Evidence for a Ligand CO That Is Required for Catalytic Activity of CO Dehydrogenase from *Rhodospirillum rubrum*†

Jongyun Heo,‡ Christopher R. Staples,‡ Cale M. Halbleib, and Paul W. Ludden*

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

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ABSTRACT: Radiolabeling studies support the existence of a nonsubstrate CO ligand (CO_L) to the Fe atom of the proposed [FeNi] cluster of carbon monoxide dehydrogenase (CODH) from *Rhodospirillum rubrum*. Purified CODH has variable amounts of CO_L dissociated depending on the extent of handling of the proteins. This dissociated CO_L can be restored by incubation of CODH with CO, resulting in a 30−40% increase in initial activity relative to as-isolated purified CODH. A similar amount of CO_L binding is observed when as-isolated purified CODH is incubated with ^{14}CO: approximately 0.33 mol of CO binds per 1 mol of CODH. Approximately 1 mol of CO was released from CO-preincubated CODH upon denaturation of the protein. No CO could be detected upon denaturation of CODH that had been incubated with cyanide. CO_L binds to both Ni-containing and Ni-deficient CODH, indicating that CO_L is liganded to the Fe atom of the proposed [FeNi] center. Furthermore, the Ni in the CO_L-deficient CODH can be removed by treatment with a Ni-specific chelator, dimethylglyoxime. CO preincubation protects the dimethylglyoxime-labile Ni, indicating that CO_L is also involved in the stability of Ni in the proposed [FeNi] center.

Carbon monoxide dehydrogenase (CODH)[1] oxidizes substrate CO (CO_S) to CO_2, allowing *Rhodospirillum rubrum* to grow anaerobically in the dark with CO as the energy source (*1, 2*). The structure of the active site of CODH from *R. rubrum* has been proposed to contain a binuclear [FeNi] cluster (*3*). An $[Fe_4S_4]$ cluster, which will be referred to as $[Fe_4S_4]_B$, functions as the conduit for electrons to pass to the external electron acceptor, CooF (*3*). Three distinguishable redox states of the [FeNi] cluster have been proposed: $[Fe^{2+}-Ni^{2+}]^{4+}$, $[Fe^{3+}-Ni^{2+}-H^-]^{4+}$, and $[Fe^{2+}-Ni^{2+}-H^-]^{3+}$ (*4*). The EPR signals attributed to the [FeNi] component of as-isolated CODH were observed to be spectroscopically heterogeneous (*4*). This heterogeneity could be removed upon CO treatment of the reduced CODH, and the loss of heterogeneity was correlated with 30−40% increase in the initial CO-oxidation activity relative to the activity of as-isolated CODH. Another class of Ni-containing enzymes, the [NiFe] hydrogenases, contain CO and cyanide ligands in their NiFe binuclear clusters (*5, 6*). These observations suggest that CO might serve as a ligand (CO_L) to the [FeNi] cluster in addition to serving as the substrate for the enzyme. This article confirms the presence of intrinsic CO_L by using a ^{14}CO-binding assay with heterogeneous CODH and chemical detection of the enzyme bound CO_L. This article also shows that CO_L is involved in the stability of Ni in the [FeNi] center. Additionally, the effect of cyanide (CN^-) on the intrinsic CO_L is investigated. CODH purified by a protocol that minimizes loss of activity and metals during heat treatment results in an enzyme containing 9 Fe atoms/Ni atom in CODH.

A nickel-deficient form of CODH from *R. rubrum* can be obtained with an apparently full complement of Fe and presumably a [Fe−□] center, where □ = a vacant site normally occupied by Ni (*7, 8*). Ni-deficient CODH has negligible CODH activity. However, incubation of reduced Ni-deficient CODH with Ni^{2+} results in an enzyme with 40% wild-type CODH activity due to reconstitution of Ni into the vacant site of the [Fe−□] center (*7, 9*). This suggests that although Ni has been incorporated into Ni-deficient CODH to 1 Ni atom/monomeric enzyme, either (i) only 40% of the CODH molecules are active or (ii) the in vitro reconstituted [FeNi] site has only 40% activity of CODH activated in vivo. Nevertheless, the availability of Ni-deficient CODH from *R. rubrum* allows for testing of the possibility that CO_L binds to a site other than the Ni atom. Data presented in this article indicates that CO_L and slow binding CN^- interact with Ni-deficient CODH in a manner nearly identical to that of holo-CODH.

MATERIALS AND METHODS

Buffers and Experimental Conditions. All media and buffers used for the culture and growth of Ni-deficient cells

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* Corresponding author. Telephone: 608-262-6859. Fax: 608-262-3453. E-mail: ludden@biochem.wisc.edu.

‡ These two authors contributed equally to the work contained herein.

[1] Abbreviations: CODH, carbon monoxide dehydrogenase; EPR, electron paramagnetic resonance; CN^-, cyanide; CO_S, substrate CO; CO_L, ligand CO; MOPS, 3-(*N*-Morpholino)propanesulfonic acid; DTH, sodium dithionite; BSA, bovine serum albumin; MV, methyl viologen; ICP-MS, inductively coupled plasma mass spectrometry; SHE, standard hydrogen electrode; IC, indigo carmine; EDTA, Ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; Hb, hemoglobin; DE-52, diethylaminoethyl cellulose; dpm, disintegration/min.

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were passed down a metal-chelating column of Chelex-100 cation-exchange resin (Bio-Rad) before the addition of the necessary metals, as were all buffers used in protein purification of both Ni-containing and Ni-deficient CODH. MOPS (3-(*N*-Morpholino)propanesulfonic acid, USB) buffer (metal-free 100 mM MOPS, pH 7.5) was used in all protein purifications, in sequential treatments of enzyme-bound columns, and in assays of CODH activity. All experiments were performed under N_2 (<2 ppm O_2) in a Vacuum Atmospheres Dri-Lab glovebox (model HE-493) unless otherwise stated.

Growth of R. rubrum on Ni-Supplemented and Ni-Depleted Media. Wild-type strain (UR2) *R. rubrum* was cultured according to established procedures in medium (*8*) that was either completely lacking nickel for Ni-deficient cells or was supplemented with 0.05 mM $NiCl_2$ (Sigma) for Ni-supplemented cells.

