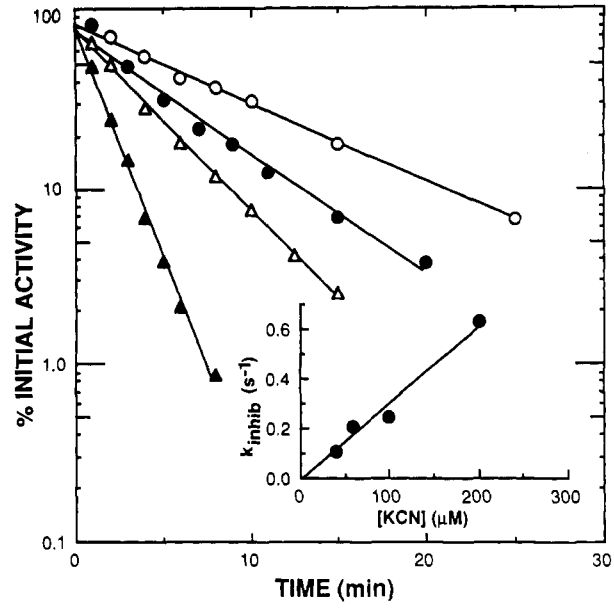

FIGURE 2: Effect of cyanide concentration on the inhibition of dithionite-reduced CO dehydrogenase. Incubation vials were prepared as described under Methods. Incubations were initiated by addition of 2 μL (10.21 mg of protein/mL) of enzyme solution to 1 mL of anaerobic buffer. Samples (2–10 μL) were removed at the indicated times, and their CO oxidizing activity was measured with the methylviologen reduction assay. The inhibitor concentrations in the incubation vials were (○) 40, (●) 60, (△) 100, and (▲) 200 μM potassium cyanide. Apparent first-order rate constants were calculated from the data. The inset shows a plot of k_{inhib} vs [KCN].

°C. CO concentrations in anaerobic solutions were determined according to the hemoglobin binding assay as previously described (Bonam et al., 1984).

Scintillation Spectrometry. Samples were counted in a Packard Minaxi Tri-Carb 4000 scintillation counter (Packard Instrument Co., Downers Grove, IL) in Bio-Safe II scintillation cocktail. Quench correction was made by comparison to a standard curve of counting efficiency vs spectral index of external standard for a set of ^{14}C quenched standards in Bio-Safe II cocktail.

Data Analysis. Kinetic constants were determined by nonlinear least-squares fit to appropriate models (Elsevier Biosoft, Cambridge, England) as described under Results.

RESULTS

Figure 1A shows the effect of various cyanide concentrations on the activity of CO dehydrogenase under turnover conditions (i.e., in the presence of CO and electron acceptor). The resultant inhibition is a time-dependent process with loss of CO oxidizing activity approaching 100% at the higher cyanide levels within the time frame of this experiment. Semilog plots of percent activity vs time for several cyanide levels are shown in Figure 1B. Loss of activity in these plots remains linear over the entire time course, demonstrating that the inhibition is a pseudo-first-order process. Furthermore, each plot extrapolates back to 100% activity at $t = 0$, demonstrating that the initial rate of CO dehydrogenase activity is unaffected by the level of cyanide added. The pseudo-first-order rate constants (k_{inhib}) for the inhibition were derived from fits to the plots in Figure 1B. The interrelation between these rate constants and the concentrations of cyanide present is shown in the inset to Figure 1B. This plot is linear and tends toward the origin. The slope of this plot gives the true second-order forward rate constant of 72.2 ± 1.75 M^{-1} s^{-1} for cyanide inhibition under turnover conditions.

The rate of cyanide inhibition was found to vary as a function of the concentration of methylviologen (the electron acceptor for CO oxidation) present. Lowering the concentration of methylviologen over a range of values from 10 mM (normal assay condition) down to 0.5 mM at a constant level of cyanide resulted in a linear decrease in the rate of inhibition. The half-lives for the rate of inhibition by 500 μM KCN at methylviologen levels of 1, 2, 3, 5, 7.5, and 10 mM were 35.1, 29.6, 26.2, 24.4, 22.6, and 17.2 s, respectively.

