Function and CO Binding Properties of the NiFe Complex in Carbon Monoxide Dehydrogenase from *Clostridium thermoaceticum*†

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Received June 11, 1992; Revised Manuscript Received September 1, 1992

ABSTRACT: Adding 1,10-phenanthroline to carbon monoxide dehydrogenase from *Clostridium thermoaceticum* results in the complete loss of the NiFeC EPR signal and the CO/acetyl-CoA exchange activity. Other EPR signals characteristic of the enzyme (the $g_{av} = 1.94$ and $g_{av} = 1.86$ signals) and the CO oxidation activity are completely unaffected by the 1,10-phenanthroline treatment. This indicates that there are two catalytic sites on the enzyme; the NiFe complex is required for catalyzing the exchange and acetyl-CoA synthase reactions, while some other site is responsible for CO oxidation. The strength of CO binding to the NiFe complex was examined by titrating dithionite-reduced enzyme with CO. During the titration, the NiFeC EPR signal developed to a final spin intensity of 0.23 spin/$\alpha\beta$. The resulting CO titration curve (NiFeC spins/$\alpha\beta$ vs CO/$\alpha\beta$) was fitted using two reactions: binding of CO to the oxidized NiFe complex, and reduction of the CO-bound species to a form that exhibits the NiFeC signal. Best fits yielded apparent binding constants between 6000 and 14 000 M^{-1} ($K_d = 70$–165 μM). This sizable range is due to uncertainty whether CO binds to all or only a small fraction (~23%) of the NiFe complexes. Reduction of the CO-bound NiFe complex is apparently required to activate it for catalysis. The electron used for this reduction originates from the CO oxidation site, suggesting that delivery of a low-potential electron to the CO-bound NiFe complex is the physiological function of the CO oxidation reaction catalyzed by this enzyme.

Carbon monoxide dehydrogenase (CODH)[1] from *Clostridium thermoaceticum* is the central enzyme in the acetyl-CoA or Wood pathway, an autotrophic pathway used by acetogenic bacteria to generate cellular carbon from CO_2 and H_2 (Ragsdale et al., 1988, 1990; Wood & Ljungdahl, 1991). It catalyzes the synthesis of acetyl-CoA from CO, coenzyme A, and a methyl group bound to the corrinoid/iron–sulfur protein (CoFeS):

$$CH_3\text{–[CoFeS]} + CO + CoASH \rightleftharpoons CH_3\text{–}\overset{O}{\overset{\|}{C}}\text{–SCoA} + [CoFeS]^- + H^+ \quad (1)$$

the reversible oxidation of CO to CO_2:

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

and a variety of exchange reactions including CO/acetyl-CoA exchange:

$$CH_3\text{–}\overset{O}{\overset{\|}{{}^{14}C}}\text{–SCoA} + CO \rightleftharpoons CH_3\text{–}\overset{O}{\overset{\|}{C}}\text{–SCoA} + {}^{14}CO \quad (3)$$

Although all of CODH's activities are destroyed by oxygen, the synthase and exchange activities are far more sensitive to oxygen than is CO oxidation (Ragsdale & Wood, 1985; Raybuck et al., 1988). The exchange activity is also inhibited by a variety of agents which are not inhibitors of CO oxidation activity. From these differential effects, Raybuck et al. (1988) proposed that the synthase and exchange reactions are catalyzed at one site on the enzyme, while CO oxidation is catalyzed at another.

CODH has an $(\alpha\beta)_3$ quaternary structure composed of two types of subunits (Ragsdale et al., 1983a). Recently, both subunits were cloned and their molecular masses were determined to be 72 928 and 81 730 Da (Morton et al., 1991). Each $\alpha\beta$ dimer[2] contains 2 nickels, 11–13 irons, and approximately 14 sulfide ions (Ragsdale et al., 1983a). The metal ions are organized into incompletely understood, highly unusual complexes and clusters.

One of the most unusual complexes in the enzyme, the NiFe complex,[3] may consist of a mononuclear Ni ion complexed predominantly to sulfur ligands and chemically linked to an Fe_4S_4 cluster (Ragsdale et al., 1982, 1983b, 1985; Cramer et al., 1987; Bastian et al., 1988; Lindahl et al., 1990a,b; Fan et al., 1991). The NiFe complex can be stabilized in two states. When reduced, it has $S = {}^1/_2$ and exhibits the NiFeC EPR signal, with $g = 2.08$, 2.075, and 2.028. Reduction to this form can occur by any of three procedures: (1) addition of excess CO to either oxidized or reduced CODH (Ragsdale, 1982, 1983b); (2) addition of CO_2 to CODH samples electrochemically poised below –450 mV using viologens as mediators (Lindahl et al., 1990a); or (3) addition of acetyl-CoA to CODH electrochemically poised in a similar manner (Gorst & Ragsdale, 1991). The presence of hyperfine broadening in the NiFeC signal of samples reduced with ^{13}CO demonstrates that CO or some derivative thereof is bound to the complex in this EPR-active state (Fan et al., 1991). Gorst and Ragsdale (1991) have suggested that CO itself, rather than a derivative of CO, is the bound species.

† This work was supported by the Robert A. Welch Foundation and the Center for Energy and Mineral Resources at Texas A&M University. The EPR spectrometer was obtained with a grant (CHE-8912763) from the National Science Foundation.