Purification of Ni-Supplemented- and Ni-Deficient-CODH in the Presence of Glycerol. Holo- (Ni-containing) and Ni-deficient-CODH were purified as described previously for the wild-type enzyme (*8*) except that the heat treatment steps were modified to 10 min at 60 °C and 2 min at 80 °C; furthermore, glycerol was added to a final concentration of 5%, and sodium dithionite (DTH) was not present during the heat treatment. Anaerobic, DTH-free buffer was stored at least 24 h in the Vacuum Atmospheres glovebox prior to use. "As-isolated" CODH refers to purified CODH that has not been incubated with CO following extraction from the cell.

Protein Assays. The purity of CODH samples was determined to be greater than 99% by SDS and native PAGE analysis. Protein content was determined using bicinchoninic acid (*10*) and bovine serum albumin (BSA grade A, Sigma) as a standard. BSA solution was standardized against carbonic anhydrase prior to use. Total protein concentration was determined for each sample described in this study from 10 replicate protein assays. The replicate BSA standard curves were generated for each protein assay. Protein assays were performed in the absence of DTH.

CODH Activity Assays. CO-oxidation activities were determined by the ability of CODH to reduce methyl viologen (MV, Sigma) in a CO-saturated assay mixture containing 10 mM MV and 1 mM EDTA in 100 mM MOPS; a trace amount of DTH was added to the assay solution (*8*).

CO-oxidation activity is expressed as units which are defined as μmol of CO oxidized min^{-1} mg $protein^{-1}$. If the trace of DTH is omitted, a short lag in activity is observed.

Metal Analysis. The metal contents of the enzyme samples were determined at the University of Georgia by inductively coupled plasma mass spectrometry (ICP-MS). Enzyme samples were passed through a Sephadex G-25 column (0.5 × 10 cm, Pharmacia) equilibrated in metal-free 100 mM MOPS buffer at pH 7.5 prior to analysis. Calculation of metal contents expressed as moles of metal per mole of monomeric enzyme was based upon protein concentration and the calculated molecular weight of CODH (66.9 kDa) from the deduced amino acid sequence (*11*).

Preparation of Redox-Poising Agents and Other Solutions. Thionin ($E_m{}^{\circ\prime} = +64$ mV versus SHE, Aldrich), indigo carmine ($E_m{}^{\circ\prime} = -125$ mV versus SHE, Sigma), and DTH ($E_m{}^{\circ\prime} = -530$ mV versus SHE, Fluka) were prepared in MOPS buffer. Potassium cyanide (KCN, Sigma) solution was prepared in a 0.01 M NaOH (Fisher) solution immediately prior to use. The Ni-specific chelator, dimethylglyoxime (Sigma), ethylenediaminetetraacetic acid (EDTA, Fisher), sodium dodecyl sulfate (SDS, Fisher), NaCl (Fisher), and $NaHCO_3$ (Fisher) were each prepared in MOPS buffer. CO-saturated buffer was prepared in a serum-stoppered vial by sparging MOPS buffer with CO for 20 min in the hood. The hemoglobin (bovine hemoglobin (Hb), Sigma) assay solution was prepared by following a previous method (*12*).

Preparation of Indigo Carmine Oxidized-CODH. As-isolated CODH (in MOPS buffer containing 2 mM DTH) was loaded onto a DE-52 column (0.5 cm × 2 cm, diethylaminoethyl cellulose, Whatman), and excess oxidized indigo carmine (IC) solution ($E_{calc} \cong 0$ mV) was applied. IC was removed by washing with excess MOPS buffer. IC-poised CODH was eluted with MOPS containing 400 mM NaCl and immediately passed through a Sephadex G-25 (0.5 cm × 15 cm) column equilibrated with MOPS buffer to remove salt.

Preparation of Reduced, CO-Treated CODH. Purified, as-isolated CODH was incubated with CO for 30 min under reducing conditions (2 mM DTH). CO-treated CODH was applied to a DE-52 column (0.5 cm × 2 cm), and DTH was removed by washing with MOPS buffer. A 100 mM solution of oxidized indigo carmine solution was passed over the column-bound CODH and subsequently was removed by washing with MOPS buffer. IC-poised CODH was eluted using MOPS buffer containing 200 mM NaCl, and the eluent was passed down a Sephadex G-25 column (0.5 cm × 15 cm) to remove NaCl. CO-treated, IC-poised CODH was retreated with 2 mM DTH to reduce the enzyme.

Preparation of ^{14}CO. ^{14}C-formate was obtained from American Radiolabeled Chemicals (St. Louis, MO). ^{14}CO was produced by addition of concentrated H_2SO_4 to a double serum-stoppered vial (25 mL size, 100% unlabeled CO gas atmosphere) containing ^{14}C-formate (56 mCi/mmol) (*1*). The gas phase (10 mL) was removed while compensating the pressure by adding unlabeled CO gas. The ^{14}CO gas was then injected into an evacuated vial (25 mL) containing 10 mL of MOPS buffer, and the pressure was adjusted to approximately 1 atm by addition of unlabeled CO. The specific activities of the prepared ^{14}CO solution (16.2−81.4 mCi/mol of CO) were determined by counting the total radioactivity from an aliquot (5 μL) and measuring the corresponding CO concentration by Hb-binding assay (*1*).

^{14}CO Binding Study by Gel Filtration. CODH samples of varying concentration were incubated with ^{14}CO. Each CODH sample was applied to a Sephadex G-25 column (0.5 cm × 8 cm) and chromatographed at a flow rate of 0.2 mL/min. Upon elution, the entire enzyme fraction was collected directly into a gastight scintillation vial (20 mL) that contained a scintillation cocktail solution, and the radioactivity was measured by scintillation counting. As a control, a BSA sample, at a concentration corresponding to each concentration of CODH, was prepared and treated as described for CODH. The amount of ^{14}CO bound to each sample of CODH was determined from the radioactivity in the sample (subtracting the counts in the BSA control), given the known specific activity of the ^{14}CO and the concentration of CODH in the sample.

