Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 6811–6814, October 1985
Biochemistry

Evidence that an iron–nickel–carbon complex is formed by reaction of CO with the CO dehydrogenase from *Clostridium thermoaceticum*

(electron spin resonance/hyperfine interaction/acetate biosynthesis/acetyl-CoA synthesis/acetogenic bacteria)

STEVE W. RAGSDALE†, HARLAND G. WOOD†, AND WILLIAM E. ANTHOLINE‡

†Department of Biochemistry, School of Medicine, Case Western Reserve University, Cleveland, OH 44106; and ‡National Biomedical ESR Center, Medical College of Wisconsin, Milwaukee, WI 53226

Contributed by Harland G. Wood, June 24, 1985

ABSTRACT **The interaction between carbon monoxide and the CO dehydrogenase from *Clostridium thermoaceticum* was studied by electron spin resonance (ESR) techniques. When the enzyme reacts with CO, a paramagnetic complex is formed which previously was shown, by isotope substitution, to be due to a nickel–carbon species. In this paper, we demonstrate that iron is also a component of this ESR-detectable complex. When the iron in the enzyme is replaced with ^{57}Fe, a broadening of 18 G in the $g_{\parallel}$ and 7 G in the $g_{\perp}$ region is seen. This hyperfine interaction is probably due to more than one iron atom in the complex. Coenzyme A influences this ESR spectrum. In the absence of CoA, the ESR spectrum consists of two superimposed signals, which were simulated using the following ESR parameters: signal 1, with $g = 2.074$ and $g = 2.028$, and signal 2 with $g_x = 2.062$, $g_y = 2.047$, and $g_z = 2.028$. CoA converts signal 2 into signal 1. Since iron, nickel, and carbon all are part of this ESR-detectable complex, we propose that these atoms exist in a spin-coupled complex with net spin = 1/2, analogous to other iron–sulfur centers in which the metals are bridged by acid-labile sulfide.**

Carbon monoxide dehydrogenase is present in high concentration in the acetogenic bacteria (1, 2). The CO dehydrogenase of *Clostridium thermoaceticum* contains 1 Zn, 2 Ni, 11 Fe, and 14 inorganic S per mol of dimeric enzyme ($M_r \approx$ 150,000) (3). Four atoms each of iron and inorganic sulfide are part of a $[4Fe\text{–}4S]^{1+/2+}$ center for which electron spin resonance (ESR) measurements give g values at 2.04, 1.94, and 1.90 (4). Four more iron and inorganic sulfide atoms may exist in another [4Fe–4S] center with g values at 1.75, 1.86, and 2.01 (4) or 1.96; however, the exact nature of this center is unclear. The remaining two nickel and three iron atoms have not yet been characterized. These irons are not present as classical [2Fe–2S], [3Fe–xS], or [4Fe–4S] centers.

The enzyme from *C. thermoaceticum* has been shown to catalyze several essential reactions in the metabolism of the acetogenic bacteria. It catalyzes the oxidation of CO to CO_2 or the reduction of CO_2 to CO (2, 3, 5) (Eq. **1**). In addition, it recently has been found to be the central component in the synthesis of acetyl-CoA (6). It catalyzes exchange reactions between CO and the carbonyl of acetyl-CoA (Eq. **2a**) (6, 7) and between CoA and the CoA moiety of acetyl-CoA (Eq. **2b**) (8).

$$CO \leftrightharpoons C_1 \overset{H_2O}{\leftrightharpoons} CO_2 + 2e^- + 2H^+ \quad [1]$$

$$CH_3\text{-}{*}CO\text{-}SCoA + CO \leftrightharpoons CH_3\text{-}CO\text{-}SCoA + {*}CO \quad [2a]$$

$$CH_3\text{-}CO\text{-}SCoA{*} + CoASH \leftrightharpoons CH_3\text{-}CO\text{-}SCoA + {*}CoASH. \quad [2b]$$

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

For the last reaction (Eq. **2b**), dithiothreitol or disulfide reductase and a source of electrons (NADPH or reduced ferredoxin) are required (8). Our results indicate that CO dehydrogenase catalyzes the final steps in the synthesis of acetyl-CoA by condensing the methyl group of a methylated corrinoid protein with CoA and CO (or other C_1 donors) to form acetyl-CoA (6). Thus when CO binds to the enzyme, a CO dehydrogenase–C_1 complex is formed which can undergo oxidation to CO_2 in the presence of suitable electron-transfer components (2, 3) or which can react with the methyl of the methylated corrinoid protein and CoA to form acetyl-CoA. It is not clear how the enzyme controls whether the CO goes to CO_2 or the C-1 of acetyl-CoA, but pH is a major factor.

