Nickel Is Required for the Transfer of Electrons from Carbon Monoxide to the Iron–Sulfur Center(s) of Carbon Monoxide Dehydrogenase from *Rhodospirillum rubrum*†

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ABSTRACT: The role of nickel in CO oxidation and electron flow was investigated in carbon monoxide dehydrogenase from *Rhodospirillum rubrum*. The Fe–S centers of oxidized, nickel-containing (holo) CO dehydrogenase were completely reduced within 1 min of exposure to CO. The Fe–S centers of oxidized, nickel-deficient (apo) CO dehydrogenase were not reduced during a 35-min incubation in the presence of CO. Apo-CO dehydrogenase Fe–S centers were reduced by dithionite. The Fe–S centers of cyanide-inhibited, holo-CO dehydrogenase were not reduced in the presence of CO but were reduced by dithionite. Treatment of apo-CO dehydrogenase with cobalt(II), zinc(II), and iron(II) resulted in association of these metal ions (0.70, 1.2, and 0.86 mol of M^{2+}/mol, respectively) with the protein but no increase in specific activity. Purified holo-CO dehydrogenase contained 1.1 mol of nickel/mol of protein and could not be further activated upon addition of $NiCl_2$, suggesting the presence of one catalytic nickel site on the enzyme. The M^{2+}-treated enzymes could not be further activated by addition of $NiCl_2$ as opposed to the untreated apoenzyme, whose activity was stimulated 50–100-fold to the level of holoenzyme upon addition of $NiCl_2$. When placed under CO, the Fe–S centers of the cobalt-treated enzyme became reduced over a 35-min time course, as opposed to the zinc- and iron-treated enzymes, which remained oxidized. We conclude that nickel, or an appropriate nickel analogue in the nickel site, mediates electron flow from CO to the Fe–S centers of CO dehydrogenase.

Carbon monoxide dehydrogenase [carbon monoxide:(acceptor) oxidoreductase; EC 1.2.99.2; CODH[1]] 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). The purified protein is a monomer of 61 800 molecular weight which contains seven to eight iron, one to two nickel, and less than stoichiometric (0.2–0.3) levels of zinc (Bonam & Ludden, 1987; Ensign et al., 1989). CODH from *R. rubrum* may be purified in an inactive, nickel-deficient form (apo-CO dehydrogenase; apo-CODH) from cells exposed to CO and grown on medium depleted of nickel (Bonam et al., 1988). This nickel-deficient enzyme, which is identical with the holoenzyme except for the nearly complete absence (<0.1 mol/mol of protein) of nickel, is rapidly activated upon the addition of $NiCl_2$. CO dehydrogenase from *R. rubrum* is the only nickel-containing enzyme that has been obtained in a nickel-deficient form that can be activated by nickel. The occurrence of nickel in enzymes has recently been reviewed by Hausinger (1987) and Walsh and Orme-Johnson (1987).

CO dehydrogenases have also been purified from acetogenic (Drake et al., 1980; Ragsdale et al., 1983a,c) and methanogenic (Kryzycki & Zeikus, 1984; Terlesky et al., 1986) bacteria. These enzymes are large (200 000–440 000 molecular weight) multimeric nickel- and iron-containing proteins. In addition to oxidizing CO to CO_2, these enzymes also catalyze the reduction of CO_2 to CO and the synthesis and/or degradation of acetyl-CoA (Ragsdale & Wood, 1985; Wood et al., 1986; Terlesky et al., 1987). Accordingly, the enzymes are more properly designated as CO dehydrogenase/acetyl-CoA synthases.

The nickel site of the purified enzyme from the acetogen *Clostridium thermoaceticum* has been extensively characterized by EPR spectroscopy. An EPR-observabable signal arising from a nickel-carbon species is formed in the presence of CO (Ragsdale et al., 1982, 1983b), and this signal has been proposed to arise from a mixed nickel–iron center on the basis of isotopic iron and nickel substitutions (Ragsdale et al., 1985). Accordingly, this center has been proposed as the site of CO binding. EPR studies of the purified enzyme from the methanogen *Methanosarcina thermophila* demonstrate the presence of an analogous center in the presence of CO (Terlesky et al., 1987). Two additional subsites distinct from but in close proximity to the CO binding site have been implicated in the binding of coenzyme A (Shanmugasundaram et al., 1988) and a methyl group (Pezacka & Wood, 1988) to the acetogenic enzyme.