Incubation of CO dehydrogenase with cyanide under nonturnover conditions (in the absence of CO and electron acceptor) results in a time-dependent inhibition analogous to that seen under turnover conditions. Figure 2 shows a semilog plot of percent activity remaining vs time for dithionite-reduced enzyme incubated with various cyanide levels. Because of a trend toward a constant level of activity as equilibrium is approached, the percent activity remaining has been modified by compensating for the residual activity level reached upon attainment of equilibrium according to

$$\% \text{ compensated activity} = (v - v_s)/(v_0 - v_s) \quad (1)$$

where v is the velocity of the enzyme at time t, v_s is the steady-state (equilibrium) velocity, and v_0 is the initial velocity (velocity in the absence of inhibitor). The inhibition thus plotted follows pseudo-first-order kinetics, and the plot of k_{inhib} vs cyanide concentration is linear, tending toward the origin (see inset, Figure 2). The calculated rate constant for cyanide

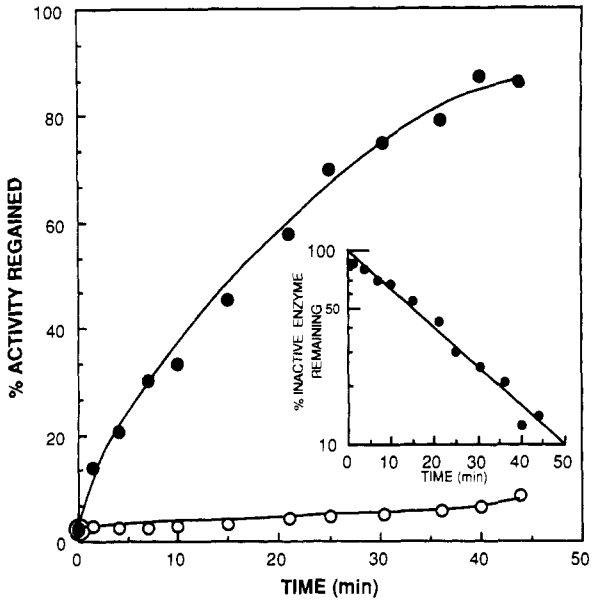

FIGURE 3: Reversibility of cyanide inhibition of CO dehydrogenase. 20 μL of a 0.1 M stock of KCN was added to 650 μL of a solution (2.36 mg of protein/mL) of holo-CO dehydrogenase to give a final KCN concentration of 3 mM. After 25-min incubation a sample was withdrawn and CO oxidizing activity measured spectrophotometrically. Cyanide-inhibited protein was then subjected to anaerobic gel filtration chromatography on a 0.5 × 25 cm column of Sephadex G-25 to separate protein and unbound cyanide. Desalted protein was assayed for CO oxidizing activity. 20 μL of desalted protein was then diluted into 980 μL of anaerobic buffer in incubation vials under 101-kPa N_2 or 101-kPa CO. Samples (1–6 μL) were withdrawn at the indicated times over a 45-min time course, and their CO oxidizing activity was measured. (●) Incubation under 101-kPa CO; (○) incubation under 101-kPa N_2. The inset shows a semilog plot of percent inactive enzyme remaining vs time of incubation for the incubation under CO. A rate constant for the reactivation of cyanide-inhibited enzyme ($k_{reverse}$) was derived from the equation of this line.

inhibition under these conditions is 48.9 ± 7.14 M^{-1} s^{-1}. When oxidized CO dehydrogenase is incubated with cyanide under conditions identical with those present for the reduced protein, a similar inhibition pattern is seen. However, the rate of inhibition is significantly higher for the oxidized enzyme, with a calculated rate constant of 79.5 ± 4.91 M^{-1} s^{-1}.