When CO dehydrogenase is reacted with CO, an ESR-detectable species with g values at 2.074 and 2.028 is formed which has been described as a nickel(III)-carbon radical (4) and a nickel(III)-carbon species (9). This signal is seen at temperatures up to 170 K. In contrast, the [4Fe–4S] centers in the enzyme are significantly broadened at temperatures >40 K. The [4Fe–4S] centers contribute insignificantly to the ESR spectra, recorded at 100 K, that are reported in this paper. Clearly the ESR spectrum of the paramagnetic CO dehydrogenase–C_1 complex is due to nickel and carbon since hyperfine splitting was detected upon isotopic substitution with ^{61}Ni ($I = 3/2$) and also when ^{13}CO ($I = 1/2$) reacted with the enzyme (9). The major effects of the isotope substitutions were a splitting of the $g = 2.028$ ($g_{\parallel}$) component by ^{13}CO and a broadening of the 2.074 ($g_{\perp}$) component by the ^{61}Ni nucleus. In this paper we show that iron contributes also to this ESR signal. Thus the ESR signal that appears after addition of CO to the CO dehydrogenase apparently is due to an exchange-coupled complex consisting of nickel, carbon, and iron.

We have recently reported another ESR signal, at $g = 2.05$, that can be observed upon reaction of CO dehydrogenase with CO (6) and that is affected by treatment of the enzyme with CoA. We now have studied the $g = 2.05$ signal in detail and have found that it is one of the components of a rhombic signal (g_x, g_y, and g_z are distinct) that can be converted into the 2.074/2.028 signal by treatment with CoA or acetyl-CoA. Thus the reaction of CO dehydrogenase with CO yields a complicated ESR spectrum consisting of two superimposed signals: one with g values at 2.074 and 2.028 and one with g values at 2.062, 2.047, and 2.028. Treatment with CoA converts the latter signal into the former one. Since these signals are interconvertible and have similar g values and lineshapes, they are proposed to originate from similar paramagnetic centers.

MATERIALS AND METHODS

C. thermoaceticum. (DSM 521) was grown as described (10). For growth in ^{57}Fe-containing medium, iron(III) oxide (93.31% isotopic purity, Oak Ridge National Laboratories) was first dissolved in concentrated HCl and added to the medium to a final concentration of 26 μM. The final calcu-

lated isotope purity was 80.8% because 5 μM ^{56}Fe was present as a contaminant in the medium before addition of the ^{57}Fe. The initial specific activity of the enzyme from cells cultured in this medium was 6 μmol of CO oxidized·min^{-1}·mg^{-1} (6 units/mg).

CO dehydrogenase was purified to homogeneity (specific activity ≈400 units/mg) as described (6). The enzyme was purified and maintained under anaerobic conditions in a type B Coy anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI).

ESR experiments were performed with a Varian E-112 spectrometer equipped with an E-102 X-Band microwave bridge. The temperature was kept constant with a Varian variable temperature-controller. Double integration was performed manually as described by Fee (11). The field was calibrated by use of a Varian gaussmeter and by measurement of the six lines of Mn^{2+} in strontium oxide powder (100 mesh, Alfa Products), as described by Bolton *et al.* (12). The g values were calibrated with 1,1-diphenyl-2-picrylhydrazyl powder (g = 2.0037) (12). Frequency was measured using a digital frequency meter (model 548; EIP Microwave Inc., San Jose, CA). Solid-phase powder spectra were simulated at the National Biomedical ESR center on a PDP-11 computer by the program of Pilbrow and Winfield (13). Other details of the ESR experiments are in the figure legends.

