Identification of a Cyanide Binding Site in CO Dehydrogenase from *Clostridium thermoaceticum* Using EPR and ENDOR Spectroscopies

Mark E. Anderson,† Victoria J. DeRose,‡
Brian M. Hoffman,*,‡ and Paul A. Lindahl*,†

Departments of Chemistry
Texas A & M University
College Station, Texas 77843-3255
Northwestern University
Evanston, Illinois 60208-3313

Received July 2, 1993

Carbon monoxide dehydrogenase from *Clostridium thermoaceticum* ($CODH_{Ct}$) catalyzes the synthesis of acetyl-CoA and the reversible oxidation of CO to CO_2.[1] The enzyme has an $(\alpha\beta)_3$ subunit structure containing two Ni and 11–13 Fe per $\alpha\beta$.[2] The metal ions are organized into approximately four autonomous complexes/clusters including: (i) the NiFe complex, thought to contain one Ni ion chemically linked to an iron–sulfur cluster;[3,4] (ii) an $[Fe_4S_4]^{2+/1+}$ cluster; (iii) a single iron known as ferrous component II;[4] and (iv) a cluster, comprised of at least two irons, that yields a rhombic EPR signal ($g_1 = 2.01$, $g_2 = 1.81$, $g_3 = 1.65$, $g_{av} = 1.82$) in its one-electron reduced form ($E^{\circ\prime} = -220 \pm 35$ mV vs NHE).[4] Circumstantial evidence suggests that Ni is part of this cluster, but this is not known with certainty.[4] We shall refer to this cluster as the C-cluster.[5] The NiFe complex almost certainly functions as the active site for acetyl-CoA synthesis, while another, unidentified species appears to serve as the active site for CO oxidation.[1,6]

Cyanide inhibits the CO oxidation activities of all CODHs investigated so far.[2,7,8] Because the substrate CO reverses inhibition by CN^-, Grahame and Stadtman inferred that CN^- and CO compete for the same binding site.[8] A similar conclusion was reached from a thorough investigation of the effects of CN^- on the CODH from *Rhodospirillum rubrum* ($CODH_{Rr}$).[9] CN^- was found to be a slow-binding inhibitor of CO oxidation that binds at the active site. We have sought to identify the CO oxidation active site of $CODH_{Ct}$ by identifying its CN^- binding site.[10] We report here that CN^- alters the $g_{av} = 1.82$ signal and unambiguously show by 35-GHz ^{13}C ENDOR[11] spectroscopy that CN^- binds directly to the C-cluster.

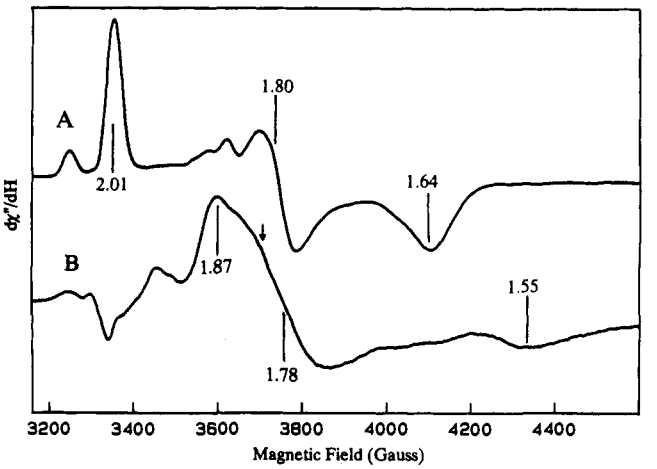

Figure 1. EPR of partially oxidized CODH before (A) and after (B) addition of KCN. The $g_{av} = 1.82$ signal in A quantified to 0.3 spin/$\alpha\beta$. The $g_{av} = 1.72$ signal in B quantified to 0.1 spin/$\alpha\beta$. Both spectra contain minor signals from uncharacterized species. Arrow in B indicates the g-value at which ENDOR data were collected. Conditions: temperature, 10 K; microwave frequency, 9.428 GHz; microwave power, 20 mW; modulation amplitude, 11 G. The signal was simulated using the program XPOW[24] and $g_1 = 1.87$, $g_2 = 1.78$, $g_3 = 1.55$.

