CO Dehydrogenase from Clostridium thermoaceticum

EPR AND ELECTROCHEMICAL STUDIES IN CO2 AND ARGON ATMOSPHERES

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The EPR and redox properties of the metal complexes in CO dehydrogenase (CODH) from Clostridium thermoaceticum were studied. Controlled potential coulometric reductive titrations of CODH were performed under argon and CO2 atmospheres. In the titrations performed under argon, five to eight electrons/dimer were required for reduction, and four distinct EPR signals appeared. These included a signal with $g_{xx} = 1.82$ ($E_0 = -220$ mV), two signals with the same $g$ values but different linewidths at $g_{xx} = 1.94$ ($E_0 = -440$ mV), and a signal at $g_{xx} = 1.86$ ($E_0 = -530$ mV). All of the $S = 1/2$ EPR signals had low spin concentration values, whereas $0.2$ and $0.3$ spins/dimer were typically obtained for each signal. Features between $g = 6$ and 4, typical of $S = 3/2$ states, were also observed, and these may account, at least to some degree, for the low spin concentration values.

Under CO2, and at negative potentials, CODH served as an electrocatalyst in the reduction of CO2 to CO. The apparent half-maximal activity for this reduction at pH 6.3 occurred at $-430$ mV, a potential near the thermodynamic value. An EPR signal, arising from a complex containing Ni, Fe, and the carbon from CO/CO2 developed along with this activity. The reduction of this complex is probably the last step to occur prior to the catalysis of CO2 reduction.

Carbon monoxide dehydrogenase (CODH) from Clostridium thermoaceticum catalyzes the following two reactions (1-3):

$$
\text{CO} + \text{H}_2\text{O} \rightarrow \text{CO}_2 + 2\text{H}^+ + 2\text{e}^-
$$

$$
\text{CH}_3\text{H}_2\text{O}[\text{Co}] + \text{CO} + \text{CoASH} \rightarrow \text{CH}_3\text{C(Oi)SCoA} + [\text{Co}].
$$

In the first reaction, carbon monoxide is oxidized to carbon dioxide, while in the second, carbon monoxide, a methyl group, and coenzyme A are oxidized by CODH to form acetyl-CoA. The ability of CODH to catalyze the second reaction has only recently been discovered, and this has established the enzyme as a central participant in the synthesis of acetate from CO2 and H2 in acetogenic bacteria (4). We have proposed that CODH might be more appropriately called acetyl-CoA synthase (4). The pathway responsible for this reaction involves at least three additional enzymes, including methyltransferase, a corrinoid/Fe-S protein ([Co]), and disulfide reductase. Recently, it was found that the genes coding for CODH, methyltransferase, and the corrinoid/Fe-S protein are clustered within a 10-kilobase region of DNA (5).

In this pathway, CO2 is reduced to 5-methyltetrahydrofolate via a series of reactions involving formate dehydrogenase and tetrahydrofolic acid-related enzymes (1, 6). Methyltransferase catalyzes the transfer of this methyl group to the corrinoid/Fe-S protein, forming enzyme-bound methylcobalamin (7).

The corrinoid-bound methyl group is then transferred to a site, apparently a cytochrome residue (8), on CODH. Finally, CODH binds CO and CoASH and acetyl-CoA is formed (4). At some point in the final steps, a disulfide reductase may be involved (9). A low potential electron carrier, such as an eight-iron ferredoxin, is required for efficient catalysis of acetyl-CoA synthesis (4, 10).

CODH from the acetogenic bacterium, C. thermoaceticum, has been purified to apparent homogeneity (11). Polyacrylamide gel electrophoresis of the enzyme under denaturing conditions yields two protein bands of equal intensity, corresponding to $\alpha$ and $\beta$ subunits of $M = 71,000$ and 78,000 (4, 11). Gel filtration studies indicate that CODH has a molecular weight of 440,000, suggesting an $\alpha_6\beta_6$ heminergic protein subunit structure (11). Pore-limit electrophoresis suggests that the $\alpha$ dimer may be active as well (12). Metal and sulfide analyses yield about 11 irons, 1.8 nickels, 1 zinc, and 14 acid-labile sulfide ions/dimer (11). Upon prolonged incubation with disulfide reductase, $\sim 17$ sulphydryl groups/dimer are formed (9).