Given the complexity of the acetogenic CODH and the differential labilities of the CO dehydrogenase and acetyl-CoA synthase activities to oxygen sensitivity (Ragsdale & Wood, 1985; Raybuck et al., 1988) and inhibition by arginine-selective reagents, coenzyme A (Ragsdale & Wood, 1985), and cyanide (Diekert et al., 1984), it has been proposed that two distinct sites, possibly each containing nickel, may exist on the enzyme for these two activities (Raybuck et al., 1988). In contrast to the acetogenic enzyme, the only reaction known to be catalyzed by CO dehydrogenase from *R. rubrum* is the oxi-

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[1] Abbreviations: CODH, carbon monoxide dehydrogenase; EPR, electron paramagnetic resonance; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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dation of CO to CO_2 (Bonam & Ludden, 1987). The monomeric nature of the enzyme from *R. rubrum*, the simplicity of the reaction it catalyzes, and the purification of a nickel-deficient form make it an ideal model enzyme for studying the role of nickel in CO oxidation. In this paper we present a UV–visible spectrophotometric study on the flow of electrons from CO to the Fe–S centers of apo- and holo-CO dehydrogenase from *R. rubrum* and present preliminary results on the incorporation of other transition metals, specifically iron, cobalt, and zinc, into the apoenzyme in place of nickel.

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). Ultrapure $NiCl_2$, $CoCl_2$, $Fe(NO_3)_3$, $CaCl_2$, and $MgSO_4$ were obtained from Aldrich Chemical Co. (Milwaukee, WI). Methylviologen and indigo carmine were obtained from Sigma Chemical Co. (St. Louis, MO).

Methods

Protein Purification. Holo- and apo-CO dehydrogenase were purified as described previously (Bonam & Ludden, 1987; Bonam et al., 1988; Ensign et al., 1989). The final step of purification, preparative-scale native gel electrophoresis, yields two bands of CODH activity referred to as peaks 1 and 2 (Bonam & Ludden, 1987). These forms are identical in molecular weight on SDS–PAGE but differ in Fe content and specific activity, peak 1 being more active than peak 2. The majority of CODH purifies as peak 1, and this is the form that has been used for the experiments described in this paper. Purified peak 1 holo-CO dehydrogenase had a specific activity of 4440 units/mg (1 unit = 1 μmol of CO oxidized/min) when assayed at pH 7.5 with methylviologen as electron acceptor according to the spectrophotometric assay described previously (Ensign et al., 1989). Purified peak 1 apo-CO dehydrogenase had a specific activity of 46 units/mg, which was activated 50–100-fold upon treatment with 5 mM $NiCl_2$. Protein concentration was determined by the method of Peterson (1977) using Sigma grade A bovine serum albumin dried over P_2O_5 as standard.

Treatment of Apo-CO Dehydrogenase with Co(II), Zn(II), and Fe(II). Apo-CO dehydrogenase (5–10 mg) was bound to a 0.5 × 1.5 cm column of DE-52 anion-exchange resin (Whatman) equilibrated in 50 mM NaCl in 100 mM MOPS, pH 7.5, containing 1 mM dithionite. For treatment with $CoCl_2$, the protein and column were reequilibrated in anaerobic 50 mM NaCl/MOPS buffer containing 0.05 mM dithionite, as Co(II) was slowly reduced to Co(0) at higher (0.5–2 mM) dithionite levels. Column-bound apoenzyme was treated with 5 mL of anaerobic 5 mM $CoCl_2$ in 100 mM MOPS, pH 7.5, passed through the column at a flow rate of 0.2 mL/min. Excess $CoCl_2$ was then removed by passage of several column volumes of 50 mM NaCl/MOPS (0.05 mM dithionite) over the column, followed by reequilibration in buffer containing 1 mM dithionite. Cobalt-treated apo-CODH was washed with 75 mL of buffer followed by elution in 400 mM NaCl/MOPS buffer. The protein was further separated from exogenous cobalt by passage over a 0.5 × 25 cm column of Sephadex G-25, followed by passage over a 1.5 × 10 cm column of Bio-Rad Chelex-100 resin. The protein was then reconcentrated on a 0.5 × 1.0 cm column of DE-52 prior to oxidation. Apoenzyme was treated with $ZnCl_2$ in an identical manner, except 20 mL of 0.5 mM $ZnCl_2$ in MOPS, pH 7.5, at a flow rate of 1 mL/min was passed over the column. Apoenzyme was treated with 10 mL of a 1 mM Fe(II) solution, which was obtained by degassing and reducing a 1 mM solution of $Fe(NO_3)_3$ in 100 mM MOPS, pH 7.5, with 2 mM dithionite. Exogenous iron and zinc were removed as described for the cobalt treatment.