The $g_{av} = 1.82$ signal (Figure 1A) was generated from a sample[12] of dithionite-free, partially oxidized $CODH_{Ct}$. After addition of KCN, the $g_{av} = 1.82$ signal transformed into a new signal (Figure 1B) with $g_{av} = 1.72$ ($g_1 = 1.87$, $g_2 = 1.78$, and $g_3 = 1.55$).[15] Signals of similar appearance were obtained in spectra of all 50 CN^--inhibited samples examined. In contrast to the response of the C-cluster to CN^-, the g-values and line shapes of EPR signals from the NiFe complex and the $[Fe_4S_4]^{1+}$ cluster were unaffected by the cyanide treatment.[16]

To determine whether CN^- binds directly to the C-cluster, a sample[17] prepared similarly but with $^{13}CN^-$ was examined for ^{13}C ($I = {}^1/_2$) ENDOR signals. The ENDOR spectrum (Figure 2A), taken at a field where the EPR consisted only of the g_{av} =

* To whom correspondence should be addressed.
† Texas A & M University.
‡ Northwestern University.

(1) Ragsdale, S. W. *CRC Crit. Rev. Biochem. Mol. Biol.* **1991**, *26*, 261–300.
(2) Ragsdale, S. W.; Clark, J. E.; Ljungdahl, L. G.; Lundie, L. L.; Drake, H. L. *J. Biol. Chem.* **1983**, *258*, 2364–2369.
(3) (a) Ragsdale, S. W.; Ljungdahl, L. G.; DerVartanian, D. V. *Biochem. Biophys. Res. Commun.* **1983**, *115*, 658–665. (b) Ragsdale, S. W.; Wood, H. G.; Antholine, W. E. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 6811–6814. (c) Fan, C.; Gorst, C. M.; Ragsdale, S. W.; Hoffman, B. M. *Biochemistry* **1991**, *30*, 431–435. (d) Bastian, N. R.; Diekert, G.; Niederhoffer, E. C.; Teo, B. K.; Walsh, C. T.; Orme-Johnson, W. H. *J. Am. Chem. Soc.* **1988**, *110*, 5581–5582.
(4) (a) Lindahl, P. A.; Münck, E.; Ragsdale, S. W. *J. Biol. Chem.* **1990**, *265*, 3873–3879. (b) Lindahl, P. A.; Ragsdale, S. W.; Münck, E. *J. Biol. Chem.* **1990**, *265*, 3880–3888.
(5) This cluster has been previously called the "$g_{av} = 1.82$" species.[4,6] Since it also gives rise to a signal with $g_{av} = 1.72$ and most likely to one with $g_{av} = 1.86$,[4] another name was chosen to avoid confusion. The "C" in C-cluster highlights its relevance in CO oxidation.
(6) (a) Shin, W.; Lindahl, P. A. *Biochemistry* **1992**, *31*, 12870–12875. (b) Shin, W.; Lindahl, P. A. *J. Am. Chem. Soc.* **1992**, *114*, 9718–9719. (c) Shin, W.; Anderson, M. E.; Lindahl, P. A. *J. Am. Chem. Soc.* **1993**, *115*, 5522–5526.
(7) (a) Abbanat, D. R.; Ferry, J. G. *J. Bacteriol.* **1990**, *172*, 7145–7150. (b) Eikmanns, B.; Fuchs, G.; Thauer, R. K. *Eur. J. Biochem.* **1985**, *146*, 149–154. (c) Smith, M. R.; Lequerica, J. L.; Hart, M. R. *J. Bacteriol.* **1985**, *162*, 67–71. (d) Rajagopal, B. S.; Lespinat, P. A.; Faugue, G.; LeGall, J.; Berlier, Y. M. *Appl. Environ. Microbiol.* **1989**, *55*, 2123–2129. (e) Diekert, G. B.; Thauer, R. K. *J. Bacteriol.* **1978**, *136*, 597–606. (f) Drake, H. L.; Hu, S.-I.; Wood, H. G. *J. Biol. Chem.* **1981**, *255*, 7174–7180. (g) Möller-Zinkhan, D.; Thauer, R. K. *Arch. Microbiol.* **1990**, *153*, 215–218. (h) Ragsdale, S. W.; Ljungdahl, L. G.; DerVartanian, D. V. *J. Bacteriol.* **1983**, *155*, 1224–1237. (i) Thauer, R. K.; Fuchs, G.; Kaufer, B.; Schnitker, U. *Eur. J. Biochem.* **1974**, *45*, 343–349.
(8) Grahame, D. A.; Stadtman, T. C. *J. Biol. Chem.* **1987**, *262*, 3706–3712.
(9) (a) Ensign, S. A.; Hyman, M. R.; Ludden, P. W. *Biochemistry* **1989**, *28*, 4973–4979. (b) Stephens, P. J.; McKenna, M. C.; Ensign, S. A.; Bonam, D.; Ludden, P. W. *J. Biol. Chem.* **1989**, *264*, 16347–16350. (c) Ensign, S. A.; Bonam, D.; Ludden, P. W. *Biochemistry* **1989**, *28*, 4968–4973. (d) Hyman, M. R.; Ensign, S. A.; Arp, D. J.; Ludden, P. W. *Biochemistry* **1989**, *28*, 6821–6826. (e) Bonam, D.; McKenna, M. C.; Stephens, P. J.; Ludden, P. W. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 31–35.
(10) $CODH_{Ct}$ and $CODH_{Rr}$ are similarly affected by CN^-,[16] and thus CN^- probably binds at the CO oxidation active site of $CODH_{Ct}$.
(11) Hoffman, B. M. *Acc. Chem. Res.* **1991**, *24*, 164–170.
(12) $CODH_{Ct}$ was prepared, purified, and characterized as described,[2,13] with CO/acetyl-CoA exchange and CO oxidation activities of 0.15 and 240 units/mg, respectively. The sample used for Figure 1A was prepared by transferring a dithionite-free sample into a double-septum-sealed optical cuvette with a pathlength of ~1 mm and then adding thionin incrementally until A_{420} ceased increasing.[14] The CN^--inhibited sample was prepared by adding 12 μL of 7.8 mM stock KCN (prepared in 50 mM NaOH) to the thionin-oxidized sample. Final concentrations were 75 μM $CODH_{Ct}$ $\alpha\beta$, 205 μN thionin, and 220 μM CN^-.
(13) (a) Lundie, L. L., Jr.; Drake, H. L. *J. Bacteriol.* **1984**, *159*, 700–703. (b) Ramer, S. E.; Raybuck, S. A.; Orme-Johnson, W. H.; Walsh, C. T. *Biochemistry* **1989**, *28*, 4675–4680. (c) Shin, W.; Lindahl, P. A. *Biochim. Biophys. Acta* **1993**, *1161*, 317–322.
(14) Shin, W.; Stafford, P. R.; Lindahl, P. A. *Biochemistry* **1992**, *31*, 6003–6011.
(15) At 5 K, the $g_{av} = 1.82$ signal was half-saturated at *ca.* 50 mW, while the $g_{av} = 1.72$ signal did not saturate significantly up to 200 mW.
(16) Anderson, M. E.; Lindahl, P. A., submitted for publication.