The metals are arranged into a variety of complexes with poorly understood structures. Upon incubation of CODH with CO, an axial EPR spectrum (the NiFeC signal) with effective $g$ values of $g_\parallel = 2.08$ and $g_\perp = 2.02$ is observed (13). Owing to its slow electronic spin relaxation rate, the signal is observable at temperatures as high as 170 K. This signal arises from a species containing Ni, Fe, and the carbon from CO, as demonstrated by the hyperfine broadening of the signal when samples are enriched in either $^{61}$Ni (14), $^{13}$C0 (14), or $^{57}$Fe (15). Therefore, the complex responsible for this EPR signal has been referred to as a NiFeC complex (15).

A rhombic signal with $g$ values of 2.062, 2.047, and 2.028 is sometimes observed in spectra of CO-treated samples (15). It is thought to arise from an altered form of the NiFeC complex (15). Addition of CoASH converts the rhombic signal into the

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§ The abbreviations used are: CODH, CO dehydrogenase; CPC, controlled potential coulometry; mW, milliwatts.

2 Very recently, Ramer et al. (48) suggested that the subunit stoichiometry of the CODH holoenzyme is actual $\alpha_6\beta_6$ and that the $\gamma$ subunit is the disulfide reductase polypeptide.
EPR and Electrochemistry of CO Dehydrogenase

axial form, which led to the suggestion (15) that CoASH binds near or at the complex. When CODH is treated with N bromosaccharinimide, an agent known to modify tryptophan residues, only the rhombic signal is observed (16). From fluorescent quenching studies, it has been suggested that a tryptophan residue may be involved in the binding of CoASH to CODH (17).

The reduced enzyme exhibits a g = 1.94-type EPR spectrum, as well as another spectrum with g values at 1.97, 1.87, and 1.75 (gav = 1.86) (13, 18). It has been suggested that these signals arise from [Fe₅S₅]⁺⁺ clusters (21). Another signal with g values of 2.01, 1.81, and 1.65 (gav = 1.82) has been observed with both dithionite-reduced samples or CO-treated samples to which the oxidized corrinoid/Fe-S protein was added (18, 19).

Both iron and nickel EXAFS of the enzyme have been reported (20, 21). Analysis of the iron EXAFS spectrum indicates the presence of iron-sulfur clusters. The reported Fe-Fe coordination number of 2 ± 0.6 is most consistent with the presence of [Fe₅S₅] clusters (21). The nickel ions have been proposed to be bound to either four sulfurs, at 2.16 Å, (21) or to a combination of sulfur and oxygen/nitrogen ligands (20).

The CODHs from other anaerobic bacteria contain nickel and iron complexes as well and exhibit related but not identical EPR signals. The enzyme from the acetogen Acetobacterium woodii has the same (αβ)₅ subunit structure (12) and physiological role (15) as the C. thermoacetaticum enzyme. The reduced form of the enzyme from A. woodii yields EPR signals similar to the gav = 1.94 and NiFeC signals of C. thermoaetaticum (12). The CODH from Methanosarcina thermophila exhibits a NiFeC-type EPR signal similar to the acetogenic enzymes (22). The reduced CO dehydrogenase from Rhodospirillum rubrum exhibits two gav = 1.94 signals, a gav = 1.82-type spectrum (termed signal A), and a gav = 1.56-type spectrum (termed signal B) (23, 24). No NiFeC-type signal has been reported for the R. rubrum enzyme. A nickel-deficient inactive form of the R. rubrum CODH exhibits g = 1.94 signals identical to those in the holoenzyme (25). Unlike the enzyme from C. thermoacetaticum, the R. rubrum enzyme is not involved in acetate synthesis.