Assay of Nickel Activation of Apo-CO Dehydrogenase. Assay of activation of CO dehydrogenase by $NiCl_2$ was carried out as previously described (Bonam et al., 1988) by diluting aliquots of enzyme into 9-mL serum vials containing 1 mL of 10 mM MOPS buffer, pH 7.5, 0.05 mM dithionite, and 5 mM $NiCl_2$ under an atmosphere of 100% N_2. Maximal levels of activation were obtained after 15–20-min incubation.

Protein Oxidation. Oxidations of dithionite-reduced apo-CODH-, metal-treated apo-CODH, and holo-CODH were carried out 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. Proteins were oxidized following the procedure of Eady et al. (1987) with several modifications. Proteins were stripped of dithionite by passage over a 1 × 10 cm column of Sephadex G-25. A 4-cm layer of indigo carmine bound to AG 1-X8 ion-exchange resin (Bio-Rad) was placed above a 5-cm layer of indigo carmine free AG 1-X8, which was located above a 1 × 15 cm column of Sephadex G-25. Dithionite-free proteins were applied to this column and left in contact with the indigo carmine layer for several minutes before elution. All column buffers were preevacuated/flushed with N_2 and allowed to equilibrate in the anaerobic box for several days; 100 mM MOPS buffer, pH 7.5, was used in all steps.

Cyanide Treatment of Holo-CO Dehydrogenase. Oxidized holo-CODH (4440 units/mg) was incubated with 3 mM KCN for 25 min. Excess KCN was removed by passage over a 0.5 × 25 cm column of Sephadex G-25 gel filtration resin. All manipulations were performed in the anaerobic box with dithionite-free 100 mM MOPS buffer.

UV–Visible Absorbance Measurements. Spectra and fixed-absorbance measurements were recorded on a Shimadzu UV-160 UV–visible spectrophotometer. Oxidized protein samples were transferred via gas-tight syringes to stoppered, anaerobic quartz cuvettes containing 101-kPa N_2 or CO. Reduction of Fe–S centers was monitored by following loss of absorbance at 418 nm. Extinction coefficients for oxidized (34.2 mM^{-1} cm^{-1}) and CO-reduced (17.5 mM^{-1} cm^{-1}) CO dehydrogenase at 418 nm were reported previously (Bonam & Ludden, 1987). Spectra of oxidized and reduced protein samples were recorded from 200 to 600 nm.

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.

Results and Discussion

The UV–visible absorbance spectra of oxidized holo-CODH under N_2 under CO, and after reduction by 1 mM dithionite are presented in Figure 1A. The spectrum of the oxidized enzyme under N_2 exhibits a broad absorbance shoulder from 350 to 480 nm, characteristic of absorbance due to oxidized Fe–S centers. The spectrum is very similar to that seen for the purified acetogenic (Ragsdale et al., 1983a,c) and methanogenic (Krzycki & Zeikus, 1984; Terlesky et al., 1986) CODHs. Exposure of the oxidized enzyme to CO leads to a rapid loss of absorbance in the 350–480-nm region, interpreted as a reduction of the Fe–S center(s) of the enzyme (Bonam & Ludden, 1987). This loss in absorbance is rapid, the re-