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[1] Abbreviations: CODH, carbon monoxide dehydrogenase; $NiFe_{ox}$, oxidized form of NiFe complex; $NiFe_{red}$, one-electron-reduced form of $NiFe_{ox}$; NiFeC, form of NiFe complex yielding the NiFeC EPR signal; $NiFe_{ox}$-CO, CO-bound form of $NiFe_{ox}$; CoA, coenzyme A, FCII, ferrous component II; CoFeS, the corrinoid iron–sulfur protein from *Clostridium thermoaceticum*; phen, 1,10-phenanthroline.

[2] Molar ratios involving CODH will be referred to on a per $\alpha\beta$ dimer basis.

[3] The name "NiFe complex" rather than "NiFeC complex" is used to avoid confusion in distinguishing CO-bound from unbound forms of this complex.

0006-2960/92/0431-12870$03.00/0

The other known state of the complex, designated "$NiFe_{ox}$", is more oxidized than NiFeC and is diamagnetic ($S = 0$). The irons in this state yield a quadrupole doublet with parameters (ΔE_Q = 1.15 mm/s, δ = 0.44 mm/s) typical of $[Fe_4S_4]^{2+}$ clusters (Lindahl et al., 1990b). This state is present when the enzyme is oxidized, but is not easily studied under these conditions because essentially all of the other iron-containing complexes and clusters in CODH are also diamagnetic and yield quadrupole doublets which overlap that from $NiFe_{ox}$. The $NiFe_{ox}$ quadrupole doublet can be isolated from the others by reducing CODH with either sodium dithionite or low-potential, electrochemically reduced viologens in the absence of CO or CO_2 (Lindahl et al., 1990a,b). Under these conditions, all other known metal sites in CODH are reduced, resulting in half-integer spin states and Mössbauer features with magnetic hyperfine interactions. The only site unable to be reduced by these reductants is $NiFe_{ox}$, allowing the $NiFe_{ox}$ quadrupole doublet to be easily distinguished from the other spectral components.

Gorst and Ragsdale (1991) recently monitored the development of the NiFeC signal as the potential of a solution of CODH containing acetyl-CoA was varied electrochemically. From this potentiometric titration, they found that the NiFeC signal develops in a one-electron reduction process in accordance with a midpoint potential of −541 mV.[4] They concluded that the observed value was near to the true reduction potential of whatever redox couple was involved (not specified), but that there might be differences in the two values due to effects of CO binding.

Lu and Ragsdale (1991) found that CODH needs to be reductively activated in a one-electron process before it can catalyze CoA/acetyl-CoA exchange and that the midpoint potential of the site requiring activation was below −486 mV. They suggested that this site was the NiFe complex. This assignment was supported by an experiment in which the ^{13}C-labeled carbonyl group of [1-^{13}C] acetyl-CoA was transferred onto the NiFe complex during CO/acetyl-CoA exchange (Gorst & Ragsdale, 1991). The same authors found that the NiFeC state was kinetically competent to be an intermediate in acetyl-CoA synthesis. These experiments provide strong evidence that the NiFe complex is the site of the synthase and exchange reactions.

CODH contains other metal complexes and clusters besides the NiFe complex. These yield various EPR signals, including the g_{av} = 1.94, g_{av} = 1.86, g_{av} = 1.82, and g_{av} = 2.03 signals (Lindahl et al., 1990a; Shin et al., 1992). The g_{av} = 1.94 signals arise from an $[Fe_4S_4]^{1+}$ cluster, while the others arise from species that are not well understood. The enzyme also contains an EPR-silent species consisting of a single iron, characterized in its reduced state by a distinct quadrupole doublet known as FCII (Lindahl et al., 1990b).

An unusual characteristic of CODH EPR signals is that their intensities are significantly less than the expected values of 1 spin/$\alpha\beta$ (Lindahl et al., 1990a,b). The NiFeC signal, for example, has a maximal intensity corresponding to 0.1–0.4 spins/$\alpha\beta$. The reason for these low values is not understood. Lindahl et al. (1990b) suggested that they may be low because some purified CODH molecules lack one or more metal ions, but this is not known with certainty.

Our long-term goal is to formulate a unified model describing the structures and functions of the metal complexes and clusters in the enzyme. Such a formulation is impeded by the large number of such species present, the diverse reactions catalyzed by the enzyme, and the unknown nature of the low EPR signal intensities. Fortuitously, we discovered a means of selectively destroying the CO/acetyl-CoA exchange activity of CODH and found that this procedure selectively eliminated the NiFeC EPR signal. This has allowed us to assign functions to some of the metal complexes. We have also developed a means of studying the CO-binding properties of the NiFe complex. By adding CO to dithionite-reduced CODH, we determined that CO binds to the NiFe complex with an apparent binding constant comparable to some well-characterized nickel-containing complexes. To analyze the CO titration curve, we formulated two sequential reactions which explicate the known CO binding and redox chemistry of the NiFe complex. In this paper, we describe and analyze these experiments and their implications, including a proposal why CODH catalyzes both CO oxidation and the synthesis of acetyl-CoA.