We have studied the magnetic and redox properties of the metal complexes in CODH by combining EPR spectroscopy with controlled potential coulometry (CPC). Besides providing estimates of the reduction potentials for each EPR-active complex, we identify low field EPR signals between g = 6 and 4 and report that the enzyme can serve as an efficient electron catalyst in the reduction of CO₂ to CO. We also highlight some major problems facing future biophysical studies of this enzyme. In the following paper we report the results of a Mössbauer study of this enzyme.

EXPERIMENTAL PROCEDURES

C. thermoacetaticum (DSM 521) cells were grown in 20 liters of medium as described earlier (26). CODH was prepared under anaerobic conditions in a chamber (Coy Laboratory Products) in the presence of 5 mM dithiotreitol as described (4). The six preparations used in these experiments had an average specific activity of 380 ± 80 units/mg in the oxidation of CO to CO₂, and 130 ± 50 units/mg in the exchange of [1-¹⁴C]acetate-Fe₄S₄ (ICN Radiochemicals) with CO (4). The specific activity (CO oxidation) of the protein used in the argon-atmosphere titration shown in Fig. 2 and the CO₂-atmosphere titration (Figs. 3 and 4) was 350 units/mg. Protein was determined by the method of Lowry et al. (28) using five iron standards to generate the standard curve. Sample was prepared for metal analysis by digestion in polyethylene tubes at 84 °C for 18 h in 2 M metal-free hydrochloric acid. The tops of the tubes were sealed with epoxy to prevent evaporation.

Nanometer chloride and dithiotreitol were removed from sample solutions by anode gel filtration chromatography (Sephadex G-25 in 100 mM Tris, pH 7.6). Samples were oxidized by slow, stepwise additions of excess thionin (~2 mM) (Sigma) to stirred solutions. After ~20 min of incubation, they were chromatographed (Sephadex G-25) to remove the dye and concentrated with an Amicon (W. R. Grace) unit (PM-30 membrane).

The electrochemical cell and procedures used for the titrations are described in the following paper (30). The saturated calomel reference electrode was found to have a potential of ±250 mV versus NHE, using the methyl viologen couple (Eₐ = -440 mV) as a standard. All potentials quoted in the text are versus NHE, and were obtained at intermediate pH values. A typical redox buffer solution poised under an argon atmosphere had a pH of 7.2, while that poised under a CO₂ atmosphere had a pH of 6.2. Reduction potentials were obtained by fitting spin concentration versus potential data by the least squares method to the Nernst equation,

\[ E_i = E_m - \frac{0.059}{n} \log \frac{S_i}{S_j} - \frac{S_j}{S_i} \]

(30) where S, and E, are the spin concentrations and the solution potential, respectively, for each run, n is the number of electrons transferred, E, is the midpoint potential at the pH specified, and S_j is the spin concentration of the fully reduced signal. We must caution the reader that nothing is known about the reversibility of these redox processes; we have performed reductive titrations only. Unless otherwise mentioned, we estimate an uncertainty of ±35 mV for the calculated midpoint potentials. The large uncertainty arises for two reasons. First, the number of points used in the fitting procedure was small (~6). Second, the concentrations for each signal were quite difficult to obtain precisely because many signals overlapped. The intensities had to be estimated by simulating each signal and adding the simulations together in the proportions required to fit the observed spectra.

Signals were simulated using the XPOW program (31–35). EPR spectra were manipulated and signal intensities quantitated using a program (EPRDAT) written using the ASYST® (McMillan Co.) language. For spin quantitations, the second integral values of the spectra were manipulated and signal intensities quantitated using a program (ELECTRO), also written in ASYST. All spin and coulometric quantitations are reported on a per 12Fe basis. Errors in protein concentration and molecular weight determined.
minations can be ignored when quantitations are reported in this way. Our choice of the value 12 arises from our view that there are probably two nickels/holoenzyme, and from the reported average ratio of $\approx 61$ Fe/Ni in CODH samples. Given the uncertainties involved in the determination of metal contents in enzymes, other values in the neighborhood of 12 could have been used as well.