Preparation of the Equilibrium Dialysis Unit and Its Application. An equilibrium dialysis unit for measuring ^{14}CO-

binding to Ni-deficient- and holo-CODH was slightly modified from published methods (*13*). The equilibrium dialysis unit consists of an upper chamber (the cylindrical portion of a 1.5 mL microfuge tube) and a lower chamber (the lid of the 1.5 mL microfuge tube) with a dialysis membrane (molecular weight cut off 12 000−14 000) between the two portions. Prior to the experiment, the dialysis membrane was treated in a solution of 1 mM EDTA and 1 mM $NaHCO_3$ and boiled at 100 °C for 10 min. The EDTA-treated dialysis membrane was then washed with an excess amount of double distilled water. The cylindrical upper chamber of the equilibrium dialysis unit was capped with a rubber serum stopper (Wheaton) to seal the system anaerobically and to make it accessible by syringe. The final bed volume of the modified upper chamber is 1000 μL, and that of the lower chamber is 300 μL. Prior to the binding study of ^{14}CO to CODH, Hb was used to test the sensitivity and accuracy of this method. The Hb solution (in the range of 0.2−20 nmol) was loaded in the lower chamber, and buffer solution containing dissolved ^{14}CO was injected into the upper chamber. After 60 min the sample had reached equilibrium, and nearly identical values for the amount of CO binding to Hb were obtained using this method compared to results obtained by monitoring the shift of the Soret band upon CO binding to Hb (*14*). As a control for this method, buffer was added to the lower chamber in place of the protein solution.

Detection of a Bound Form of CO_L. Chemical detection of a bound CO molecule was adapted from a previously published method (*6*). CODH was denatured by SDS/EDTA/heat treatment, and the amount of CO released was measured by adding Hb and then determining the percent shift of the Soret absorption indicative of CO binding to Hb (*1, 12*). For the experiments, MV-treated CODH was loaded onto a DE-52 column (0.5 cm × 1 cm) and washed with 10 mL of MOPS buffer. Excess thionin (1 mL of 100 mM) was applied to the DE-52-bound CODH and subsequently removed by washing with 50 mL of MOPS buffer. Oxidized CODH was eluted with 400 mM NaCl in MOPS buffer and desalted by passage down a Sephadex G-25 column (0.5 cm × 10 cm). The CO-oxidation activity was measured as described in the CODH activity assays. A fraction of CO-treated CODH sample was loaded onto a DE-52 column, and subsequently a CN^-/DTH solution (1 mL of 1 mM CN^- with 2 mM DTH) was loaded and incubated for 30 min. Excess CN^- was removed by washing with 10 mL of MOPS buffer. CN^--treated, DE-52 bound CODH was eluted with 400 mM NaCl in MOPS buffer and was desalted by passage down a Sephadex G-25 column (0.5 cm × 10 cm). The activity of the eluted CODH was measured as described previously. To denature the samples of CODH, SDS (0.1 mL of 20%) and EDTA (0.1 mL of 2 mM) were added to each CODH sample (0.2 mL of 0.45 μmol of CODH) and each was immediately sealed in a 2 mL vial. The vials were heated to 95 °C for 10 min and then chilled on ice for 30 min. The gas phase of each sample was removed using a gastight microsyringe while compensating for gas pressure with another syringe filled with N_2. The gas phase of each sample was introduced anaerobically into a Hb assay solution. A CO-saturated solution (880 μM) was used as a standard.

Ni Activation of Ni-Deficient CODH, and Treatment of Holo-CODH with a Ni-Specific Chelator. Immobilized enzyme samples (CODH samples were loaded to DE-52 columns) were treated sequentially with effectors (i.e., CO, CN^-, Ni, or Ni-specific chelator) as a mobile phase.

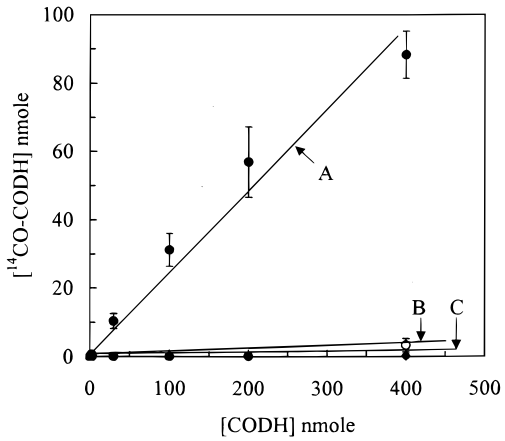

FIGURE 1: Study of ^{14}CO binding by gel filtration. As-isolated (●), CO-treated (○), and indigo carmine oxidized CODH (◆) (100 μL) were incubated with the ^{14}CO solution (200 μL, 30.8 mCi/mol) in serum-stoppered vials (2 mL size) and incubated for 30 min. Aliquots of each sample (2−10 μL) were removed and used to determine protein concentration. The concentration of CODH in each sample was varied from 0 to 4 mM. ^{14}CO-treated samples (300 μL total) were passed through Sephadex G-25 columns, and the total proteins were collected directly into the scintillation vials which contained scintillation solution. Typically all proteins were eluted when the collecting volume had reached 500 μL. The moles of ^{14}CO bound to each sample of CODH was determined as described under Materials and Methods. The data were fitted to linear regression, and the slopes represent the fraction of bound mol of CO/mol of CODH. Slope A shows reduced, as-isolated CODH bound CO molecule 0.25 ± 0.08 mol of CO/mol of CODH. Oxidized or ^{12}CO-treated CODH showed negligible binding of additional CO (slopes B and C). Each experiment was repeated 3 times.