EXPERIMENTAL PROCEDURES

Enzyme Preparation and Characterization. Clostridium thermoaceticum was grown as described (Lundie & Drake, 1984). Purification and preparation of samples were performed in an anaerobic glovebox (Vacuum/Atmospheres HE-453) containing an argon atmosphere with less than 1 ppm oxygen as detected by an oxygen analyzer (Teledyne Model 310). Rubber septa were stored in the box for 3 days prior to use. CODH (EC 1.2.99.2) was purified and assayed essentially according to published procedures (Ragsdale & Wood, 1985; Raybuck et al., 1988; Ramer et al., 1989). Protein concentrations were determined by the Biuret method (Pelley et al., 1978). Samples used for the 1,10-phenanthroline experiments were homogeneous according to polyacrylamide gel electrophoresis and had 250 and 0.32 units/mg for CO oxidation and CO/acetyl-CoA exchange specific activities, respectively. That used for the CO binding experiment was 95% pure, contained 1.9 Ni and 11.7 Fe per $\alpha\beta$, and had 193 and 0.27 units/mg specific activities.

Treatment of CODH with 1,10-Phenanthroline. 1,10-Phenanthroline (Sigma) was added (1 mM final concentration) to CODH (1.9 mg/mL) solutions containing 50 mM Tris, pH 8.0, and 2 mM dithionite. After mixing, solutions were transferred to EPR tubes and then frozen after 9 h.

CO Titration of Dithionite-Reduced CODH. Modified EPR tubes (4-cm × 5-mm o.d.) had two 1-mm holes drilled opposite each other, 2 mm from the open ends. Small quartz spheres (~3-mm diameter) were inserted into the tubes. The open ends and the holes were sealed with rubber septa. Using a needle, septa were intentionally punctured 5–6 times. The sealed tubes were filled with 18.3 μM CODH, so that no gas phase remained in the tubes. This condition was ensured by injecting enough solution for some to leak through the punctures in the septa. Enough freshly-prepared 0.1 M sodium dithionite was injected into the tubes to yield 2 mM final concentration. Solutions were stirred by repeated inversion of the sphere-containing tubes. From 0 to 45 μL of CO-saturated buffer at 25.5 °C [0.90 mM CO (Budavari, 1989)] was added 3–5 min after the dithionite injection, and solutions were stirred again. All but the top 4 mm of the samples were frozen by dipping into liquid nitrogen-cooled isopentane. Septa were removed, and unfrozen liquid was discarded. A 20-cm × $^1/_4$-in. delrin rod was trimmed at one end to leave two 1-mm pin-like protuberances opposite each other and emanating orthogonal to the axis of the rod. The protuberances were inserted into the holes of the modified EPR tubes, and the assembly was lowered into an Oxford Instruments ER910A cryostat. EPR spectra were obtained with a Bruker ESP300 spectrometer, calibrated with an HP5352B frequency counter

[4] Unless mentioned otherwise, potentials quoted in text are vs NHE.

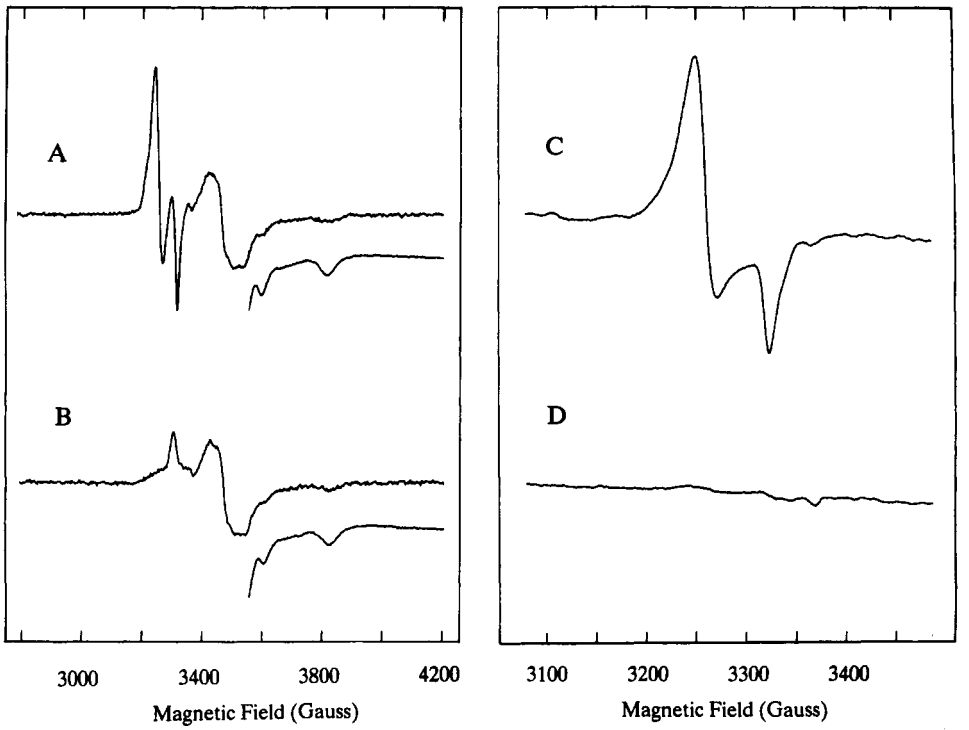


FIGURE 1: EPR of 1,10-phenanthroline-treated CODH. (A) Before adding phen, 10 K; (B) after adding phen, 10 K; (C) same as (A), except at 130 K; (D) same as (B), except at 130 K. Other EPR conditions were as follows: 9.425-GHz microwave frequency; 11.5-G modulation amplitude; 0.05-mW microwave power for (A) and (B); 80-mW microwave power for (C) and (D). Spectra are the average of 5 scans for (A) and (B) and 4 scans for (C) and (D). Shown below the 0.05-mW spectra of (A) and (B) are 20-mW scans that serve to highlight features from the g_{av} = 1.86 signals.

and Bruker ER035M NMR gauss meter. Signal intensities were quantified as described (Orme-Johnson & Orme-Johnson, 1987). The enzyme concentration for the titration was taken as the nickel concentration (atomic absorption spectrometry) divided by 2, correcting for the dilution caused by adding CO-saturated buffer. A molecular mass of 154 700 Da for the CODH $\alpha\beta$ dimer was used in calculations.