RESULTS

CPC Titrations—CPC reductive titrations of thionin-oxidized CODH were performed under the inert gas argon, and under CO$_2$, a substrate for the enzyme. The potentials at which the buffered solutions were controlled varied from 0 to $-650$ mV. Between five and eight electron equivalents/12 Fe were required to reduce the oxidized enzyme in the titrations under argon. Our results are summarized in Table I. The number of equivalents used for reduction increased monotonically without obvious structure as the solution potential was lowered, suggesting a rather even distribution of reduction potentials among the different redox-active sites.

The results of the titration performed under the substrate CO$_2$ were significantly different from those under argon. In CO$_2$, and at potentials $< -550$ mV, the cell current never returned to background levels, rendering the determination of the number of electron equivalents used impossible. (We will argue below that this steady-state current is due to the catalytic reduction of CO$_2$ to CO.) However, at potentials between 0 and $-300$ mV, currents did return to background levels, and quantitations were possible. Two electron equivalents reduced the oxidized enzyme in this potential region.

The electrochemically poised samples obtained from the titrations were analyzed by EPR spectroscopy. We observed a variety of signals, which are summarized in Fig. 1 and Table II and are characterized in the following sections.

$g = 1.94$ Signal—in earlier studies, a $g = 1.94$ signal was observed in spectra of reduced CODH (13). We have found that, depending on the protein preparation used, two $g = 1.94$ signals, with the same $g$ values (2.04, 1.94, 1.90) but different linewidths may be observed. The narrow signal, $g_N = 1.94$, is shown in Fig. 1A, while the broad one, $g_B = 1.94$, is shown (along with $g_N = 1.94$) in Fig. 1B. The proportion of each signal observed depends somewhat on the sample and the EPR conditions. The $g_N = 1.94$ signal saturates at lower powers ($\leq 10$ microwatts versus $> 5$ mW, at 10 K) and shows relaxation broadening at higher temperatures ($\sim 60$ versus $\sim 20$ K) than the $g_B = 1.94$ signal. At higher temperatures and lower microwave powers, the $g_N = 1.94$ signal is most conspicuous. We do not presently know why some preparations yield more of the broad form than others.

FIG. 1. Summary of CO dehydrogenase EPR signals. A, CODH ("Fe-enriched") after addition of dithionite. Only the $g_N = 1.94$ signal (0.1 spin/12Fe) is observed.3 EPR conditions: 0.05 mW microwave power, 10 K sample temperature, 9.24 GHz microwave frequency, 10 G modulation amplitude. The dashed line is a simulation using a Gaussian lineshape. The $g$ values (linewidths in gauss) were 2.04(11), 1.94(13), and 1.90(15). B, thionin-oxidized CODH poised at $-640$ mV. Both the $g_N = 1.94$ and $g_B = 1.94$ signals (0.85 spin/12Fe) are observed. EPR conditions were as above. The spectral region near $g = 2.0$ has been removed (a large radical signal due to the redox mediators was present) and replaced by a simulation generated from the least squares fit of a quadratic polynomial to the regions adjacent to that deleted. The dashed line is the sum of two simulations; one with the parameters listed in A (for $g_N = 1.94$) and another with the same $g$ values but with linewidths of 18, 22, and 48 G (for $g_B = 1.94$). The simulations were summed, respectively, in a 1:5 ratio of their second integral values. C, dithionite-reduced CODII. Both the $g = 1.94$ (0.22 spins/12Fe) and the $g = 1.82$ signal (0.55 spins/12Fe) are observed. EPR conditions were the same as in A, except $T = 13$ K. The dashed line is the sum of two simulations. The parameters used in the simulation of the $g = 1.94$ signal were similar to that used in A. The other simulation used $g$ values (linewidths) of 2.01(13), 1.81(28), and 1.65(30) and a Gaussian lineshape. D, thionin-oxidized CODH poised at $-600$ mV in an argon atmosphere. Both the $g_N = 1.94$ (0.43 spins/12Fe) and $g_B = 1.94$ signals (0.36 spins/12Fe) are observed. EPR conditions were the same as in A. The dashed line through the data is the sum of two simulations. The parameters used to simulate $g_B = 1.94$ were similar to that used in A. The other simulation used $g$ values (linewidths) of 1.97(13), 1.86(15), and 1.75(19) and a Gaussian lineshape. E, thionin-oxidized CODH poised at $-600$ mV in a CO$_2$ atmosphere. In this spectrum, the NiFeC (0.12 spins/12Fe), $g_N = 1.94$ (0.24 spins/12Fe), and $g_B = 1.94$ (0.30 spins/12Fe) signals are observed. EPR conditions were the same as in A. The dashed line is the sum of three simulations. The NiFeC signal was simulated using the $g$ values (linewidths) of 2.08(19), 2.07(12), and 2.03(9) and a Lorentzian lineshape. The parameters used for $g_N = 1.94$ and $g_B = 1.86$ were similar to those used in A and D, F, CO-reduced CODH ("Fe-enriched"). The NiFeC signal (0.25 spins/12Fe), the $g_N = 1.94$ (0.22 spins/12Fe), $g_B = 1.94$ (0.22 spins/12Fe), and 1.86 (0.22 spins/12Fe) signals are observed. EPR conditions were the same as in A except for a sample temperature of 12 K. The dashed line is the sum of four simulations, using the parameters described above for the respective signals.