RESULTS

Direct Observation of the Binding of ^{14}CO to As-Isolated CODH. CODH purified from *R. rubrum* grown on Ni-supplemented medium is isolated in a heterogeneous form. The heterogeneity is most clearly demonstrated by the line shapes of the component of the EPR signals arising from the [FeNi] cluster in the state referred to as C_{red1} (*4*). Heterogeneity of EPR signal can be removed by incubation of the CODH sample with CO, concurrent with an approximate 40% increase in initial activity. Thus, an activating CO ligand (CO_L) was proposed to present at the [FeNi] cluster (*4*). The analysis of ^{14}CO binding to holo-CODH by gel filtration shows that 0.25 mol of CO binds per 1 mol of reduced, as-isolated CODH; binding to either oxidized or ^{12}CO-pretreated CODH is not observed (Figure 1). The control, BSA, showed negligible CO binding (data not shown). This result indicates that the site for CO_L binding is accessible only when CODH is reduced. To confirm the gel filtration result, the binding of ^{14}CO to CODH was measured by an equilibrium dialysis method. Results of equilibrium dialysis (Table 1) show that both as-isolated, holo-CODH and as-isolated, Ni-deficient CODH bind approximately 0.3 mol of CO/mol of CODH but only under reducing conditions. Consistent with the gel-filtration result, no ^{14}CO binding was observed in the equilibrium dialysis system when using ^{12}CO-pretreated CODH or oxidized CODH. The ^{14}CO studies suggest that CO_L binds to the Fe

Table 1: ^{14}CO Binding Study of Wild-Type holo- and Ni-Deficient-CODH[a]

pretreatments		mol of ^{14}CO/mol of CODH	
^{12}CO treatment	redox state (mV)	holo	Ni-deficient
A. −	0	−0.04 ± 0.02	−0.01 ± 0.01
B. −	−530	+0.36 ± 0.06	+0.33 ± 0.02
C. +	0	−0.09 ± 0.01	−0.01 ± 0.00
D. +	−530	−0.01 ± 0.00	−0.01 ± 0.00

[a] IC-oxidized, as-isolated samples (line A; 0.15 μmol of holo CODH and 0.37 μmol of Ni-deficient CODH), reduced as-isolated samples (line B; 0.16 μmol of holo-CODH and 0.37 μmol of Ni-deficient-CODH), IC-oxidized, CO-preincubated samples (line C; 0.19 μmol of holo-CODH and 0.21 μmol of Ni-deficient-CODH), and reduced, CO-preincubated samples (line D; 0.19 μmol of holo-CODH and 0.21 μmol of Ni-deficient-CODH) were placed in the lower chamber of the equilibrium dialysis unit and covered with the dialysis membrane as described under the Materials and Methods. The dialysis unit was sealed by snapping the stoppered upper chamber over the dialysis-membrane-covered lower chamber. ^{14}CO-saturated MOPS buffer (46.2 mCi/mol CO) was introduced into the upper chamber through the rubber stopper using a gastight microsyringe, and pressure was released by removing gas simultaneously. After incubation (60 min) at room temperature, the samples (50 μL) from the lower and upper chamber were taken separately using a gastight microsyringe (the lower chamber was accessed by puncturing the plastic cap) and the radioactivity in each was determined. The mol of ^{14}CO/mol of CODH was calculated from the differences of the dpm of the radioisotope of the upper and lower chamber and the known concentration of CODH. All equilibrium dialysis experiments were repeated 3 times.

site of the putative [FeNi] cluster, as a similar amount of CO was observed to bind to both holo-CODH and Ni-deficient CODH under reducing conditions (Table 1, line B). The rate of binding is slow, reaching completion at 25 min (data not shown).

Direct Detection of a CODH-Bound CO Ligand Using the Hemoglobin Assay. CODH samples were denatured by SDS in the presence of EDTA and then heat treated to liberate bound CO. The entire gas phase of this sample was then added to a solution containing hemoglobin, and a shift in the Soret peak was used as a marker for the binding of CO to the heme group. The Hb-CO measured was used to estimate the amount of CO_L present in the CODH sample. As-isolated CODH that was not pretreated with either CO or CN^- had a specific activity of 7000 units. Quantitation of intrinsic CO liberated by SDS/EDTA/heat treatment of this as-isolated CODH yielded a value of 0.20 CO atoms/monomer CODH. CODH preincubated with CO in the presence of DTH was similarly analyzed, yielding a value of 0.82 CO atoms/monomer CODH. Analysis of CO-pretreated CODH samples before and after subjection to gel-filtration chromatography yielded the same values. Conversely, when DTH-reduced CODH was pretreated with CN^- for 30 min after the CO pretreatment, only 0.03 mol of CO/mol of CODH were observed to be present after gel filtration. This result indicates that CN^- nearly completely displaces CO_L under reducing conditions. As stated above, the specific activity of the DTH-reduced CODH sample could be increased 42% by CO pretreatment on the high end of the typical amount of activation achieved by CO pretreatment. This may indicate that the starting CODH sample contained a significant percent of nonfunctional enzyme. We have previously proposed that the nonfunctional CODH population is associated with CO_L-unbound forms that can be activated by CO binding. The CODH forms are termed "ready" and "unready" dependent upon the presence of CO_L (*4*).

CN^- Does Not Affect Ni Insertion into Ni-Deficient CODH but Alters the Activity of the Resulting Holo-Form (Table 2). In this experiment, as-isolated samples of Ni-deficient CODH (in either the oxidized or the reduced state) were sequentially treated with CN^- and CO while bound to a DE-52 column. After these treatments, the column-bound, treated CODH was reduced by DTH and incubated with $NiCl_2$. CODH was then eluted from each column, and the activity of each sample was tested. Ni-deficient CODH treated only with $NiCl_2$ served as the standard for activity and had a specific activity of 4870 ± 16 units (Table 2, row A). Treatment of Ni-deficient CODH in the oxidized form with CN^- resulted in no change in the level of activity following

Table 2: Effect of Ni Activation of Ni-deficient-CODH Treated with CO and CN^- at Different Redox States (Sequence of Treatments in Order 1−4)

column no.	(1) redox states of column (DE-52) bound Ni-deficient-CODH (mV)	(2) CN^- (100 μM)	(3) washing buffer[a]	(4) Ni activation[b,c] ($V_{max, CO}$)
A	0	−	−CO	4870 ± 16
B	0	+	−CO	4773 ± 154
C	0	+	+CO	5114 ± 3
D	−530	−	−CO	4490 ± 31
E	−530	+	−CO	0 → E* +CO: 4042 ± 216
F	−530	+	+CO	4188 ± 94