RESULTS AND DISCUSSION

Fig. 1 shows the ESR spectrum of the CO dehydrogenase after addition of CO in the presence of CoA. This spectrum will be referred to as signal 1 and has been reported previously (4); to obtain more accurate ESR parameters, we have simulated the ESR spectra. Excellent agreement of the simulated and the experimental spectra was obtained by assuming an axially symmetric system. The effect of CoA on the enzyme was studied because CoA is a substrate of the CO dehydrogenase in the synthesis of acetyl-CoA. When the enzyme reacts with CO in the absence of CoA, a variable amount of another signal, at g = 2.05 and which will be referred to as signal 2, is seen (Fig. 2*A*). In different preparations of the enzyme we have seen from 0 to 80% of the total signal intensity in signal 2. To view this second species in isolation, we subtracted a fraction of the spectrum of the CoA-treated enzyme (signal 1) from that of the nontreated enzyme. We have never seen signal 2 by itself; it has so far always been found in the presence of signal 1. Acetyl-CoA has an effect similar to CoA; thiols, such as dithiothreitol, cannot substitute for CoA in effecting this change in ESR properties. It is unclear whether the effect of CoA is due to allosteric changes or whether it results from direct bonding of CoA to the paramagnetic site. Clearly, however, our studies indicate that binding of CoA does induce changes in geometry or coordination number of the metal–carbon center and that signals 1 and 2 are from very similar metal–carbon centers. For simulating the spectrum shown by the dotted line in Fig. 2*B*, we assumed rhombic distortion. Both signals 1 and 2 appear to belong to viable intermediates in the transformation of CO by the enzyme, since they are present in total concentration approximately equal to the concentration of

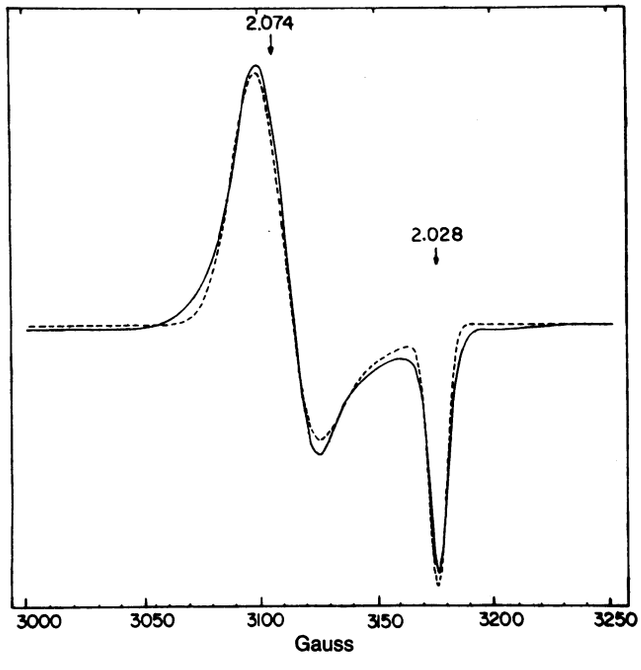

FIG. 1. ESR spectrum of CO dehydrogenase–CO complex in the presence of CoA. ESR spectra of enzyme at 30 mg/ml were obtained at 100 K at 9.015 GHz. The enzyme, in 50 mM Tris/HCl, pH 7.6/1 mM dithiothreitol/1 mM CoA, was allowed to react with CO for 20 min and then was frozen in liquid nitrogen. ESR parameters were as follows: power, 15 mW; scan range, 400 G; scan rate, 100 G/min; time constant, 0.5 sec; modulation amplitude, 5 G; modulation frequency, 100 KHz. A simulated spectrum (----) consistent with the experimental spectrum (—) was obtained by using the following parameters: g_x = 2.074, g_y = 2.074, g_z = 2.028, W_x = 11.8 G, W_y = 11.8 G, W_z = 3.7 G.