The reversibility of cyanide inhibition was investigated with cyanide-inhibited, reduced CO dehydrogenase. After removal of unbound cyanide by gel filtration chromatography, the enzyme remained inactive (97.5% inactive after gel filtration vs 99.5% before). Ninety percent of pre-cyanide activity was recovered over 45 min when the cyanide-inhibited enzyme was incubated under an atmosphere of CO (see Figure 3). Incubation under an atmosphere of N_2 over the same time course led to no significant increase in enzyme activity.

A semilog plot of percent inactive enzyme remaining vs time of incubation under CO is shown in the inset to Figure 3. The reactivation is shown to follow first-order kinetics, allowing a direct determination of the rate constant for reactivation under CO. This rate constant was calculated to be 7.70 × 10^{-4} s^{-1}.

We further investigated the possible competitive nature of CO and cyanide binding by studying the effect of various CO levels on the rate of enzyme inhibition at a fixed cyanide level. Figure 4 shows the level of protection against inhibition which CO affords after a 25-min incubation in the presence of 100 μM KCN. The protection response is hyperbolic in nature, being most dramatic at lower CO levels. An apparent dissociation constant (K_D) for CO of 60.3 μM was calculated by fitting the data to a hyperbola.

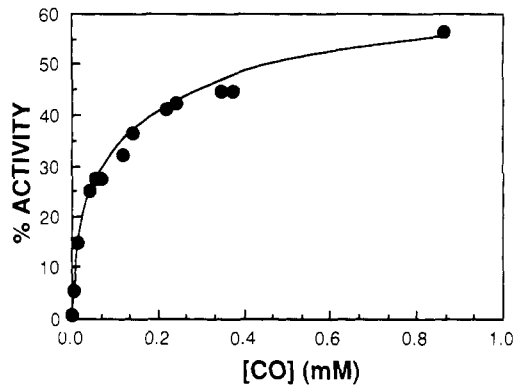

FIGURE 4: Protection of CO dehydrogenase against cyanide inhibition by CO. Incubation vials (9 mL) were set up containing ~2 mL of anaerobic buffer under N_2 as described under Methods. 0–12 mL of CO was added as overpressure via gas-tight syringes to the vials, the gases were allowed to mix, and the vials were then vented to atmospheric pressure. An additional vial was sparged vigorously with CO to give a saturated solution. After 10-min shaking to equilibrate CO in the liquid and gas phase, 3–10 μL of buffer was withdrawn and the concentration of dissolved CO measured as described under Methods. The CO concentration in the buffer was measured at several additional time points to ensure that a constant dissolved CO concentration was present throughout the course of the incubation. Potassium cyanide was added to each vial to give a final concentration of 100 μM. Incubations were initiated by the addition of 3 μL (10.21 mg of protein/mL) of an enzyme solution to give a total volume of 2 mL. Samples were withdrawn after 25-min incubation, and their CO oxidizing activity was measured.

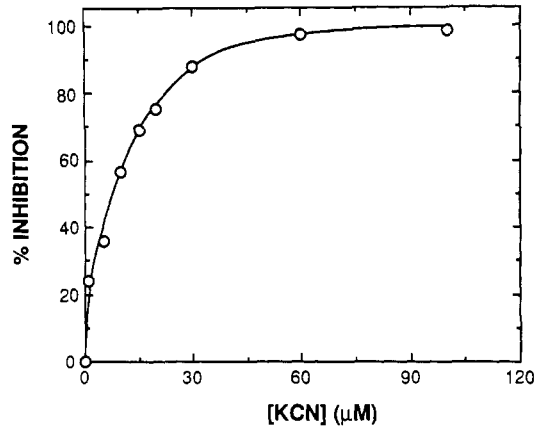

FIGURE 5: Extent of CO dehydrogenase inhibition as a function of cyanide concentration. Incubation vials were prepared as described under Methods, containing 2 mL of MOPS buffer, pH 7.5, and 0–100 μM KCN. Incubations were initiated by the addition of 3 μL (10.21 mg of protein/mL) of an enzyme solution. Samples were withdrawn after 25-min incubation, and their CO oxidizing activity was measured spectrophotometrically.