[a] Washing buffer was either saturated CO (+CO) or lacked CO (−CO). [b] Activity of Ni-constituted CODH is given as units (μmol CO oxidation min^{-1} mg of $protein^{-1}$). [c] The Ni activation phase of this experiment step (4) was performed at −530 mV regardless of the redox potential of steps 1−3. The order of treatments is crucial in this experiment to test the effect of redox state and CN^- on Ni activation of Ni-deficient CODH. Step 1: preparation of Ni-deficient-CODH at different redox states. In the Vacuum Atmospheres glovebox, CO-treated, Ni-deficient-CODH samples (5 mg/mL) were bound to DE-52 columns (0.5 cm × 1 cm). Excess thionin solution (100 mM) was applied to all the enzyme bound DE-52 columns, and thionin was removed by washing with MOPS buffer. Reduction of the enzymes bound onto the columns for samples D−F was performed by applying 10 mL of DTH solution (0.1 mM). Step 2: treatments of CN^-. Some column-bound samples (columns B, C, E, and F) were treated with 1 mL of CN^- solution (100 μM) and incubated for 30 min. Excess CN^- was subsequently removed by washing with 20 mL of MOPS buffer. Step 3: treatments of CO. Columns C and F were washed with 20 mL of CO-saturated buffer (+CO). Other columns were washed with the same volume of buffer containing no CO (−CO). Step 4: Ni activation. Ni activation was performed by applying 10 mL of Ni-activating buffer containing 1 mM $NiCl_2$ and 0.1 mM DTH in MOPS buffer to each column. Note that all column-bound CODH samples become reduced by DTH during this step. Nonspecifically-bound Ni was removed by washing with excess MOPS buffer. Ni-treated CODH was eluted with 400 mM NaCl in MOPS buffer, and CO oxidation activity was assayed as described previously. Because treatment E had no CO oxidation activity, sample E was reapplied to DE-52 column and treated with CO-saturated buffer (20 mL) in the presence of DTH (0.1 mM) (E*). The unbound CO was removed by washing with 100 mL of MOPS buffer. The sample was eluted with 400 mM NaCl in MOPS buffer, and CO oxidation activity was determined.

Table 3: Effects of Ni-Specific Chelator on Holo-CODH Treated with CO at Various Redox States

Column Number	(1) Redox states[a] → (2) CO[b] → (3) DM[c] (mV)			(4) $V_{max,CO}$[d] −CO	+CO	Metal contents[e] [Ni]	[Fe]	(5) Ni[f] → $V_{max,CO}$[d]	+CO	Metal contents[e] [Ni]	[Fe]
A	0	−	−	6,327	9,937	0.95	8.92				
B	−530	−	−	6,526	9,261	0.95	8.92				
C	0	−	+	6,426	6,311	0.95	8.98	C* +	9,812	1.07	8.98
D	−530	−	+	6,301	9,865	0.60	8.66	D* +	9,894	1.06	8.65
E	0	+	−	10,360	10,411	0.95	8.98				
F	−530	+	−	10,372	10,412	0.92	8.94				
G	0	+	+	10,009	10,101	0.92	8.90				
H	−530	+	+	9,896	10,039	0.91	8.90				

[a] Redox states of column-bound (DE-52) holo-CODH. [b] CO treatment. [c] Dimethylglyoxime. [d] Maximum CO oxidation activity was expressed as units. The assays were repeated 3 times, and the standard errors were within 60 units. [e] Metal contents are expressed as mol of metal/mol of CODH. [f] Ni incorporation. Standard errors of all metal analyses were within 0.04 mol of metal/mol of CODH on the basis of the protein assays. All the sequential treatments were performed on column-bound (DE-52) CODH, and the flow rate of all the treatment was 0.1 mL/min, unless otherwise noted. Step 1: As-isolated, holo-CODH samples (1 mL of 4.2 mg/mL) were loaded to DE-52 columns (0.5 cm × 1 cm) and poised with indigo carmine or DTH as described previously. Step 2: Column-bound CODH samples were treated with CO-saturated (+CO), or CO-free (−CO) MOPS buffer (10 mL). Step 3: Dimethylglyoxime buffer (20 mL of 100 μM dimethylglyoxime) was applied to the column-bound CODH samples, and the column was subsequently washed with MOPS buffer (100 mL). To maintain the given redox states during the treatment of dimethylglyoxime, dimethylglyoxime buffer containing 0.1 mM DTH was used for the treatment of columns B, D, F, and H. DTH-free dimethylglyoxime buffer was used for the treatment of columns A, D, E, and G. The enzyme samples were eluted with MOPS buffer containing 400 mM NaCl and subsequently passed through a Sephadex G-25 column (0.5 cm × 10 cm) to remove salt. Step 4: The maximum CO-oxidation activity ($V_{max,CO}$) and metal contents of each sample were determined as described previously. After the above sequential treatments (steps 1−4), sample D had a low Ni content (63% of control, line A) and low activity that could not be increased by CO treatment. This sample was subjected to Ni incorporation (D*). Sample C* was a control for the Ni incorporation of D. Step 5: Samples C* and D* were reloaded to DE-52 columns and subsequently treated with Ni as described in Table 2. Eluents were CO-treated, and then $V_{max,CO}$ and metal contents were determined as described earlier.

Ni insertion (Table 2, row B). Treatment of CODH in the oxidized form with CN^- followed by CO resulted in a very small increase in activity (Table 2, row C) compared to row A of Table 2. The results shown in rows B and C of Table 2 indicate that the slow tight-binding of CN^- to CODH does not occur when CODH is in the oxidized form. The increase in activity resulting from Ni insertion into Ni-deficient CODH starting in the reduced state (Table 2, row D) is the same as in row A of Table 2 because DTH is present during the Ni insertion phase of the experiment (samples for rows A and D of Table 2 are essentially the same during the Ni insertion phase of the assay). When column-bound, Ni-deficient CODH is treated with CN^- in the reduced state (Table 2, row E), the CODH has no activity after Ni treatment. However, when the sample from row E of Table 2 was subsequently treated with CO in the noncolumn-bound reduced state, 83% of sample activity shown in row D of Table 2 was obtained. This result indicates that Ni was inserted into Ni-deficient CODH while on the DE-52 column, but in a step prior to Ni-insertion a change occurred with the CN^- treatment that resulted in an inactive holo-form. We propose that the change (resulting in inactivation) is the displacement of a CO ligand by CN^- in the Ni-deficient form of CODH. We interpret these results as indicating that the CO ligand in holo-CODH is bound to the iron atom of the putative [FeNi] cluster. This result is consistent with the result obtained in the ^{14}CO binding study. The observed pattern of the sample shown in row E of Table 2 is repeated in the sample shown in row F, where column-bound, reduced CODH is first treated with CN^- and then treated with CO prior to Ni insertion.