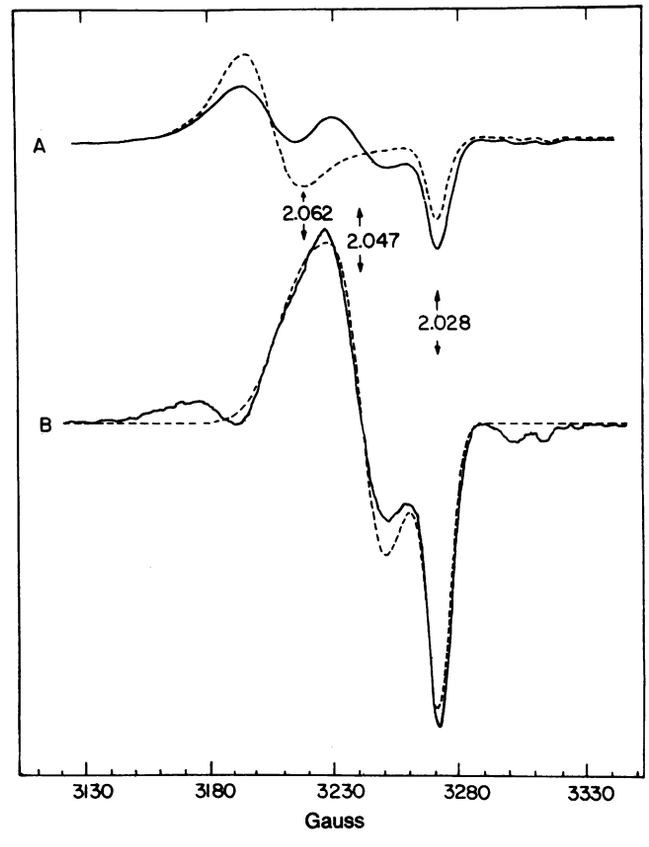

FIG. 2. ESR difference spectrum of the non-CoA-treated CO dehydrogenase–CO complex. ESR conditions were as described in the legend to Fig. 1, except that the microwave frequency was 9.286 GHz. Spectra A: Non-CoA-treated (—) and CoA-treated (----) CO dehydrogenase. Spectra B: Experimental (—) and simulated (----) difference spectra. The experimental difference spectrum was obtained by subtracting 62% of the spectrum of the CoA-treated enzyme from that of the non-CoA-treated enzyme. The simulations used the following ESR parameters: g_x = 2.062, g_y = 2.047, g_z = 2.028, W_x = 11.8 G, W_y = 7.25 G, W_z = 4.50 G.

enzyme. The signals are observed at maximum intensity within 1–2 min after reaction with CO at room temperature (data not shown).

The 2.074/2.028 signal has previously been shown to originate from a nickel–carbon complex (9). When the iron in the enzyme is replaced with ^{57}Fe (I = 1/2), substantial hyperfine broadenings are seen (Fig. 3). This broadening is 18 G in the $g_{\parallel}$ (2.028) region and 7 G in the $g_{\perp}$ (2.074) region. When ^{13}CO was substituted for ^{12}CO, we observed an 11 G splitting in the $g_{\parallel}$ region and a 9 G broadening in the $g_{\perp}$ region (spectra not shown). The hyperfine parameters determined for ^{61}Ni, ^{57}Fe, and ^{13}CO are listed in Table 1. Thus we must now describe the signal as a Ni–Fe–C signal, since spin density is delocalized among these atoms. The $A_{\perp}(^{13}C)$ observed in this work is significantly different from the value published in ref. 9. We do not understand this discrepancy and have therefore initiated further studies. We have also recorded ESR spectra of the enzyme reacted with $C^{17}O$ (36% purity; I = 3/2); no significant broadening due to ^{17}O was observed. That there is a strong hyperfine interaction with $^{13}C^{16}O$ and not with $^{12}C^{17}O$ demonstrates that the bond between metal and the CO-derived species is a metal-carbon and not a metal-oxygen bond. Incubation of the enzyme in $^{2}H_2O$ also did not significantly change the linewidth of the ESR spectrum.

Our goal is to determine the structure of the species giving rise to the g = 2.074/2.028 ESR spectrum. Clearly the signal results from a center containing iron, nickel, and carbon derived from CO. That inorganic sulfide is also a part of this center is suggested by earlier studies (3, 14) which have consistently demonstrated that the number of inorganic sulfide atoms is larger than the number of iron atoms and, in fact, is approximately equal to the total number of iron plus nickel atoms per mole of dimeric enzyme. Although inorganic sulfur analyses have been inaccurate in some Fe–S proteins (15), these determinations in the CO dehydrogenase are consistent with a model in which the nickel and iron sites are linked by inorganic sulfur in a manner analogous to the [2Fe–2S], [3Fe–xS], and [4Fe–4S] clusters.