0002-7863/93/1515-12204$04.00/0

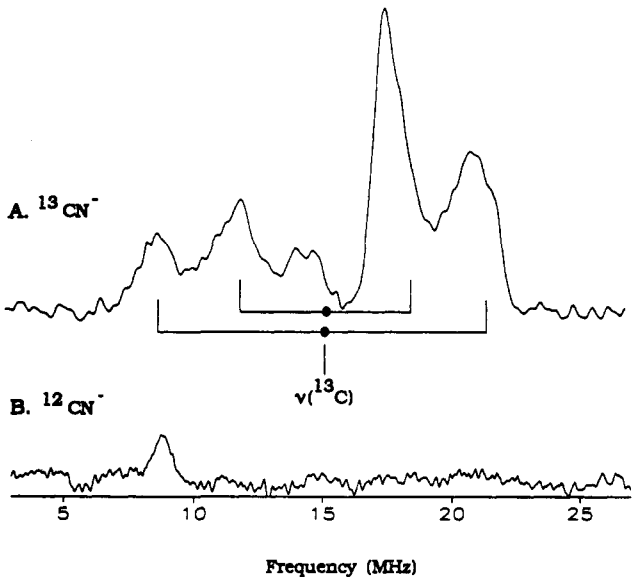

Figure 2. Q-band ENDOR of the $g_{av} = 1.82$ signal from $CODH_{Ct}$ prepared with $^{13}CN^-$ (A) and $^{12}CN^-$ (B). Data were taken at $g = 1.81$. The ^{13}C ENDOR pattern shows two doublets centered at the Larmor frequency for ^{13}C ($\nu(^{13}C)$, filled circle) and split by hyperfine interactions A_i (connecting lines). The spectrum of the $^{12}CN^-$-inhibited sample shows only a single peak at 8.5 MHz, possibly due to ^{14}N.[18] Conditions: temperature, 2 K; scan rate, 1 MHz/s; radio frequency power, 30 W; time constant, 32 ms; microwave frequency, 35.35 (A) and 35.18 GHz (B); microwave power, 0.8 mW; 100-KHz field modulation; modulation amplitude, 1.2 G.