Relationship of the Removable Ni and CO_L (Table 3). Table 3 shows that 37% of Ni from as-isolated (not CO-pretreated) *R. rubrum* CODH can be removed by treatment with the Ni-specific chelator, dimethylglyoxime, when CODH is in the reduced state (Table 3, line D). Ni can also subsequently be reconstituted into the dimethylglyoxime-pretreated enzyme as shown in Table 3 line D*. However, CO preincubation of CODH prior to dimethylglyoxime treatment results in an enzyme population with a negligible population of labile Ni, indicating that the presence of CO_L results in a more stable Ni site (Table 3, line F). The CODH sample that was pretreated with dimethylglyoxime (Table 3, line D) did not increase $V_{max,CO}$ upon treatment with CO prior to measurement of the activity. Furthermore, the activity of the non-CO-treated (as-isolated) CODH was identical to the activity of CODH with the labile Ni removed. A previous report also showed that 30% of the population of CODH from *Clostridium thermoaceticum* contains a labile Ni (*15*). This Ni can be removed by treatment with 1,10-phenanthroline, and Ni can subsequently be reconstituted into the 1,10-phenanthroline-pretreated enzyme.

Quite different from the case of Ni-deficient CODH purified from *R. rubrum* grown on Ni-deficient medium, reconstitution of the dimethylglyoxime-treated CODH sample with Ni and subsequent treatment with CO (Table 3, D*) results in a CODH sample with $V_{max,CO}$ nearly identical to that of CO-treated CODH that was not subjected to dimethylglyoxime treatment (Table 3, G and F). The data indicate that the labile Ni originates from the [FeNi] cluster that lacks CO_L.

Metal Contents and Activities of holo- and Ni-Deficient-CODH. The earlier protocol for the purification of CODH from *R. rubrum* included heat treatments of 5 min at 60 °C and 5 min at 80 °C in the presence of 2 mM DTH (*1*). However, we discovered that heat treating as-isolated CODH at 80 °C with 2 mM DTH for 1 min results in a significant amount of denatured CODH (~10% for holo-CODH and ~50% of Ni-deficient-CODH; data not shown). When the CODH solution contains 1−5% of glycerol and DTH is not used, the heat treatment steps resulted in loss of approximately 1% of holo- and 10% of Ni-deficient-CODH. All the heat treatment steps for the purification of holo-CODH were performed with 5% glycerol for 10 min at 60 °C and 2 min at 80 °C in the absence of DTH. In addition to the observed overall yield, the protein obtained using this modified method had greater activity, and the yield of purified enzyme was nearly 2-fold higher. Measured Fe contents of CODH purified using the traditional method (heat treatment at 80 °C with 2 mM DTH) were 7.42 ± 0.28 mol of Fe/mol of CODH, which is similar to previously reported data (*16*). However, the Fe content of the enzyme purified with glycerol present (5% glycerol), a shortened heat treatment time at 80 °C, and an absence of DTH was 8.96 ± 0.12 mol of Fe/mol of CODH. Analysis by native and SDS−PAGE followed by native preparative gel purification revealed the absence of CooF contamination. The Ni content was not changed significantly. EPR analysis of several CODH samples prepared by the previous and current protocols indicates that a variable population of $[Fe_4S_4]$ can be damaged by heat treatment. With the treatments, approximately 1 atom of Fe is lost and the EPR spectrum oftentimes shows the presence of $[Fe_3S_4]$ clusters, indicating that one Fe atom of a $[Fe_4S_4]$ cluster is heat-labile (data not shown).

The activity of holo-CODH obtained by using the new protocol is >6000 units and can be further increased to >10 000 units by CO incubation. Previously reported activity of holo-CODH purified by the traditional method was no more than 7000 units even after CO incubation. Application of the new protocol to the purification of Ni-deficient CODH increases CODH activity dramatically after Ni incorporation (~5000 units using the new protocol compared to previous report; ~2000 units using the old protocol), and Fe contents are also similar to that of holo-CODH (8.41 ± 0.39 mol Fe/mol of Ni-deficient-CODH). However, there is a negligible effect of CO pretreatment on the activity of Ni-incorporated CODH (Ni-constituted Ni-deficient CODH). For example, CO-oxidation activity of as-isolated holo-CODH increases a minimum of 30% upon CO treatment (i.e., from 7000 to 12 000 units). However, the activity of as-isolated, Ni-incorporated CODH increases only ~5% upon CO pretreatment (i.e., from 4870 to 5114 units, Table 2). This result suggests that Ni-deficient-CODH may have another defect in addition to the lack of Ni.

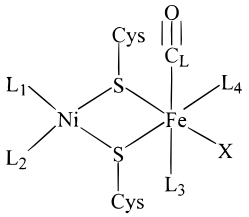

FIGURE 2: Proposed model structure of the active site of CODH from *R. rubrum*. The binding site of Ligand CO (CO_L, $C_L{\equiv}O$) is assigned to the Fe site of the putative [Fe−Ni] cluster on the basis of this study. L_1, L_2, L_3, and L_4 represent possible unknown ligands. A ligand "X" denotes a putative bridging ligand to $[Fe_4S_4]_C$ proposed by the Mössbauer study (*3*). The backbone of the [FeNi] cluster is adapted from the model structure of [NiFe] hydrogenase, *C. vinosum* (*5*). The spatial position of CO_L is assigned arbitrarily.