The g values of the nickel–iron–carbon center are very close to 2, suggesting that the ESR signal belongs to a species with net electronic spin S = 1/2. In addition, double integration of this ESR spectrum has yielded a value of one spin per mole of enzyme dimer, which is consistent with an S = 1/2 ground state (however, because we do not know how

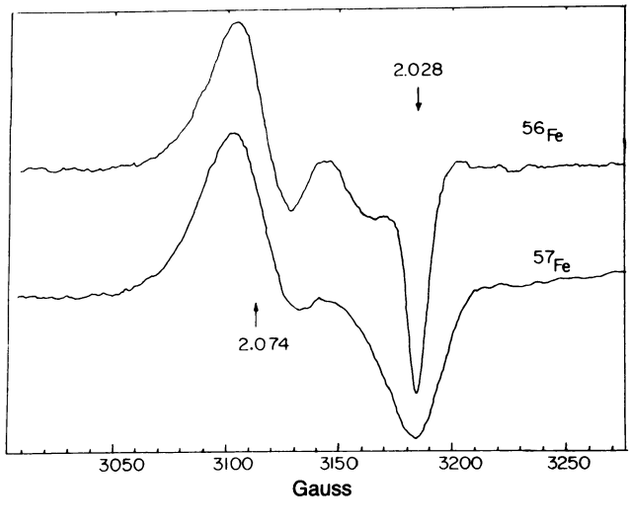

FIG. 3. ESR spectra showing hyperfine broadening from ^{57}Fe. ESR conditions were as described in the legend to Fig. 1, except that the microwave frequency was 9.034 GHz. The enzyme was treated with CoA before reaction with CO for 20 min.

Table 1. Hyperfine parameters for nuclei involved in the 2.074/2.028 ESR signal

Nucleus	$A_{\parallel}$, MHz	$A_{\perp}$, MHz	Reference
^{13}C	26	13	This work
	25	6	9
^{61}Ni	1.4	8.1	9
^{57}Fe	51*	20*	This work

The parameters were all obtained by measurement of the broadening or splitting of the lines in the ESR spectra. The conversion from gauss to MHz was as follows: A (MHz) = $(\beta/h) \times g \times a$ (gauss) = $1.39962 \times g \times a$ (gauss).

*Only one iron is assumed, but more than one is a clear possibility. If there were, for example, two, then the splitting would be determined by $A_1 + A_2$. Thus, A could be significantly smaller than these values.

many clusters of this type are in the enzyme, we do not know to what value the spectra should integrate). These results, in conjunction with the lack of any evidence for an isolated CO· radical, Fe, or Ni species, show that the ESR spectrum originates from a spin-coupled system with spin S = 1/2 containing Ni, Fe, carbon from CO, and presumably sulfide. Mixed-metal centers have a biological precedent in the Mo–Fe cofactor of nitrogenase (16).

It would be interesting to take the hyperfine parameters (A values) of Table 1 and deduce the oxidation states of the iron and nickel. However, this is not possible because we do not know how many atoms of each metal are present in the CO-binding center. The observed parameters are effective A values, which refer to the system spin and not to the individual component metals. These effective A values can be quite different from those that can be observed for the uncoupled atoms. For example, a spin-coupling model has been developed for oxidized 3Fe centers in which the hyperfine parameters for the individual uncoupled iron sites are equivalent; the effective values for the coupled system, however, are very different and depend strongly upon the orientation of the individual spins relative to the system spin (17). Thus, in CO dehydrogenase, the small A values for nickel relative to those observed for nickel(III) complexes (18–20) may reflect that the spin (S) of this individual nickel atom could be almost perpendicular to the net magnetic moment. Another possible explanation for the small A values is that the nickel could be in an even-electron state, such as Ni^{2+}. Mössbauer and electron–nuclear double-resonance studies have been initiated to determine the number of metals in this novel Ni–Fe–C center. We believe that the center contains more than one iron atom, since we do not observe symmetric splitting patterns at $g_{\parallel}$ as for ^{13}CO, even though $A_{\parallel}(^{13}C)$ is less than the apparent $A_{\parallel}(^{57}Fe)$. However, other explanations to account for the unsymmetrical features are possible; e.g., $\tilde{A}(^{57}Fe)$ and $\tilde{g}$ may not have the same principle axis system.