Analysis of CO Titration. The CO titration and the potentiometric titration of Gorst and Ragsdale (1991) were simulated using programs written in the ASYST (McMillian Co.) language. Equilibrium concentrations of the $NiFe_{ox}$, $NiFe_{ox}$-CO, and NiFeC forms of the NiFe complex were determined for solutions of CODH containing CO at various concentrations or poised at different solution potentials, in accordance with reactions 5 and 6. Equilibrium concentrations were determined by iteratively solving equilibrium expressions for one reaction, and then using the concentrations thereby obtained as the starting values for the other. A similar procedure was used for the thermodynamic reaction cycle. The equilibrium constants K_2 and K_4 corresponding to the reduction potentials $E^{\circ\prime}_2$ and $E^{\circ\prime}_4$ were obtained from the relation $K_{2(4)} = \exp(\Delta E n \mathcal{F}/RT)$, where $n = 1$, $\mathcal{F}$ = 96 487 C/equiv, R = 8.314 J/(mol·K), T = 300 K, $\Delta E = E^{\circ\prime}_{2(4)} - E^{\circ\prime}_d$, and $E^{\circ\prime}_d$ is the reduction potential for 2 mM sodium dithionite at pH 8, taken as –0.60 V (Mayhew, 1978). We could not measure the potential of the solution used in the titration (containing 2 mM dithionite at pH 8.0), but another CODH solution similarly prepared exhibited a potential of –0.82 V vs Ag/AgCl, corresponding to –0.60 V vs NHE.

RESULTS AND DISCUSSION

Enzymatic Function of the NiFe Complex. The bidentate ligand 1,10-phenanthroline (phen) was added to CODH as described in Experimental Procedures. The CO/acetyl-CoA exchange and CO oxidation activities were 0.32 and 250 units/mg, respectively, prior to addition and 0.0 and 240 units/mg after addition. Thus, adding phen to CODH completely inactivated the CO/acetyl-CoA exchange activity, but had no effect on CO oxidation activity. This demonstrates that CODH can catalyze CO oxidation with *complete independence* of its ability to catalyze CO/acetyl-CoA exchange and supports the conclusion of Raybuck et al. (1988) that CO oxidation and the exchange/synthase reactions are catalyzed at different sites in CODH.

Prior to adding phen, EPR spectra of CO-reduced CODH samples at 10 K exhibited the NiFeC, g_{av} = 1.94, and g_{av} = 1.86 signals, with intensities of 0.21, 0.54, and 0.17 spin/$\alpha\beta$, respectively (Figure 1A). These values are typical of CODH reduced by CO. After adding phen, spectra of the CO-reduced samples, shown in Figure 1B, exhibited the g_{av} = 1.94 and g_{av} = 1.86 signals with intensities (0.53 and 0.16 spin/$\alpha\beta$) essentially the same as obtained prior to adding phen. Most importantly, *the NiFeC signal was absent* in spectra from the treated samples. This is most clearly seen in high-temperature EPR spectra (Figure 1C,D). Thus, adding phen to CODH caused the complete loss of the NiFeC signal, but had no effect on the other CODH EPR signals. This strongly suggests that phen bound to or reacted with the NiFe complex and that it did not bind/react with the other EPR-active species in CODH. These experiments indicate that *the NiFe complex is required for exchange and synthase activities and is not required for CO oxidation.*

CO oxidation must therefore occur at a site other than the NiFe complex, which we shall designated the "CO oxidation site". This site binds CO, oxidizes it to CO_2, and delivers the electrons obtained from this oxidation to other metal complexes and clusters in CODH, yielding the g_{av} = 1.94, g_{av} = 1.86, and (as we shall argue below) the NiFeC signals.

CO Titration of Dithionite-Reduced CODH. For most metal complexes which exhibit measurable changes upon binding CO, binding constants can be obtained simply by measuring the extent of those changes at different partial

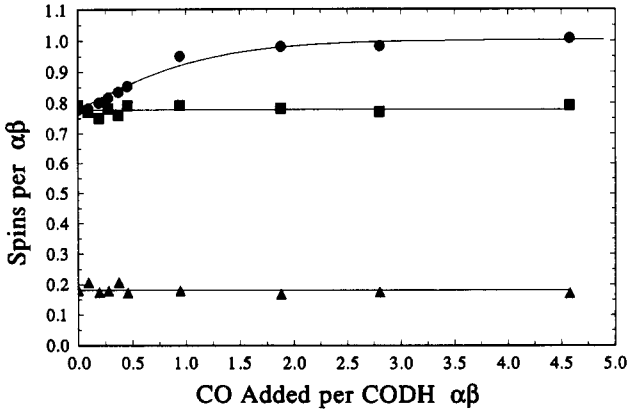


FIGURE 2: EPR signal intensities of CO titration samples. Triangles indicate g_{av} = 1.86 signal intensities, in spins/$\alpha\beta$. Solid line through the triangles, at 0.18 spin/$\alpha\beta$, represents the average intensity. Squares indicate g_{av} = 1.94 signal intensities offset by 0.18 spin/$\alpha\beta$. Solid line through the squares, at 0.78 spin/$\alpha\beta$, represents the sum of the average g_{av} = 1.94 and g_{av} = 1.86 intensities. Circles indicate NiFeC signal intensities offset by 0.78 spin/$\alpha\beta$. Solid curve through circles was the same as in Figure 3, except offset by 0.78 spin/$\alpha\beta$. EPR conditions were as in Figure 1.