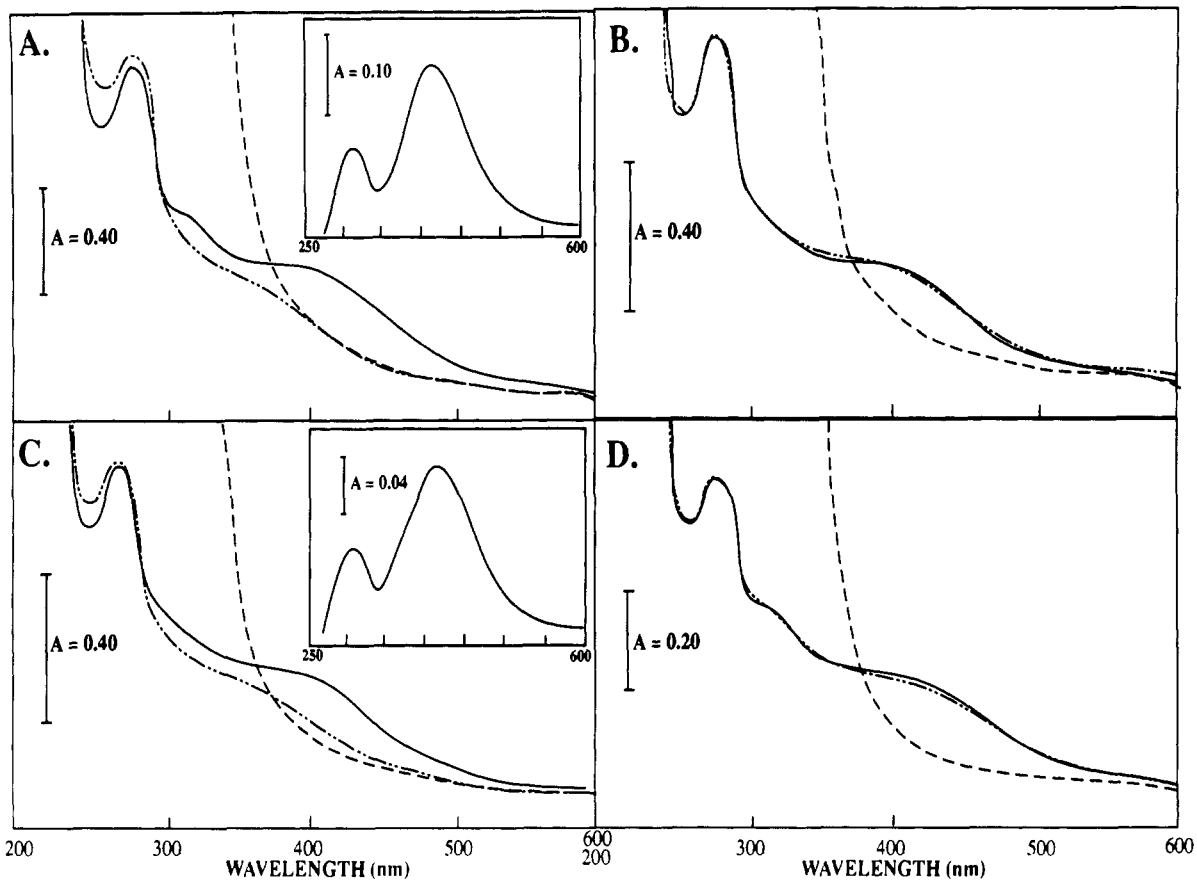

FIGURE 1: UV-visible spectra of holo-CODH, apo-CODH, and cobalt- and zinc-treated apo-CO dehydrogenase. Spectra are of indigo carmine oxidized protein samples under 100% N_2 (—), under 100% CO after 35-min incubation (– – –), and after addition of 1 mM dithionite (– –) vs buffer (100 mM MOPS, pH 7.5). The insets show the difference spectrum for oxidized minus CO-reduced enzyme. (A) Holo-CODH (0.918 mg/mL); (B) apo-CODH (0.881 mg/mL); (C) cobalt-treated apo-CODH (0.683 mg/mL); (D) zinc-treated apo-CODH (0.483 mg/mL).

duction being essentially complete within 1 min of exposure to CO. The difference spectrum for oxidized minus CO-reduced CODH (inset to Figure 1A; Bonam & Ludden, 1987) shows a ΔA peak centered at 418 nm, with a less dramatic peak at 315 nm. Addition of dithionite to CO-reduced CODH results in no further loss of absorbance (Figure 1A), indicating that CO is able to reduce the Fe–S centers to the same level as dithionite.