The K_D for cyanide binding to CO dehydrogenase was calculated in an analogous way, by incubating enzyme for a fixed time (25 min) in the presence of various cyanide levels. The resultant plot of percent inhibition vs cyanide concentration was also found to be hyperbolic (Figure 5). Fitting these data to a hyperbola gave a K_D for cyanide of 8.46 μM.

The apparent K_D of the enzyme for CO was measured in the presence of cyanide; compensation must be made for the level of cyanide present and the K_D of the enzyme for cyanide in calculating the true K_D for CO according to

$$K_{D,apparent} = K_{D,true}(1 + [KCN]/K_{D,KCN}) \quad (2)$$

This compensation gives a K_D for CO of 4.70 μM. By way of comparison, the K_m for CO at pH 7.5 was determined to be 32 μM.

The protection that CO affords against cyanide inhibition and the requirement of CO for reactivation of cyanide-in-

Table I: Inhibition of Apo- and Holo-CO Dehydrogenase by Cyanide[a]

	apo-CODH	holo-CODH
pre-KCN treatment		
activity (control)	57.9	4165
activity after Ni activation	2054	4170
activity increase due to Ni	1996	5
x-fold nickel activation	35.1	1.0
post-KCN treatment		
activity (% control)	0.463 (0.80)	22.91 (0.55)
activity after gel filtn (% control)	3.92 (6.77)	104.7 (2.51)
activity after Ni activation	2033	121.9
activity increase due to Ni	2029	17.2
x-fold nickel activation	519	1.16

[a] Incubation vials were prepared containing ~3 mg of apo- or holo-CO dehydrogenase and 3 mM KCN in MOPS buffer containing 1 mM sodium dithionite. After 25-min incubation, samples were withdrawn, and activity was measured spectrophotometrically as described under Methods. Cyanide-treated proteins were then subjected to anaerobic gel filtration chromatography on a 0.5 × 25 cm column of Sephadex G-25 to separate protein and unbound cyanide. CO oxidizing activity measurements, protein determinations, and activation of holo- and apoenzymes by $NiCl_2$ were performed as described under Methods. Activity is reported as specific activity [μmol of CO oxidized min^{-1} (mg of protein)$^{-1}$].

Table II: Metal Content of Apo- and Holo-CO Dehydrogenase and Results of $K^{14}CN$ Labeling

protein sample	metal content[a]	activity (units/mg) pre-KCN	activity (units/mg) post-KCN	mol of $^{14}CN^-$ bound/mol of protein[b]
holo-CODH	7.0 Fe	4234	19.2	
	1.6 Ni			0.52
	0.3 Zn			
apo-CODH	7.0 Fe	87.0	1.0	
	0.01 Ni			0.021
	0.2 Zn			
lysozyme				0.0076
bovine serum albumin				0.088

[a] Reported as moles of metal per mole of protein. Metal analyses and protein determinations were performed as described under Methods. [b] Incubation vials containing ~50 μmol of protein in 0.5 mL of buffer were prepared as described under Methods. 5 mM $K^{14}CN$ (2200 dpm/nmol of CN^-) was added to each vial. After 25-min incubation, CO oxidizing activity of apo- and holo-CO dehydrogenase was measured as described under Methods. Proteins were then separated from unbound cyanide by gel filtration chromatography on a 0.5 × 25 cm column of Sephadex G-25. Proteins were eluted in several fractions and additional fractions collected until all radioactivity had eluted from the column. Protein determinations were performed as described under Methods. $^{14}CN^-$ was quantitated by liquid scintillation spectrometry as described under Methods.

hibited enzyme indicate that binding of CO and cyanide are mutually exclusive and suggest a common binding site. As mentioned earlier, nickel has been proposed as the CO binding site of CO dehydrogenase. The nickel dependency of cyanide inhibition was investigated by comparing the effects of cyanide on holo- and apo-CO dehydrogenase. The results of these experiments are summarized in Table I.