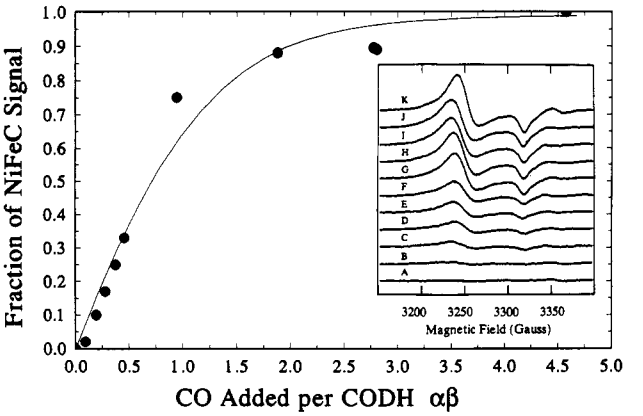


FIGURE 3: CO titration of dithionite-reduced CODH. Circles indicate NiFeC signal intensities, normalized to 0.23 spin/$\alpha\beta$. Solid line is a simulation, using reactions 5 and 6, with K_d = 80 μM and $E^{\circ\prime}_2$ = −540 mV. Inset shows NiFeC signals of the samples used in the titration. The number of moles of CO added per mole of CODH $\alpha\beta$ was as follows: (A) 0.00; (B) 0.09; (C) 0.19; (D) 0.28; (E) 0.37; (F) 0.46; (G) 0.94; (H) 1.88; (I) 2.80; (J) 4.58; (K) 48.9 (CO saturated).

pressures of CO. But due to the extraordinary complexity of CODH, this method could not be used here; even at low partial pressures, CO will be actively removed from the atmosphere by the enzyme and will be used to reduce all of the complexes housed therein. A plot of the NiFeC signal intensity obtained by adding CO incrementally to oxidized enzyme would also not readily yield the desired CO binding constant, because some of the added CO would be used to reduce other sites in CODH, and the resulting curve would not solely reflect CO binding to the NiFe complex.

How could the binding of CO to NiFe be studied in isolation from other binding or reduction reactions? Our strategy was to add CO incrementally to *dithionite-reduced* CODH and follow the development of the NiFeC signal. Dithionite reduces all known metal sites in CODH except the $NiFe_{ox}$ form of the NiFe complex. We anticipated that all of the CO added to dithionite-reduced CODH would be used in binding, not in reduction processes.

Different amounts of CO were added to aliquots of a CODH solution containing an excess of sodium dithionite. The spin intensities of the EPR signals from the resulting samples are summarized graphically in Figure 2. As expected, the g_{av} = 1.94 and 1.86 signals were present in all spectra, with average intensities of 0.60 and 0.18 spin/$\alpha\beta$, respectively. These values are typical of CODH fully-reduced by dithionite.

The NiFeC signal developed with added CO, as shown in the inset of Figure 3, to a final intensity corresponding to 0.23 spin/$\alpha\beta$. The CO titration curve in Figure 3 was obtained by plotting the NiFeC signal intensities from each sample versus the number of CO/$\alpha\beta$ added.

Analysis of the CO Titration. Since the NiFe complex was in the $NiFe_{ox}$ form at the beginning of the CO titration and the NiFeC form at the end, one might suppose that this titration could be described by

$$NiFe_{ox} + CO \rightleftharpoons NiFeC \quad (4)$$

However, the chemistry occurring must be more complex. A comparison of the spin state of $NiFe_{ox}$ ($S = 0$) with that of NiFeC ($S = {}^1/_2$) indicates that the two states differ by an odd number of electrons. Gorst and Ragsdale (1991) measured a midpoint potential of −541 mV and $n = 1$ behavior for the reduction of the NiFe complex, indicating that the NiFeC state is one electron more reduced than $NiFe_{ox}$. Thus, the generation of the NiFeC state from $NiFe_{ox}$ must involve both CO binding and one-electron reduction.

The inability of dithionite or electrochemically-reduced low-potential viologens to reduce $NiFe_{ox}$ by one electron suggests that $NiFe_{ox}$ must bind CO *before* it becomes reduced, as shown in the following reactions:

$$NiFe_{ox} + CO \rightleftharpoons NiFe_{ox}\text{-}CO \quad (5)$$

$$NiFe_{ox}\text{-}CO + e^- \rightleftharpoons NiFeC \quad (6)$$

$NiFe_{ox}$-CO is the CO-bound product of $NiFe_{ox}$. We designate K_1 to be the CO binding constant for reaction 5 and $E^{\circ\prime}_2$ the reduction potential for the half-cell reaction 6 at neutral pH.