3It is not clear why only the $g_N = 1.94$ signal is observed in this spectrum. The sample used was from an earlier preparation and was not characterized as well as later samples. We use this spectrum here because it provides an unobscured illustration of this signal. The spectrum could have arisen if the potential of the sample was about $-450$ mV (see the spectrum of such a sample in Fig. 2) or if the temperature of the sample in the spectrometer was about 5 K higher than that indicated.
The conditions indicate the gas under which the signals were observed. Other details are given in the text and figure legends.

| Signal          | Conditions | $g$ values | $E_m$ (±35 mV) | Spins/12Fe |
|-----------------|------------|------------|---------------|------------|
| NiFeC           | CO$_2$ or CO | 2.08, 2.02 | $-350 > E_m > -520$ | 0.3 ± 0.2 |
| $g_{ave} = 1.94$ | CO$_2$ or Ar | 2.04, 1.94, 1.90 | $-440$ | 0.64 ± 0.14 |
| $g_{ave} = 1.86$ | Ar         | 2.01, 1.81, 1.65 | $-290$ | 0.18 ± 0.06 |
| $g_{ave} = 1.86$ | CO$_2$ or Ar | 1.97, 1.87, 1.75 | $-530$ (Ar) | 0.32 ± 0.10 |
| $g_{ave} = 1.86$ | Ar         | 1.97, 1.87, 1.75 | $-360$ (CO$_2$) | 0.32 ± 0.10 |

We have attempted to assess the reduction potentials of the $g_{ave} = 1.94$ species. From plots of signal intensities versus potential, an average reduction potential of $-440$ mV was obtained for the different titrations. The midpoint potential associated with the $g_N = 1.94$ species appears to be somewhat more positive than that yielding the $g_N = 1.94$ signal, but further studies and more experience with enzyme preparations are required to quantify this difference.

The values obtained for the spin concentrations of these signals were low; the sum of both signals had an average of 0.64 ± 0.14 spins/dimer (17 different samples). When the separate contributions from each signal was assessed, the narrow signal yielded an average of 0.26 ± 0.05 spins/dimer, and the broad one 0.34 ± 0.14 spins/dimer (11 samples).

**Low Field EPR Features**—The EPR spectra of [Fe,S]$_4$$^{1+}$ clusters in $S = 3/2$ spin states exhibit low intensity features between $g$ values of 0 and 4 (37-40). In spectra of reduced CODH, we noticed similar features. The features became more intense at negative potentials, but they did not develop in a simple Nernstian fashion. Some intensity remained in spectra which lacked the $g_{ave} = 1.94$ signals. It may be that the low field features reflect contributions from more than one complex.