DISCUSSION

Existence of a Non-Amino Acid Ligand (CO_L) on the Fe Site of the [FeNi] Cluster. The direct ligand binding studies clearly demonstrate the presence of CO_L on CODH. The upper limit of detectable CO_L from CO-treated CODH (0.82 mol of CO_L/mol of CODH) indicates that 1 mol of CO_L binds to 1 mol of CODH stoichiometrically. We propose that the binding site of CO_L is the Fe atom of the proposed [FeNi] cluster and that the structure of the [FeNi] cluster from *R. rubrum* CODH would be $[(CO_L)Fe{-}Ni]$ for holo-CODH and $[(CO_L)Fe{-}\square]$ for Ni-deficient-CODH (where $\square$ = the vacant site normally occupied by Ni) (*9*). The direct observation of CO_L in this study suggests our previous hypothesis, based upon the removal of heterogeneity from the EPR spectrum of CODH in the redox state producing C_{red1} by CO pretreatment, that CO_L is a ligand to the putative [FeNi] cluster (*4*).

The [NiFe] hydrogenase from *Chromatium vinosum* has been demonstrated to contain a CO ligand on the Fe site of its proposed [NiFe] cluster (*5*, *6*). On the basis of this study and on comparison to the active site structure of *C. vinosum* [NiFe] hydrogenase, we propose a model structure of the active center of CODH from *R. rubrum* (Figure 2). The bridging cysteines are speculative. To maintain Fe in a low-spin conformation with two sulfur ligands might require the presence of other strong field ligands such as CN^-. Conversely, the bridging ligands might be nitrogen- or oxygen-based (e.g., histidine or glutamate), in which case a single strong field ligand such as CO_L may be enough to maintain a low-spin configuration.

Cyanide Displaces CO_L. CN^-, a slow, tight-binding inhibitor of CODH displaces CO_L. Inhibition can be recovered upon extended CO treatment (*8*). The slow-binding inhibition by CN^- occurs only in the reduced state of CODH. This slow-binding CN^- is proposed to bind to the putative [FeNi] cluster (*17*). The results presented in Table 2 are interpreted as evidence that CO_L is present in Ni-deficient CODH (i.e., $[(CO_L)Fe{-}\square]$) and that the CO_L is displaced by CN^- to produce $[(CN^-)Fe{-}\square]$. Thus, the mode of inhibition by a ligand displacement is consistent with the slow nature of the inhibition and of reactivation (*8*). Previously, it was shown that CN^- affects the EPR signal attributed to the [FeNi] cluster but not the $[Fe_4S_4]$ clusters (*5*, *6*, *17*). Thus, it is concluded that the site of CO/CN^- binding is on the Fe of the [FeNi] cluster instead of one of the cubanes.

Given these hypotheses, previously determined kinetic constants for the slow-binding CN^- to CODH (*8*) would represent constants for the ligand displacement of CO_L by CN^-. Because both binding of CN^- to the substrate site and ligand displacement of CO_L by CN^- inhibit CODH activity, the true inhibition constants of CO oxidation activity by CN^- could not be accurately measured by monitoring CODH activity. Further study is required to determine the true inhibition constants of CN^- in CODH. Since CN^- displaces CO_L, one could imagine that free CO also can exchange CO_L. However, the analyses of the ^{14}CO binding study suggest that little CO_L was exchanged during the given experimental time (data analyses not shown). It is possible that the CO ligated complex, "CO_L−Fe" of the proposed [FeNi] cluster, is thermodynamically stable. Thus, the dissociation of CO_L from the Fe site of the [FeNi] cluster could not occur unless the thermodynamic barrier is overcome by heating.

Discrepancy of Quantified Values of CO_L by Different Methods. If CO pretreatment produces complete occupancy of CO_L on the suggested [FeNi] cluster and if 1 mol of CODH binds 1 mol of CO_L maximally on the basis of the the upper limit of the detectable CO_L of CO-treated CODH estimated by the SDS/EDTA/heat treatment, the 40% increment of CODH activity corroborates the finding from the ^{14}CO binding study that 0.36 mol of additional CO_L binds to 1 mol of as-isolated CODH (Table 1). Therefore, approximately 0.6 mol of CO_L/mol of CODH would be present on the [FeNi] cluster of as-isolated CODH: 1.0 mol of CO_L/mol of total CODH − 0.4 mol of CO_L/mol of CODH = 0.6 mol of CO_L/mol of as-isolated CODH. However, the total amount of CO_L estimated from the SDS/EDTA/heat-treated as-isolated CODH was 0.20 mol of CO_L/mol CODH. The discrepancy of estimated CO_L on the [FeNi] cluster of as-isolated CODH could arise from the difficulties of quantification of the liberated CO or/and it is an improper assumption that CO-treated CODH has 1.0 mol of CO_L/mol CODH. If one takes values of CO obtained from SDS/EDTA/heat experiments as an upper limit (the maximum occupancy of CO_L obtainable on CO-treated CODH is 0.82 mol of CO_L/mol of CODH) and as-isolated CODH binds 0.4 mol of CO_L/mol CODH additionally by CO treatment (as suggested by the ^{14}CO binding study), then as-isolated CODH is calculated to contain approximately 0.4 mol CO_L/mol of as-isolated CODH: 0.8 mol of CO_L/mol of total CODH − 0.4 mol of CO_L/mol of CODH = 0.4 mol CO_L/mol of as-isolated CODH. This calculated value is closer to the direct value obtained from SDS/EDTA/heat experiment of as-isolated CODH (0.20 mol of CO_L/mol of as-isolated CODH) than the value calculated based upon activity increase (0.6 mol of CO_L/mol of as-isolated CODH). Because 98% of CO equilibrates into the gas phase when equal volumes of gas phase and liquid phase (pure water at 1 atm, and 25 °C) are present, we assumed that most of the CO_L liberated from the denatured CODH is present in the headspace of the vial. However, because the solution contains a significant amount of metals (i.e., Fe and Ni liberated from the denatured CODH), these metals might bind CO nonspecifically, causing less CO to partition into the gas phase. An attempt to measure the dissolved CO from the solution was unsuccessful because the Soret peak of the Hb-assay solution was overwhelmed by a broad spectrum of transferred-liquid phase containing the denatured CODH. Therefore, the value of CO_L obtained by chemical quantification determining total CO content from the gas phase content may not be completely adequate. Nonetheless, from these analyses, we conclude that a significant portion of active site of as-isolated CODH lacks of CO_L, and it consequently gives less CODH activity.