The low basal level of activity present in apo-CO dehydrogenase is thought to be due to a small amount of contaminating holoprotein resulting from trace levels of nickel present in the apoprotein growth medium. After 25-min incubation in the presence of 3 mM KCN, CO oxidizing activity was nearly completely inhibited for both apo- and holo-CO dehydrogenase. Both apo- and holoenzyme remained essentially fully inhibited after removal of excess cyanide by gel filtration chromatography. Both of these inhibited enzymes were assayed for their competence to undergo activation by $NiCl_2$, and the level of activation was compared with the activation seen for the untreated apo- and holoenzymes. As indicated in Table I, the competence of apo-CO dehydrogenase to undergo full activation by addition of $NiCl_2$ is unaffected by the earlier exposure to cyanide. Holo-CO dehydrogenase, in contrast, was not activated by addition of $NiCl_2$. Incubation of either inhibited apoenzyme or inhibited holoenzyme under an atmosphere of CO resulted in full recovery of basal activity levels (data not shown), confirming that the inhibition was reversible for both enzymes.

The data presented above provide evidence that enzyme-bound nickel is the target for cyanide binding and that cyanide remains stably bound to CO dehydrogenase through gel filtration chromatography. Binding of cyanide was further investigated with $K^{14}CN$ as a radiolabel, with separation of unbound cyanide by gel filtration chromatography. The results of these experiments are summarized in Table II. Very little cyanide (<0.01 mol of CN^-/mol of protein) remained associated with lysozyme, a negative control protein, after separation of unbound cyanide. A slightly larger level of cyanide (0.088 mol of CN^-/mol of protein) remained associated with a second control protein, bovine serum albumin. A significantly larger amount of cyanide (0.52 mol of CN^-/mol of protein) remained associated with holo-CO dehydrogenase through gel filtration. In contrast, very little cyanide (0.021 mol of CN^-/mol of protein) remained associated with apo-CO dehydrogenase. The only difference between apo- and holo-CO dehydrogenase is the differing nickel contents of the two proteins (see Table II). This leads us to conclude that cyanide associates with CO dehydrogenase through enzyme-bound nickel.

Discussion

The results of these experiments define cyanide as a slow-binding inhibitor of CO dehydrogenase from *R. rubrum*. Figure 1 demonstrates the time and [KCN] dependence of cyanide inhibition. Figure 1B shows that the inhibition exhibits pseudo-first-order kinetics over the entire incubation period and at all levels of cyanide tested.

Slow-binding and slow, tight-binding inhibitors are characterized by the slow rate at which the equilibrium between enzyme, inhibitor, and enzyme–inhibitor complex is established (Morrison, 1982). The lifetime of the resultant enzyme–inhibitor complex is dominated by a slow dissociation of enzyme and inhibitor (Morrison & Walsh, 1988) in contrast to the rapid equilibria observed between enzyme and classical inhibitors (i.e., competitive and noncompetitive inhibitors).

Slow-binding inhibition can be classified into two types (Morrison & Walsh, 1988). Type I inhibition involves the slow, direct conversion of free enzyme to the final enzyme–inhibitor complex (EI) as illustrated in mechanism 1:

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI$$

Type II inhibition involves the rapid formation of an initial complex which undergoes further slow conversion to yield a final complex as illustrated in mechanism 2:

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EI^*$$

The two mechanisms are readily distinguishable by kinetic measurements (Morrison, 1982). For mechanism 1 the initial velocity of the enzyme is unaffected by the level of inhibitor present, whereas for mechanism 2 the initial velocity varies as a function of the inhibitor concentration. In addition, for mechanism 1 the relationship between the rate of inhibition and the inhibitor concentration is linear, whereas for mecha-

nism 2 the relationship between the rate of inhibition and inhibitor concentration is hyperbolic.