Our goal was to fit the potentiometric titration data of Gorst and Ragsdale (1991) and our CO titration data using reactions 5 and 6. If both CO binding and reduction occurred quantitatively, these reactions imply that the NiFeC signal should develop to an intensity of 1 spin/$\alpha\beta$. As mentioned above, it is an unexplained fact that the fully-developed NiFeC signal quantifies to only 0.1–0.4 spin/$\alpha\beta$. The final value of 0.23 spin/$\alpha\beta$ observed in our CO titration is well within the normal range. However, the appropriate analysis of the titration depends on the reason for the low values. For example, one analysis would be used if only 23% of the NiFe complexes exhibit NiFeC signals (with normal intensities) and another if 100% of the complexes exhibit signals with intensities only 23% of normal. We cannot presently distinguish between these two cases, and so we have analyzed the data assuming each case separately.

Another issue is whether the low spin intensity arose because the reductants used to effect reaction 6 were thermodynamically unable to reduce more than 23% of the $NiFe_{ox}$-CO form. This appears not to be the case; the solution potential in the presence of dithionite (−600 mV) was substantially below the calculated reduction potential of −540 mV for reaction 6, and the strength of CO binding appears substantially stronger than would be required for this scenario (the bend in the CO titration data of Figure 3 is too sharp). Our data cannot exclude the possibility that the reductants are *kinetically* able to reduce only 23% of $NiFe_{ox}$-CO. However, this situation would not affect our ability to extract the CO binding constant for reaction 5 or the reduction potential for reaction 6 from the titration data.[5]

Gorst and Ragsdale (1991) cautioned that the midpoint potential (−541 mV) obtained by fitting their potentiometric

titration curve using the Nernst equation for a single $n = 1$ half-cell reaction might differ from the true reduction potential (what we have designated $E^{\circ\prime}_2$), depending on the strength of CO binding. To determine this dependence, we simulated their potentiometric titration data using reactions 5 and 6, assuming that CO bound to each NiFe complex. The observed "midpoint potentials" of the simulations varied moderately depending on the value of K_1.[6] If CO binding was assumed fairly strong ($K_1 = 10^5$–10^7 M^{-1}), the data fit best to $E^{\circ\prime}_2$ between –540 and –550 mV. In the case of weak CO binding, satisfactory fits required more positive $E^{\circ\prime}_2$ values.

Next, we simulated the CO titration data of Figure 3 using reactions 5 and 6, again assuming that CO bound to each NiFe complex. The CO binding constants obtained were likewise only moderately dependent upon changes in $E^{\circ\prime}_2$. Simulations with $E^{\circ\prime}_2$ ranging from –510 to –560 mV fit the CO titration data best with K_1 ranging between 1.0×10^4 and 1.4×10^4 M^{-1}. Using an iterative procedure, the best fits to both sets of data were obtained with $K_1 = (1.2 \pm 0.2) \times 10^4$ M^{-1} and $E^{\circ\prime}_2 = -540$ mV. A similar analysis of the data assuming that CO binds to only 23% of the NiFe complexes yielded $K_1 = (7 \pm 1) \times 10^3$ M^{-1}.[7] We conclude that the apparent binding constant for reaction 5 (K_1) lies between 6000 and 14 000 M^{-1} (K_d = 70–165 μM), and the reduction potential at neutral pH for half-cell reaction 6 is –540 mV.

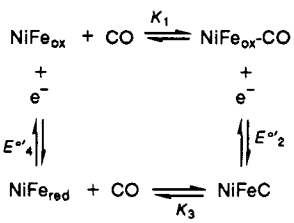
The exact site on the NiFe complex at which CO binds is unknown, but nickel and iron are likely candidates. Other nickel and iron complexes bind CO with a variety of strengths. In a series of formally Ni^{1+} tetraaza macrocycles, CO binding constants range from zero to 10^5 M^{-1} (Gagné & Ingle, 1981) ($K_d \geq 10$ μM). The hydrogenase from *Desulfovibrio gigas* binds CO at a mononuclear nickel site with $K_i = 35$ μM (Fauque et al., 1988). CO binds extremely strongly (K_d = 1.1 μM) to the oxidized states of the iron–sulfur clusters known as "H-clusters" in hydrogenases I and II from *Clostridium pasteurianum* (Adams, 1987).

Analysis of the CO-Binding and Redox Properties of the NiFe Complex Using a Thermodynamic Cycle. Our proposal that CO binds to $NiFe_{ox}$ before the complex is reduced assumes that there is a kinetic barrier preventing dithionite or low-potential viologens in the absence of CO, CO_2, or acetyl-CoA from reducing $NiFe_{ox}$. If the extent of the reduction

$$NiFe_{ox} + e^- \rightleftharpoons NiFe_{red} \qquad (7)$$

were determined by thermodynamics, the pathway (binding before reduction) used to transform $NiFe_{ox}$ to NiFeC could not be determined rigorously without studying the kinetics of the system.

We have considered this alternative situation by analyzing the CO binding and potentiometric titrations using the following thermodynamic cycle:



The cycle consists of two known states of the complex, $NiFe_{ox}$ and NiFeC, in equilibrium with two postulated states, $NiFe_{red}$ and $NiFe_{ox}$-CO. K_1 and K_3 are equilibrium constants for CO binding to $NiFe_{ox}$ and $NiFe_{red}$. $E^{\circ\prime}_2$ and $E^{\circ\prime}_4$ are thermodynamic reduction potentials for the $NiFe_{ox}$-CO/NiFeC and $NiFe_{ox}/NiFe_{red}$ couples, respectively, with corresponding equilibrium constants K_2 and K_4.

Since $NiFe_{red}$ has not been observed, even when solution potentials are –600 mV, we estimated that $E^{\circ\prime}_4 < -660$ mV and $K_4 < 0.1$. For the simulations, K_4 was varied from 0.1 to 0.0001. K_1 and K_2 were experimentally determined by fitting the CO binding and potentiometric titrations, and K_3 was determined from the relationship $K_1K_2 = K_3K_4$.