Ni Can Be Removed from the [FeNi] Center That Lacks CO_L. Data shown in Table 3 are consistent with the possibility that the Ni atom removed by dimethylglyoxime originates from an [FeNi] cluster that lacks CO_L and that CO-oxidation activity is directly correlated to both the content of Ni and the presence of CO_L at the [FeNi] cluster. CODH from *C. thermoaceticum* also exhibits labile Ni (30%), and because of this, it was suggested that the labile Ni was coordinatively unsaturated (*15*). Thus, CO_L may be involved not only in the activity of CO oxidation but also in the stability of Ni in the [FeNi] center. The absence of CO_L on the Fe site of the [FeNi] cluster could give rise to a different ligand environment, possibly causing destabilization of the Ni environment. However, it has not yet been determined if, upon Ni insertion, CO_L remains bound to the Fe site in the same manner as in Ni-deficient CODH. For example, it is possible that in holo-CODH, CO_L bridges the two metals of the [FeNi] cluster while it is a terminal ligand in Ni-deficient CODH. The purpose of CO_L is possibly to modulate the redox potential of the putative [FeNi] site. CO_L could also maintain the Fe atom of the active site in a low-spin conformation. In several other CODHs with lower activity relative to *R. rubrum* CODH, significant spectroscopic evidence exists in the literature for high-spin forms of Fe, although they have not been interpreted as such (*18*, *19*). It is possible that these high-spin forms of Fe can be removed in the other CODHs, with a concurrent increase in CODH activity, by CO-treatment.

Metal Contents and the Effect of CO_L on the Structure of CODH. The improvement of purification of CODH not only increases the activity of CO oxidation but also conserves approximately one more Fe atom per monomeric enzyme, probably as a result of protecting $[Fe_4S_4]_B$ from the effects of heat treatment. Gel shift and gel filtration experiments show that there is no significant effect of CO pretreatment on the tertiary structure of holo-CODH and Ni-deficient-CODH. However these results, in conjunction with the lack of an increase in CODH activity after CO pretreatment, suggest that the active site of Ni-deficient-CODH is unstable to heat treatment during purification, possibly due to the absence of Ni at the [FeNi] center. Thus, while the presence of CO_L significantly affects Ni stability and CO oxidation activity, it does not appear to significantly alter the overall protein conformation to a degree observable by anoxic native gel or chromatography; the overall structure of Ni-deficient-CODH protein that lacks CO_L is maintained during the heat-treatment steps, but the structure of the active site might be altered and is no longer able to catalyze even after CO pretreatment. This suggests the $V_{max,CO}$ of Ni-deficient-CODH after Ni incorporation could not be increased by pretreatment with CO. It is also possible that other *coo* proteins are required for the Ni processing to construct the proper [Fe−□] site of Ni-deficient CODH in vitro.

A previous report from our laboratory did not indicate that CN^- binds to Ni-deficient CODH. However, the earlier experiment was performed in a stoppered vial, Ni-deficient CODH was incubated with a relatively high concentration

of CN^- (3 mM), and the CODH sample was subsequently treated by gel filtration to remove unbound CN^- (*8*). Recently, we reported that holo-CODH incubated with greater than 1 mM CN^- was irreversibly inactivated and eventually denatured (*17*). Furthermore, Ni-deficient CODH is more labile to a high concentration of CN^- than holo-CODH, possibly due to lack of Ni in the [FeNi] center (data not shown). We have repeated the experiment of CN^- treatment of Ni-deficient CODH in a manner identical to the published condition (incubated Ni-deficient CODH with 3 mM CN^- in a stoppered vial). Analysis of the results showed that approximately 90% of Ni-deficient CODH sample was denatured and precipitated. The nondenatured CODH (10% of the whole CN^--treated CODH sample) can be separated from the precipitated-CODH sample by application to a gel filtration column. This 10% fraction of CN^--treated (nondenatured) CODH elutes earlier than the CN^--denatured enzyme. The final activity of the Ni-incorporated form of this 10% fraction (Ni incorporation was performed in a stoppered vial) was similar to that of the control (non-CN^--treated, Ni-deficient-CODH). This result is in agreement with the previous report that the activity of pre CN^--treated, Ni-deficient-CODH can be fully recovered by Ni incorporation. However, the rate of Ni activation of the CN^--treated, Ni-deficient-CODH in the presence of less concentrated $NiCl_2$ (<1 mM) was very slow compared to the non-CN^--treated control enzyme. When Ni activation was performed with higher concentrations of $NiCl_2$ (>5 mM) in a stoppered vial, there was no significant difference in Ni activation rate between CN^--pretreated and non-CN^--treated Ni-deficient-CODH. We suggest that the CN^- at the Fe site of Ni-deficient-CODH dissociates during the Ni insertion procedure in a Ni-concentration-dependent manner. If this possibility is correct, CN^- may dissociate from its binding site during prolonged incubations (25 min) with a high concentration of $NiCl_2$ in a stoppered vial. However, when $NiCl_2$ is applied to Ni-deficient-CODH bound to a DE-52 column, less than 10 mL of 1 mM of $NiCl_2$ is required to obtain 40% of CO-pretreated holo-CODH activity. Using the column method, CN^--pretreated, Ni-deficient-CODH retains the CN^- molecule on its binding site, and Ni was incorporated efficiently to the vacant site as shown in Table 3. These results suggest that the dissociation constant for CN^- ($K_{D,CN}$) to the column-bound, Ni-deficient-CODH is relatively low compared to the $K_{D,CN}$ of Ni-deficient CODH in a stoppered vial, possibly due to interaction of certain regions of the protein with the column matrix which would decrease the $K_{D,CN}$ of the enzyme.

CONCLUSIONS

The results presented in this article provide additional quantitative evidence for CO ligand to the proposed [FeNi] cluster and show that under reducing conditions (1) the slow-binding, inactivating CN^- molecule binds to the Fe site of the [Fe−□] center (where □ = a vacant site) producing a [(CN^-)Fe−□] center, (2) the CN^- molecule of the [(CN^-)Fe−□] center can be replaced by CO to revert back to the original [(CO_L)Fe−□] center, (3) Ni can be constituted to the [(CN^-)Fe−□] cluster producing a [(CN^-)Fe−Ni] cluster, and (4) the [(CN^-)Fe−Ni] cluster can revert back to the original [(CO_L)Fe−Ni] cluster upon CO treatment.

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