$g_{ave} = 1.82$ Signal—The $g_{ave} = 1.82$ signal, with $g$ values of 2.01, 1.81, and 1.65, is shown in Fig. 1C (along with $g_N = 1.94$). The spin concentrations of the signal, assumed to result from an $S = 1/2$ system, were significantly below unity; we obtained an average of 0.18 ± 0.06 spins/dimer (26 spectra). We found the redox behavior associated with this signal to be somewhat unusual. In argon-atmosphere titrations, it developed according to a midpoint potential of about $-220$ mV (Fig. 2), and then completely disappeared at more negative potentials. In the CO$_2$ titration (Fig. 4), and other experiments performed under CO$_2$ or CO, the signal did not develop at any potential tested. We have observed the $g_{ave} = 1.82$ signal in CO-reduced CODH samples to which oxidized corrinoid/Fe-S protein was added (18, 19).

$g_{ave} = 1.86$ Signal—The $g_{ave} = 1.86$ signal, with $g$ values of 1.97, 1.87, and 1.75, is shown in Fig. 1D (along with the $g_N = 1.94$ signal). Spin quantitations of this signal yielded an average of 0.32 ± 0.10 spins/dimer (seven samples). The redox behavior of this signal was unusual as well. In the argon atmosphere titration illustrated in Fig. 2, the signal appeared at quite negative potentials, according to a midpoint potential $E_m = -530$ mV, while in the titration performed under CO$_2$ (Fig. 4), the signal appeared when samples were poised at $-350$ mV and below, in accordance with $E_m = -360$ mV. This potential is roughly 170 mV more positive than that obtained from the titration performed under argon.

NiFeC Signal—The so-called NiFeC signal, with $g$ values of 2.08 and 2.02 is known to arise from samples prepared under CO$_2$ or CO$_2$, at negative potentials (Fig. 1E). We have observed the signal when samples were reduced electrochemically in an argon atmosphere or reduced by dithionite.

At 10 K, the NiFeC signal begins to saturate homogeneously at roughly 10 microwatts microwave power; at 50 mW, it is virtually absent. At temperatures above 77 K, saturation was not a problem. Quantitation of the signal at 100 K and 10 mW yielded an average of 0.3 ± 0.2 spins/dimer.

The CPC titration performed under a CO$_2$ atmosphere yielded, not surprisingly, results quite different from those performed under argon. With argon, the current returned to background level after the reduction of the enzyme was complete.

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Footnote:

4 In some titrations, the $g_{ave} = 1.82$ signal did not disappear at more negative potentials and the $g_{ave} = 1.86$ signal never developed. Subsequent titrations (C. M. Gorst and S. W. Ragsdale, unpublished results) suggests that the disappearance of the $g_{ave} = 1.82$ signals at negative potentials are reproducible characteristics of CODH.
EPR and Electrochemistry of CO Dehydrogenase

**Fig. 3.** Current versus time scans of the reduction of CO dehydrogenase at -600 mV in CO2 and argon atmospheres (upper); plot of steady-state current in CO2 atmosphere titration versus potential (lower). The dashed line in upper plot is of a reduction (-600 mV) performed under a CO2 atmosphere; the solid line is of one performed under argon at the same potential. In both cases, 9.8 nmol of thionin-oxidized CODH were injected after -5 min. Background currents of 2.2 pA, for the titration under CO2, and 0.7 pA, for that under argon, have been subtracted. In the lower plot, the currents present 60 min after the injection of the protein are plotted against the solution potentials. The maximum steady-state currents are being limited by a factor other than the enzyme concentration and do not reflect the turnover number intrinsic to the enzyme.

**Fig. 4.** EPR spectra of CO dehydrogenase from CPC titration in a carbon dioxide atmosphere. Details of this experiment were the same as described in Fig. 2. The two g = 1.94 species observed here are probably the most readily understood. Such signals are known to arise from either [Fe4S4]1+ or [Fe2S2]1+ clusters (42). The two signals observed in CODH, with the same g values but different linewidths, were originally considered to be a single signal and were assigned to an [Fe4S4]1+ cluster (13). From our EPR study, we were unable to determine whether the signals arise from one cluster or two. However, there is precedence for both possibilities. That two g = 1.94 signals may result from a single cluster is exemplified by the corrinoid/Fe-S protein from *C. thermoaceticum*. This protein contains a single [Fe4S4]1+ cluster which yields two g = 1.94 signals, also with different linewidths (18).