The absorbance spectra of oxidized apo-CODH under N_2, under CO, and after reduction by 1 mM dithionite are presented in Figure 1B. The spectra under N_2 and CO are virtually indistinguishable and are identical with the spectrum of oxidized holo-CODH under N_2. Apo- and holo-CODH are identical with respect to molecular weight and iron content and appear to differ only in the nearly complete absence of nickel in the apoenzyme (Bonam et al., 1988). We conclude that the absorbance shoulder in oxidized apo- and holo-CODH is due to absorbance properties of the Fe–S center(s), with no obvious contribution from nickel. The Fe–S centers of dithionite-reduced apo-CODH are indistinguishable from the Fe–S centers of holo-CODH as observed by EPR (Bonam et al., 1988), and apo-CODH becomes fully activated to a level of activity comparable to that of holoenzyme solely upon the addition of $NiCl_2$ (Bonam et al., 1988). In light of these observations, the lack of reduction of oxidized apo-CODH upon exposure to CO (Figure 1B) strongly implicates nickel, or a center formed upon the association of nickel with the enzyme, as the site of CO binding and as a mediator in the passage of electrons from CO oxidation to the Fe–S centers of the enzyme. Even after prolonged incubation in the presence of CO (>35 min) the Fe–S centers of the apoenzyme under CO remained essentially fully oxidized (Table I). The Fe–S centers were however immediately reduced upon addition of dithionite (Figure 1B).

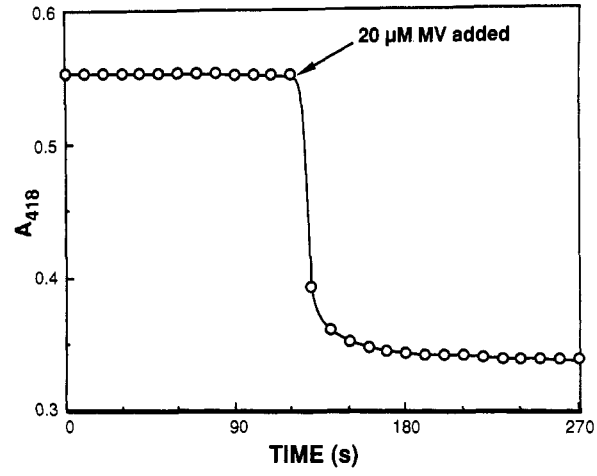

FIGURE 2: Reduction of apo-CO dehydrogenase Fe–S centers by CO. One milliliter of oxidized apo-CO dehydrogenase (1.05 mg/mL) was added to a 1.5-mL anaerobic cuvette containing a 100% CO gas phase. The absorbance at 418 nm was recorded for 120 s. One microliter of anaerobic 20 mM oxidized methylviologen was then added (final concentration = 20 μM) and A_{418} followed for an additional 150 s.

We have made use of the absorbance difference between oxidized and reduced CODH at 418 nm to measure the competence of oxidized apo- and holo-CODH to undergo CO-dependent Fe–S center reduction under various conditions. Figure 2 shows the absorbance at 418 nm vs time for oxidized apo-CODH placed under CO. No decrease in absorbance was observed over a 120-s time course in the absence of an added electron carrier. However, loss of absorbance at 418 nm was