The studies reported here are important to develop an understanding of the mechanism of action of CO dehydrogenase. This mixed metal–carbon center could be a central intermediate in the pathway of acetyl-CoA synthesis (6). Further studies clearly are necessary to determine the structure of this center.

We thank Dr. Christopher Felix (National Biomedical ESR Center) for valuable aid in all aspects of this work and Dr. Brian M. Hoffman (Northwestern University, Evanston, IL) for valuable suggestions. We thank Dr. Eckard Münck (Gray Freshwater Biological Institute, University of Minnesota, Navarre) for helpful discussions and for critical reading of the manuscript. This research was supported by Grants RR01008 and GM24913 and National Research Service Award GM10523-01 from the National Institutes of

Health. The ESR spectrometer used in these studies was provided by National Institute of General Medical Science Grant 27519.

1. Clark, J. E., Ragsdale, S. W., Ljungdahl, L. G. & Wiegel, J. (1982) *J. Bacteriol.* **151,** 507–509.
2. Diekert, G. B. & Thauer, R. K. (1978) *J. Bacteriol.* **136,** 597–606.
3. Ragsdale, S. W., Clark, J. E., Ljungdahl, L. G., Lundie, L. L. & Drake, H. L. (1983) *J. Biol. Chem.* **258,** 2364–2369.
4. Ragsdale, S. W., Ljungdahl, L. G. & DerVartanian, D. V. (1982) *Biochem. Biophys. Res. Commun.* **108,** 658–663.
5. Pezacka, E. & Wood, H. G. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 6261–6265.
6. Ragsdale, S. W. & Wood, H. G. (1985) *J. Biol. Chem.* **260,** 3970–3977.
7. Hu, S.-I., Drake, H. L. & Wood, H. G. (1982) *J. Bacteriol.* **149,** 440–448.
8. Pezacka, E. & Wood, H. G. (1985) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **44,** 671 (abstr.).
9. Ragsdale, S. W., Ljungdahl, L. G. & DerVartanian, D. V. (1983) *Biochem. Biophys. Res. Commun.* **115,** 658–665.
10. Ljungdahl, L. G. & Andreesen, J. R. (1978) *Methods Enzymol.* **53,** 360–372.
11. Fee, J. A. (1978) *Methods Enzymol.* **49,** 512–528.
12. Bolton, J. R., Borg, D. C. & Swartz, H. M. (1972) in *Biological Applications of Electron Spin Resonance*, eds. Swartz, H. M., Bolton, J. R. & Borg, D. C. (Wiley, New York), p. 100.
13. Pilbrow, J. R. & Winfield, M. E. (1973) *Mol. Phys.* **25,** 1073–1092.
14. Ragsdale, S. W., Ljungdahl, L. G. & DerVartanian, D. V. (1983) *J. Bacteriol.* **155,** 1224–1237.
15. Wahl, R. C. & Rajagopalan, K. V. (1982) *J. Biol. Chem.* **257,** 1354–1359.
16. Nelson, M. J., Lindahl, P. A. & Orme-Johnson, W. H. (1982) in *Advances in Inorganic Biochemistry*, eds. Eichhorn, G. L. & Marzilli, L. G. (Elsevier, New York), pp. 1–40.
17. Kent, T. A., Huynh, B. H. & Münck, E. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 6574–6576.
18. Sugiura, Y., Kuwahara, J. & Suzuki, T. (1983) *Biochem. Biophys. Res. Commun.* **115,** 878–881.
19. Drago, R. S. & Baucom, E. I. (1972) *Inorg. Chem.* **11,** 2064–2069.
20. Haines, R. I. & McAuley, A. (1981) *Coord. Chem. Rev.* **39,** 77–119.