On the basis of the results presented here, cyanide can be unambiguously classified as a type I inhibitor of CO dehydrogenase. As shown in Figure 1B, the semilog plots of percent activity remaining vs time extrapolate back to 100% activity at t_0 for all cyanide levels, satisfying the first of the kinetic considerations outlined above for type I inhibition. The inset to Figure 1B demonstrates the linear relationship between cyanide concentration and the pseudo-first-order rate constants for inhibition (k_{app}), satisfying the second of the kinetic considerations for type I inhibition.

Similar patterns of time-dependent inhibition were observed for enzyme incubated with cyanide under turnover conditions and for oxidized or reduced enzyme under nonturnover conditions. However, there are marked differences in the rates of the resultant inhibition. The oxidized protein was inhibited more rapidly than the dithionite-reduced protein (k_{inhib} of 79.5 for oxidized vs 48.9 M^{-1} s^{-1} for reduced) when incubated with identical cyanide levels. Under turnover conditions at 10 mM methylviologen, the rate constant for inhibition was determined to be 72.2 M^{-1} s^{-1}. As the concentration of methylviologen was decreased, the rate of inhibition decreased linearly, extrapolating to a rate constant of 41.0 M^{-1} s^{-1} at 0 mM methylviologen. The differences in the rate of inhibition under turnover conditions may be due to a higher proportion of oxidized protein being present at the higher methylviologen concentrations, resulting in more rapid inhibition. If nickel is the species binding cyanide on the enzyme, a higher oxidation state of nickel in oxidized CO dehydrogenase may have a higher affinity for cyanide than the lower oxidation state in the reduced enzyme.

Figure 3 demonstrates the reversible nature of cyanide inhibition of CO dehydrogenase. The derived first-order rate constant for the release of cyanide from the enzyme (k_2 in mechanism 1) allows a determination of the inhibition constant K_i, which is the ratio of the rate constants for the interaction of cyanide with the enzyme in the reverse and forward directions. This K_i, when compensated for the level of CO present when k_1 and k_2 are being measured (mechanism 1) and for the K_m of the enzyme for CO as in eq 2, was calculated to be 0.374 μM.

An atmosphere of CO was required for reactivation of cyanide-inhibited enzyme over a time course of 60 min. This provides the first direct physical evidence that binding of cyanide and CO to the protein is competitive. CO may facilitate reactivation of inhibited enzyme in either of two ways (Morrison & Walsh, 1988). CO may simply act to occupy the binding site vacated by cyanide upon dissociation of cyanide from the enzyme. The large excess of CO over CN^- would effectively out-compete the released CN^- for binding to enzyme, resulting in full recovery of activity. Alternatively, CO may actually promote the release of cyanide from the enzyme by beginning to interact at the site of binding before cyanide has fully dissociated.

The protection against cyanide inhibition by CO (Figure 4) provides evidence that binding of cyanide and binding of CO are competitive, mutually exclusive events and provides a convenient method for determining the K_D of the enzyme for CO, yielding a value of 4.70 μM for the dithionite-reduced protein. This value is slightly less than the K_m for CO of 32 μM at pH 7.5.

The K_D for cyanide binding was determined to be 8.46 μM. Comparison of the calculated K_D and K_i (0.374 μM) for cyanide shows an order of magnitude difference; however, direct comparison of these values is not warranted, given the different conditions under which they were calculated. The K_D was determined under reduced, nonturnover conditions, whereas the K_i was calculated as the ratio of the rate constant for inhibition (under turnover conditions) and the rate constant for reactivation (CO dependent).