Table I: Effect of CO on the Oxidized Fe–S Centers of Holo-CODH, Apo-CODH, and Cobalt-, Iron-, and Zinc-Treated CO Dehydrogenase

protein sample	metal content[a]	sp act.	x-fold activation by $NiCl_2$[b]	A_{418}/μmol of protein[c] (% reduced)[d]		
				N_2	CO	$S_2O_4^{2-}$
holo-CODH	7.37 ± 0.5 Fe 1.1 ± 0.2 Ni 0.41 ± 0.3 Zn	4440	1	34.3 (0)	20.2 (99.1)	20.1 (100)
apo-CODH	7.44 ± 0.6 Fe <0.02 Ni 0.44 ± 0.2 Zn	46.2	52	35.8 (0)	35.1 (5.0)	22.0 (100)
cobalt-treated CODH	7.44 Fe <0.02 Ni 0.44 Zn 0.70 Co	49.2	0.87	37.6 (0)	24.25 (82.6)	21.4 (100)
zinc-treated CODH	7.44 Fe <0.02 Ni 1.67 Zn	48.4	1	35.44 (0)	34.4 (6.8)	20.5 (100)
iron-treated CODH	8.30 Fe <0.02 Ni 0.44 Zn	89.0	1.2	34.8 (0)	33.2 (11.9)	21.3 (100)

[a] Reported as mol of metal/mol of protein. Metal analyses and protein determinations were performed as described under Methods. [b] Assay of nickel activation was performed as described under Methods. [c] The absorbance values reported are for the enzyme after 35 min under an atmosphere of 100% CO. [d] Percent reduced enzyme under CO was calculated as $[A_{418}(N_2) - A_{418}(CO)]/[A_{418}(N_2) - A_{418}(S_2O_4^{2-})] \times 100$.

initiated by addition of 20 μM of the oxidized redox dye methylviologen. Given the fact that apo-CODH preparations have a low level of CO-oxidizing activity (50–100 units/mg; Bonam et al., 1988) attributed to a small amount of contaminating holoenzyme, we interpret this reduction of apo-CODH as follows: CO acts as a substrate for the small amount of holo-CODH present, directly reducing the Fe–S centers, which then become reoxidized as electrons are transferred to oxidized methylviologen. Reduced methylviologen then directly reduces the oxidized Fe–S centers of apo-CODH, providing more oxidized methylviologen to act as substrate for the holoenzyme.

The results of Figure 2 allow several conclusions to be drawn. CO will not act as a direct reductant for the Fe–S centers of apo-CODH. In addition, direct protein to protein electron transfer between reduced holo- and oxidized apo-CODH does not occur, but electron transfer does occur in the presence of a suitable, diffusible electron carrier. Reduction of CODH Fe–S centers by methylviologen (or by dithionite; Figure 1B) is not dependent on the presence of nickel in the enzyme.

The role of nickel in the reduction of holo-CODH Fe–S centers was further investigated by studying the effect of enzyme-bound cyanide on this reaction. Cyanide has previously been shown to be a slow-binding inhibitor of CODH (Ensign et al., 1989). Inhibition is specific for nickel in CODH, with cyanide inhibiting, and remaining bound to, holo- but not apo-CODH after removal of unbound cyanide by gel filtration chromatography. CO protects holo-CODH from inhibition by cyanide, and reactivation of cyanide-inhibited enzyme is dependent on the presence of CO over a 60-min time course. These results suggest that binding of cyanide and binding of CO are competitive and mutually exclusive events (Ensign et al., 1989).

As shown in Figure 3, association of cyanide with holo-CODH prevents CO from directly reducing the Fe–S centers of the enzyme. This result supports the proposed pathway for electron transfer, showing that Fe–S center reduction can be blocked not only by the lack of nickel in the enzyme but also by blockage of the CO binding site on nickel. No changes in the absorption spectrum were seen for the cyanide-inhibited enzyme, and the Fe–S centers were immediately reduced upon addition of 1 mM dithionite.