Acceptable values for K_1 and $E^{\circ\prime}_2$ were similar to those obtained using reactions 5 and 6, but with larger uncertainties. For all acceptable fits, $K_1 < K_3$ and $K_2 > K_4$, which means that CO binds much tighter to $NiFe_{red}$ than to $NiFe_{ox}$ and that CO binding raises the reduction potential of the NiFe complex. This latter prediction is qualitatively in accord with the observed effects of CO binding to the H-clusters of hydrogenases I and II from *C. pasteurianum* (Adams, 1987). The reduction potentials of these clusters increase from –410 and –400 mV, respectively, in the absence of CO to –340 and –370 mV, respectively, with CO bound. Thus, CO binding increases the reduction potential of these clusters by 30–70 mV. For the NiFe complex, assuming that the thermodynamic cycle is operative, CO binding increases the reduction potential of the complex by at least 120 mV.

The NiFe reaction cycle does not specify the pathway used in the binding and reduction of $NiFe_{ox}$; $NiFe_{ox}$ may bind CO and then become reduced, or it may become reduced and then bind CO. However, the latter pathway becomes chemically unreasonable as $E^{\circ\prime}_4$ declines below approximately –700 mV. This is because the product K_1K_2 was relatively fixed experimentally by the titration curves. Therefore, as $E^{\circ\prime}_4$ declines, K_3 must increase so as to satisfy the relation $K_1K_2 = K_3K_4$. For E_4 much less than –700 mV, the CO binding constant K_3 becomes unrealistically strong ($K_d < 85$ nM). For the reduction-before-binding pathway, the miniscule proportion of $NiFe_{ox}$ that is reduced at –600 mV is bound so tightly by CO that they are quantitatively transformed to the NiFeC state. On the other hand, for the binding-before-reduction pathway, CO binds $NiFe_{ox}$ with moderate strength (K_d = 70–165 μM), comparable to many Ni complexes, and the resulting $NiFe_{ox}$-CO species is reduced at reduction potentials ($E^{\circ\prime}_2 = -540$ mV) comparable to those of some iron–sulfur clusters and Ni^{2+} complexes. Thus, without assuming any kinetic barrier for reaction 7, the binding-before-reduction pathway is chemically more reasonable.

CO Titration of Dithionite-Reduced CODH Reflects Binding to NiFe Complex Only. CO binds and reacts at two sites on CODH, the NiFe complex and the CO oxidation site. We have analyzed the CO titration curve as though all of the added CO bound exclusively to the NiFe complex. How can we be sure that some of it didn't bind to the other site? If the CO bound more tightly to another site, the NiFeC signal would not have begun to develop with the first addition of CO. If CO bound more weakly to another site, the shape of the CO binding curve (and our analysis) would not have been affected, because added CO would not have bound at the weaker site until it had bound quantitatively at $NiFe_{ox}$. Only the case where CO bound to another site with about the same binding constant as it bound to $NiFe_{ox}$ would the interpretation of the CO titration be affected. However, we doubt that this

[5] It would affect the number of electron equivalents needed for reaction 6. Only 0.23 electron equiv/$\alpha\beta$ would be used if a kinetic barrier prevented the reduction of 73% of the $NiFe_{ox}$-CO complexes.

[6] Simulations available upon request.

[7] Analysis of another CO binding titration curve, obtained at a significantly lower enzyme concentration (10 μM), yielded similar apparent binding constants.

situation occurred, for the following reasons: First, it would be unusually coincidental for CO to bind the CO oxidation site with the same strength as it binds $NiFe_{ox}$, since CO binding constants vary, depending on the structure of the complex involved. The structure of the CO oxidation site must be different than $NiFe_{ox}$ since there is only one $NiFe_{ox}$ per $\alpha\beta$. Second, since the experiment was performed in the presence of dithionite, CO would have been bound to the *reduced* form of the CO oxidation site. The reduced forms of the other known species in CODH exhibit distinct EPR or Mössbauer signals, none of which (according to the available information) shifts in position or changes shape in the presence of CO. This suggests that CO does not bind to the reduced forms of these species. Moreover, it makes more mechanistic sense for CO to bind to the oxidized form of the CO oxidation site, since its function is to remove electrons from CO. These considerations strongly suggest that the CO added during the titration bound only to $NiFe_{ox}$.

Related to these considerations is that CO is a noncompetitive inhibitor of the acetyl-CoA exchange reaction, with K_i = 400 μM (Lu & Ragsdale, 1991). Noncompetitive inhibitors bind sites on enzymes different from where substrates bind (Segel, 1975). Because CO binding at the inhibitor site is much weaker than at $NiFe_{ox}$, the CO titration should have been unaffected by CO binding at the inhibitor site.