Nickel-deficient, apo-CO dehydrogenase was used to determine the nickel specificity and mechanism of cyanide inhibition of CO dehydrogenase. Cyanide is known to form complexes with transition metals (Collman et al, 1987), and CO dehydrogenase from *R. rubrum* contains both iron and nickel. The results summarized in Table I show that cyanide inhibition requires the presence of nickel. The cyanide-inhibited enzyme remains inactive after removal of unbound cyanide by gel filtration, and radiolabeled cyanide can be shown to remain associated with the enzyme (Table II). Reversal of the cyanide inhibition can be observed under CO (Figure 3). These results suggest that CO dehydrogenase remains intact upon cyanide treatment and that the loss of activity is due to the presence of cyanide on the enzyme. When apoenzyme is treated with cyanide and excess cyanide removed by gel filtration, the enzyme is still fully competent to undergo activation by $NiCl_2$ (see Table I). Holoenzyme, in contrast, is not activated by $NiCl_2$. These results show that cyanide inhibition is not directed at the iron–sulfur centers of the enzyme, since nickel reactivates apo- but not holo-CO dehydrogenase. Likewise, the results indicate that nickel is the target of cyanide binding. The results with holoenzyme show also that cyanide has not simply chelated nickel from the enzyme and that excess nickel cannot drive cyanide off of the enzyme.

The results of $K^{14}CN$ labeling of apo- and holo-CO dehydrogenase (Table II) provide definitive evidence that cyanide remains associated with CO dehydrogenase through gel filtration and that this association is specific for the holoprotein. The binding of $^{14}CN^-$ is correlated with the presence of nickel in the protein, but the stoichiometry of binding obtained in these experiments is not understood. We are currently applying other techniques to more definitively quantitate the stoichiometry of cyanide binding to holo-CO dehydrogenase.

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References

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.

Collman, J. P., Hegedus, L. S., Norton, J. R., & Finke, R. G. (1987) in *Principles and Applications of Organotransition Metal Chemistry*, University Science Books, Mill Valley, CA.

Daniels, L., Fuchs, G., Thauer, R. K., & Zeikus, J. G. (1977) *J. Bacteriol. 132*, 118–126.

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

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

Grahame, D. A., & Stadtman, T. C. (1987) *J. Biol. Chem. 262*, 3706–3712.
Hausinger, R. P. (1987) *Microbiol. Rev. 51*, 22–42.
Krzycki, J. A., & Zeikus, J. G. (1984) *J. Bacteriol. 158*, 231–237.
Morrison, J. F. (1982) *Trends Biochem. Sci. (Pers. Ed.) 7*, 102–105.
Morrison, J. F., & Walsh, C. T. (1988) *Adv. Enzymol. Relat. Areas Mol. Biol. 61*, 201–301.
Peterson, G. L. (1977) *Anal. Biochem. 83*, 346–356.
Ragsdale, S. W., & Wood, H. G. (1985) *J. Biol. Chem. 260*, 3970–3977.
Ragsdale, S. W., Ljungdahl, L. G., & DerVartanian, D. V. (1982) *Biochem. Biophys. Res. Commun. 108*, 658–663.
Ragsdale, S. W., Clark, J. E., Ljungdahl, L. G., Lundie, L. L., & Drake, H. L. (1983a) *J. Biol. Chem. 258*, 2364–2369.
Ragsdale, S. W., Ljungdahl, L. G., & DerVartanian, D. V. (1983b) *J. Bacteriol. 155*, 1224–1237.
Ragsdale, S. W., Ljungdahl, L. G., & DerVartanian, D. V. (1983c) *Biochem. Biophys. Res. Commun. 115*, 658–665.
Terlesky, K. C., Nelson, M. J. K., & Ferry, J. G. (1986) *J. Bacteriol. 168*, 1053–1058.
Terlesky, K. C., Barber, M. J., Aceti, D. J., & Ferry, J. G. (1987) *J. Biol. Chem. 262*, 15392–15395.
Thauer, R. K., Fuchs, G., Käufer, B., & Schnitker, U. (1974) *Eur. J. Biochem. 45*, 343–349.
Walsh, C. T., & Orme-Johnson, W. H. (1987) *Biochemistry 26*, 4901–4906.
Wood, H. G., Ragsdale, S. W., & Pezacka, E. (1986) *Biochem. Int. 12*, 421–440.