The substitution of other transition metals into apo-CODH in place of nickel was investigated with the assays described

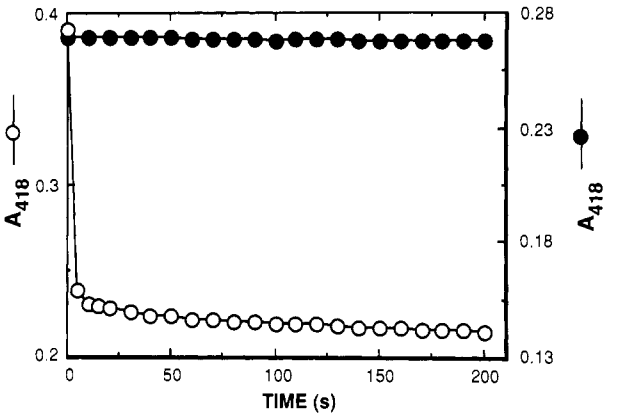

FIGURE 3: Effect of enzyme-bound cyanide on the reduction of holo-CO dehydrogenase Fe–S centers by CO. The absorbance at 418 nm was recorded over a 150-s time course for 1 mL of enzyme in a 1.5-mL anaerobic cuvette containing 100% CO. (○) Absorbance for a 0.70 mg/mL solution of oxidized holo-CODH; (●) absorbance for a 0.50 mg/mL solution of cyanide-bound holo-CODH.

above. No other transition metals tested (Mg^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, Mo^{6+}) have been able to activate apo-CODH above its residual activity level (Bonam et al., 1988). It is, however, possible that nickel analogues may substitute in catalysis, albeit at a greatly reduced rate, and any activity due to the substituted metal ion is masked by the activity of the small amount of holo-CODH present. An alternative method for determining whether these metals function in catalysis may be to monitor their ability to substitute for nickel in facilitating Fe–S center reduction by CO. Cobalt is of particular interest in this regard given its similarity to nickel in size (Pauling's metal ion radii of 0.69 Å for nickel and 0.72 Å for cobalt) and ligating properties. Iron and zinc were also chosen as possible substitutes for nickel.

The results of initial attempts to substitute these metals into the vacant nickel site of apo-CODH are shown in Table I. The specific activities of the cobalt- and zinc-treated enzymes are identical with the specific activity of the untreated apoenzyme, whereas the specific activity of the iron-treated enzyme is slightly but not significantly higher. In contrast, treatment of the apoenzyme with $NiCl_2$ following an identical protocol led to a 100-fold increase in specific activity to 4900 units/mg. Neither the zinc-, iron-, or cobalt-treated enzyme could be further activated by addition of $NiCl_2$ with the nickel activation assay, in contrast to the untreated apoenzyme which was ac-

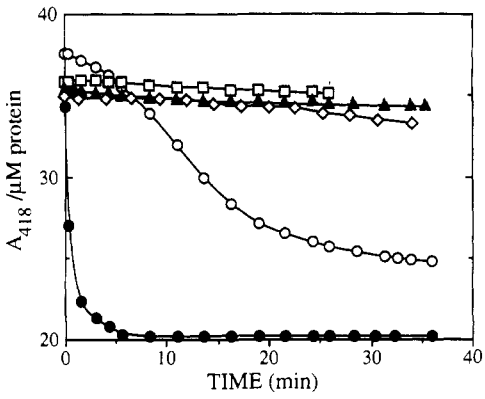

FIGURE 4: Effect of CO on the oxidized Fe–S centers of holo-CODH, and cobalt-, zinc-, and iron-treated apo-CO dehydrogenase. Indigo carmine oxidized protein samples (1 mL) were transferred via gas-tight syringes to 1.5-mL anaerobic cuvettes containing 100% CO. The absorbance at 418 nm was recorded over a 35-min time course. (●) Holo-CODH; (□) apo-CODH; (○) cobalt-treated apo-CODH; (▲) zinc-treated apo-CODH; (◇) iron-treated CODH.

tivated 52-fold (Table I). The loss of ability to be activated upon treatment with these alternate metals suggests that the metals either have become fully incorporated into the nickel site of the enzyme or have at least bound to the enzyme in such a way that nickel is prevented from ligating to the enzyme in the correct conformation to produce a catalytically active holoenzyme. Holo-CODH treated with cobalt, zinc, and iron following an identical protocol led to no loss of specific activity (data not shown), showing that CODH is not susceptible to inactivation resulting from the protocol used for these experiments.