The features observed between g = 6 and 4 probably arise from [Fe4S4]1+ clusters with S = 3/2 spin states (37-40). Similar features are observed in spectra of the nitrogenase Fe protein (37). About half the molecules of the Fe protein have an [Fe4S4]1+ cluster in an S = 1/2 spin state, the other half have S = 3/2 states. The S = 1/2 fraction gives rise to a g = 1.94 signal, while the S = 3/2 fraction yields features between g = 4 and 6. Meyer and co-workers (43) have recently shown that the S = 3/2 form of the Fe protein arises from an interaction between the cluster and its environment at low temperatures. A similar situation may apply to the clusters in CODH, but this is by no means established. Using Mossbauer
spectroscopy, we have not been able to identify the cluster(s) associated with the low field EPR features (20).

The $g_{max}$ = 1.86 and 1.82 signals have been mentioned only briefly in earlier studies (13, 18, 19), and the types of structures from which they arise have not been identified. The redox properties of the complexes yielding these signals are difficult to understand. It seems that the $g_{max}$ = 1.82 signal disappeared when samples were poised at potentials below about -400 mV because the complex yielding this signal became further reduced into an EPR silent state. Alternatively, the spin-state properties of the complex could have changed in a potential-dependent manner such that the $g_{max}$ = 1.82 signal was replaced by the $g_{max}$ = 1.86 signal. The disappearance of the $g_{max}$ = 1.82 signal could also be caused by spin-coupling to a paramagnetic species which is generated at low potential. The appearance of the $g_{max}$ = 1.82 and 1.86 signals was dependent on the presence or absence of CO$_2$/CO and, in some of our earlier titrations, on the protein preparation used. That CO$_2$ can modulate the redox properties associated with these signals, especially at potentials (e.g. -300 mV) more positive than that required for CO$_2$ reduction, indicates that at these positive potentials CO$_2$ might be binding at a site on the enzyme.

Determination of the midpoint potential of the complex yielding the NiFeC EPR signal from the CO$_2$ atmosphere titration data is difficult because the resulting EPR data reflect the redox states of the clusters during catalytic turnover (44). Consequently, the intensities do not only depend on $E_m$ but also on the rates of reduction and reoxidation of the complex, and perhaps other factors. These considerations are not relevant for samples poised at potentials $>-350$ mV, because under these conditions no turnover occurs. This potential provides an upper limit for the $E_m$ of the complex. Since some reduced signal developed in the turnover region (at $-450$ mV and below), the redox potential of the complex should be within about 70 mV of these values. With these considerations, we estimate $-350 > E_m > -520$ mV for the complex in the presence of CO$_2$ at pH 6.3. Recent studies indicate that the redox potential is slightly more negative than this estimate. We have shown in this paper that purified CODH is able to catalyze the reduction of CO$_2$ to CO. The potential at half-maximal activity, $E_{1/2} = -343$ mV, is near the thermodynamic reduction potential for the CO$_2$/CO couple at pH 6.3, ($-51.7$ mV), and the $g_{max} = 1.94$ EPR signal was found by us to be almost coincident with CO$_2$ reduction. Most likely, the last electron transfer step required before catalysis can occur is the reduction of one of the clusters yielding these signals. Since the complex producing the NiFeC signal is known to bind CO$_2$, it is probably one which must be reduced before catalysis can occur.

Much remains to be learned about the metal complexes of CODH. The plethora of EPR signals suggests that some of them arise from different states of the same complex. However, it is not yet known which of the signals is associated. It is likely that some of the complexes have unique structures, given their unusual EPR and redox properties. Mössbauer spectroscopy is well suited to address these problems (46, 47), and we have undertaken such a study, utilizing the results described here. In the following paper, the results of that study are reported, and our preliminary conclusions regarding the structures of the metal complexes in CODH are drawn.

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