Malate Dehydrogenase from the Extreme Halophilic Archaebacterium *Halobacterium marismortui*. Reconstitution of the Enzyme after Denaturation and Dissociation in Various Denaturants†

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ABSTRACT: Malate dehydrogenase from the extreme halophilic archaebacterium *Halobacterium marismortui* is a homodimer of 84 000 molecular weight. As taken from ultracentrifugal analysis, it does not show concentration-dependent dissociation down to enzyme concentrations as low as 10 ng/mL. There is no change in specific activity at concentrations between 1 and 500 ng/mL. The enzyme undergoes pH-dependent and ionic strength dependent dissociation, denaturation, and deactivation below pH 4.8 ± 0.3 and $I \leq 1.8$ M, respectively. In the range of the salt-dependent equilibrium transition (e.g., at $I \approx 0.8$ M), "structured monomers" are formed in a slow reaction. Reconstitution of this species is multiphasic with fast reassembly of "structured monomers" and subsequent folding/association to form the active dimer. At low salt, as well as at high concentrations of guanidine hydrochloride at high salt, the enzyme is fully unfolded. Therefore, reconstitution comprises both unimolecular folding and subsequent bimolecular association. In the case of the guanidine-dependent unfolding transition, salt effects and chaotropic effects are superimposed such that at low salt concentrations increased guanidine concentration leads to an apparent activation of the enzyme. Deactivation is determined by the total ionic strength: keeping the salt concentration over the whole transition range beyond 4 M, the equilibrium transition occurs at 2.3 M Gdn·HCl (2.4 M NaCl). Reactivation after guanidine denaturation follows the same mechanism as described for the reconstitution after denaturation at low salt concentration. Low urea concentrations (≤2 M), as well as short incubation at low salt, lead to an increase in the yield of reconstitution due to incomplete dissociation under unfolding conditions.

Halobacterium marismortui is an obligate halophile which requires 10–20% NaCl for optimal growth; incubation at low salt concentrations causes lysis (Larsen, 1967; Kushner, 1968; Brown, 1964). As was first reported by Ginzburg et al. (1970), *Halobacterium* from the Dead Sea possesses an intracellular salt concentration of about 4 M KCl and 2 M NaCl. The cellular inventory of the organism is adapted to these conditions. As a consequence, halophilic cytoplasmic proteins have been reported to be denatured below 1 M NaCl (Lanyi, 1974).

Native halophilic malate dehydrogenase (h-MDH)[1] from *Halobacterium marismortui* was isolated first by Mevarech et al. (1976, 1977). To avoid denaturation of the enzyme, high salt conditions were maintained during all stages of purification. As shown by ultracentrifugal analysis and SDS gel electrophoresis, h-MDH is a dimer with a molecular weight of 84 000. Its amino acid composition shows a characteristic excess of acidic over basic amino acids causing anomalous hydration (0.87 g of H_2O/g of protein) and salt binding (0.35 g/g of protein) (Eisenberg et al., 1977; Reich et al., 1982; Zaccai et al., 1986). At low salt concentration (<1 M NaCl), dissociation of the dimer, accompanied by deactivation and denaturation, was observed.

In the present study, the reconstitution behavior of h-MDH from *Halobacterium marismortui* after previous unfolding of the enzyme in various denaturants is described. The effect of low salt concentrations is compared with acid and guanidine denaturation. Under all conditions, denaturation is shown to

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[1] Abbreviations: BSA, bovine serum albumin; CD, circular dichroism; Gdn·HCl, (GdmCl in figures), guanidine hydrochloride; h-MDH and m-MDH, halophilic and mitochondrial malate dehydrogenase, respectively; OA, oxalacetic acid.

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