Metal analyses show 0.70 mol of cobalt, 1.2 mol of zinc, and 0.86 mol of iron associated with the apoenzyme as a result of the cobalt, zinc, and iron treatments (Table I). Purified holo-CODH of high specific activity contains 1.1 mol of nickel and cannot be further activated by $NiCl_2$. This indicates that there is a single specific nickel active site on CODH. In the initial purification of holo-CODH (Bonam & Ludden, 1987), CODH was found to contain 0.65 mol of nickel/mol of enzyme and specific activity of 1080 units/mg (assayed at pH 10). However, the medium used for cell growth at that time was not supplemented with $NiCl_2$, and it has since been shown that apo- and holo-CODH copurify (Bonam et al., 1988). It is likely therefore that the enzyme described in the initial purification contained a significant amount of apoenzyme and could be further activated upon treatment with exogenous nickel. We have now found that by supplementing the *R. rubrum* growth medium with 50 μM $NiCl_2$ we are able to routinely obtain holo-CODH of high specific activity (4400 units/mg when assayed at pH 7.5; ~6000 units/mg when assayed at pH 10) which cannot be further activated by addition of $NiCl_2$ and which consistently contains 1 mol of nickel/mol of enzyme as determined by plasma emission spectroscopy.

Figure 4 shows the time course for the reduction of holoenzyme, apoenzyme, and the M^{2+}-treated enzyme samples under 100% CO. The Fe–S centers of the holoenzyme were rapidly reduced, whereas the Fe–S centers of the apoenzyme and the zinc- and iron-treated enzymes remained oxidized. The Fe–S centers of the cobalt enzyme however were slowly reduced over a 35-min time course. At this point the Fe–S centers were judged to be 82.6% reduced vs 99.1% for the holoenzyme and 5.0% for the apoenzyme (Table I). These results suggest that cobalt has replaced nickel at the active site of the enzyme and is substituting for nickel in catalyzing the transfer of electrons from CO to the Fe–S centers, albeit at a greatly reduced rate.

The UV–visible spectra of the cobalt enzyme under N_2, under CO, and after full reduction by dithionite are presented in Figure 1C. The difference spectrum shown in the inset confirms that the Fe–S centers of the cobalt enzyme have been largely reduced by CO. There is no obvious contribution of cobalt to the absorption spectra, contrary to what might be expected given its spectroscopic properties. However, any contributions of cobalt to the spectrum may be hidden by the strong absorbance of the Fe–S cluster(s). The spectra of the zinc enzyme are shown in Figure 1D. The spectra of the iron enzyme were identical with those of the apoenzyme and zinc enzyme and are not shown.

Although the biochemical evidence suggests that cobalt has been incorporated into the vacant nickel site of apo-CODH, definitive proof that this is so awaits the application of more sophisticated spectroscopic tools. There is no precedent for cobalt substitution into an enzyme that naturally contains nickel. Nickel-deficient forms of other nickel enzymes have not been isolated. Cobalt has been shown to substitute for zinc in several metalloenzymes, and both cobalt and nickel are effectively incorporated into the active site of zinc-deficient apo-carbonic anhydrase B (Coleman, 1971).

SUMMARY

The results presented here demonstrate the requirement of nickel in CO dehydrogenase for CO-dependent reduction of the protein Fe–S centers. We propose that nickel, or a nickel center formed upon association of nickel with the apoenzyme, is the site of CO binding and is responsible for transferring electrons from CO oxidation to the Fe–S center(s) of the enzyme. Our previous results showing cyanide to be a nickel-specific analogue of CO also support nickel as the site of CO binding (Ensign et al., 1989). An alternate hypothesis is that nickel is only required for stabilization of a form of the enzyme in which CO (or cyanide) interacts directly with the protein Fe–S centers. We do not believe this is so, given the inability of other transition metals which interchangeably stabilize nonredox metalloenzymes (zinc, iron, and cobalt) to substitute effectively for nickel in this regard. To the contrary, it appears that nickel in CODH has the correct combination of redox potential and affinity for carbonyl ligands to bind and promote the oxidation of CO to CO_2. However, definitive proof that CO binds to a redox-active nickel center in CODH from *R. rubrum* awaits the results of EPR studies currently in progress.

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Registry No. CODH, 64972-88-9; Ni, 7440-02-0; CO, 630-08-0; Co, 7440-48-4.

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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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