Possible Function of the CO Oxidation Site in Activating the NiFe Complex. We have shown here that the NiFe complex binds CO but does not function to oxidize CO to CO_2. Thus, the CO bound at this complex appears to be used exclusively as a substrate for acetyl-CoA synthesis. It is well-known that the addition of CO to oxidized CODH (without adding another reductant) yields the NiFeC signal. In this situation, the electron used to reduce $NiFe_{ox}$-CO must have originated from a CO molecule oxidized at the CO oxidation site, not from the CO bound to $NiFe_{ox}$. Lu and Ragsdale (1991) found that CODH must be reductively activated before it is able to catalyze exchange reactions and concluded that it is the NiFe complex which must be activated. We propose that this activation corresponds to the reduction of $NiFe_{ox}$-CO, reaction 6. Taken together, these results suggest that *the physiological function of the CO oxidation site is to deliver an electron to the NiFe complex with potential low enough to activate it for acetyl-CoA synthesis.*

SUMMARY

The selective loss of exchange activity and the NiFeC signal that occurs upon addition of 1,10-phenanthroline to CODH indicates that there are two catalytic sites in the enzyme; the NiFe complex, required for catalyzing the exchange/synthase reactions, and an unidentified site, responsible for catalyzing the oxidation of CO. The CO binding curve obtained by monitoring the NiFeC signal as CO was added incrementally to dithionite-reduced CODH can be explained using two elementary reactions, CO binding (with a binding constant K_d = 70–165 μM) to the oxidized NiFe complex, followed by a one-electron reduction (with $E^{\circ\prime}$ = −540 mV) of the CO-bound species to the EPR-active NiFeC form. CO is oxidized at the CO-oxidation site, and one of the electrons resulting from that oxidation reduces $NiFe_{ox}$-CO, activating it for catalysis. Delivery of this low-potential electron to the NiFe complex is probably the physiological function of the CO oxidation activity of CODH. The proposals presented here are simple and congruent with the available evidence and thus provide a viable framework for further investigations.

ACKNOWLEDGMENT

We thank David P. Giedroc for helpful discussions regarding how best to analyze the CO binding experiments.

REFERENCES

Adams, M. W. W. (1987) *J. Biol. Chem. 262*, 15054–15061.
Bastian, N. R., Diekert, G., Niederhoffer, E. C., Teo, B. K., Walsh, C. T., & Orme-Johnson, W. H. (1988) *J. Am. Chem. Soc. 110*, 5581–5582.
Budavari, S. (1989) *The Merck Index*, p 275, Merck & Co., Rahway, NJ.
Cramer, S. P., Eidsness, M. K., Pan, W.-H., Morton, T. A., Ragsdale, S. W., DerVartanian, D. V., Ljungdahl, L. G., & Scott, R. A. (1987) *Inorg. Chem. 26* , 2477–2479.
Fan, C., Gorst, C. M., Ragsdale, S. W., & Hoffman, B. M. (1991) *Biochemistry 30*, 431–435.
Fauque, G., Peck, H. D. Jr., Moura, J. J. G., Huynh, B. H., Berlier, Y., Der Vartanian, D. V., Teixeira, M., Przybyla, A. E., Lespinat, P. A., Moura, I., & LeGall, J. (1988) *FEMS Microbiol. Rev. 54*, 299–344.
Gagné, R. R., & Ingle, D. M. (1981) *Inorg. Chem. 20*, 420–425.
Gorst, C. M., & Ragsdale, S. W. (1991) *J. Biol. Chem. 266*, 20687–20693.
Lindahl, P. A., Münck, E., & Ragsdale, S. W. (1990a) *J. Biol. Chem. 265*, 3873–3880.
Lindahl, P. A., Ragsdale, S. W., & Münck, E. (1990b) *J. Biol. Chem. 265*, 3880–3888.
Lu, W.-P., & Ragsdale, S. W. (1991) *J. Biol. Chem. 266*, 3554–3564.
Lundie, L. L., Jr., & Drake, H. L. (1984) *J. Bacteriol. 159*, 700–703.
Mayhew, S. G. (1978) *Eur. J. Biochem. 85*, 535–547.
Morton, T. A., Runquist, J. A., Ragsdale, S. W., Shanmugasundaram, T., Wood, H. G., & Ljungdahl, L. G. (1991) *J. Biol. Chem. 266*, 23824–23828.
Orme-Johnson, N. R., & Orme-Johnson, W. H. (1978) *Methods Enzymol. 52*, 252–257.
Pelley, J. W., Garner, C. W., & Little, G. H. (1978) *Anal. Biochem. 86*, 341–343.
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., Wood, H. G., & Antholine, W. E. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6811–6814.
Ragsdale, S. W., Wood, H. G., Morton, H. G., Ljungdahl, L. G., & DerVartanian, D. V. (1988) in *The Bioinorganic Chemistry of Nickel* (Lancaster, J. R., Ed.) pp 311–333, VCH, New York.
Ragsdale, S. W., Baur, J. R., Gorst, C. M., Harder, S. R., Lu, W.-P., Roberts, D. L., Runquist, J. A., & Schiau, I. (1990) *FEMS Microbiol. Rev. 87*, 397–402.
Ramer, S. E., Raybuck, S. A., Orme-Johnson, W. H., & Walsh, C. T. (1989) *Biochemistry 28*, 4675–4680.
Raybuck, S. A., Bastian, N. R., Orme-Johnson, W. H., & Walsh, C. T. (1988) *Biochemisty 27*, 7698–7702.
Segel, I. H. (1975) *Enzyme Kinetics*, p 125, Wiley-Interscience, New York.
Shin, W., & Lindahl, P. A. (1992) *Biochemistry 31*, 6003–6011.
Wood, H. G., & Ljungdahl, L. G. (1991) in *Variations in Autotrophic Life* (Shively, J. M., & Barton, L. L., Eds.) pp 201–250, Academic Press, London.

Registry No. CODH, 64972-88-9; CO, 630-08-0; AcCoA, 72-89-9.