1.72 signal, exhibited two hyperfine-split doublets centered at the ^{13}C Larmor frequency, with $A_1 = 12.7$ MHz and $A_2 = 6.5$ MHz. In contrast, the ENDOR spectrum of a $^{12}CN^-$ control (Figure 2B) consisted of only a weak feature assignable to ^{14}N.[18] The magnitudes of the ^{13}C hyperfine values are too large to arise from dipolar (through-space) coupling to $^{13}CN^-$ bound to a nearby cluster. For example, the maximum theoretical dipolar coupling to a $^{13}CN^-$ 3–4 Å from the C-cluster would be less than *ca.* 1–2 MHz.[19] Thus, the observed ^{13}C signals must reflect direct coordination to the C-cluster. The presence of *two* doublets might arise from the binding of *two* cyanides to the C-cluster, each with isotropic *A*-tensor components, or, more likely, from the binding of *one* cyanide where hyperfine anisotropy leads to a splitting of the ENDOR resonances at fields away from the edges of the EPR signal.[20] These results demonstrate that CN^- binds directly to the C-cluster and, given the body of evidence that CN^- inhibits CODHs competitively, indicate that the C-cluster is the active site for CO oxidation in $CODH_{Ct}$.

The cluster in $CODH_{Rr}$ that functions as the CO oxidation active site[9] (called the signal A cluster) has EPR, CN^--binding, and redox properties quite similar to those of the C-cluster. There is also some homology between the two polypeptides that most likely house these clusters.[21] The signal A cluster is a novel Ni–Fe–S structure in which Ni is coordinated to S and N/O in a distorted 4- or 5-coordinate complex, chemically bridged to (rather than incorporated into) an Fe–S cluster.[22] Given the similar properties of the two clusters, the C-cluster may have a similar Ni–Fe–S structure. In addition, the CODHs from *Methanosarcina barkeri* and *Methanothrix soehngenii* also contain clusters with EPR, CN^--binding, and redox properties similar to those of the C-cluster.[23] This suggests that all CODHs have CO oxidation sites with Ni–Fe–S structures that bind CN^-, engage in $n = 1$ redox chemistry with $E^{\circ\prime}$ of *ca.* -150 ± 110 mV, and, when reduced, exhibit EPR signals with $g_{av} < 2$.

Acknowledgment. This work was supported by the Robert A. Welch Foundation (A-1170) and the National Institutes of Health (GM46441 [P.A.L.], HL 13531 [B.M.H.], and a postdoctoral fellowship [V.J.D.]).

(17) The ENDOR samples (CO/acetyl-CoA exchange activities of 0.15 and 0.19 units/mg and CO oxidation activities of 240 and 246 units/mg) were prepared as described,[12] using $K^{13}CN$ (99% enriched, Cambridge Isotope Laboratories).

(18) Houseman, A. L. P.; Oh, B.-H.; Kennedy, M. C.; Fan, C.; Werst, M. M.; Beinert, H.; Markley, J. L.; Hoffman, B. M. *Biochemistry* **1992**, *31*, 2073.

(19) The dipolar interaction is calculated from the formula: $A_{dp} = g_n\beta_n g_e\beta_e[3\cos^2\theta - 1][1/r_{12}^3]$, where r_{12} is the distance between interacting centers, θ is the angle between a vector connecting the centers and the external magnetic field direction, and g and β have their usual meanings. The effects of spin coupling in a multicentered, spin-coupled cluster would increase the strength of interaction by a factor of no more than $\sim 7/3$.

(20) The number of cyanides bound could be resolved by multiple field ENDOR, but our inability to measure ENDOR at fields approaching g_3 precludes this. The limits of the spectrometer magnet restricted data collection to $g > 1.75$, corresponding to fields <14 300 G. Of secondary importance, data collection was limited to $g < 1.81$ due to overlap from residual amounts of the $g_{av} = 1.94$ signal exhibited by the sample employed.

(21) Kerby, R. L.; Hong, S. S.; Ensign, S. A.; Coppoc, L. J.; Ludden, P. W.; Roberts, G. P. *J. Bacteriol.* **1992**, *174*, 5284–5294.

(22) Tan, G. O.; Ensign, S. A.; Ciurli, S.; Scott, M. J.; Hedman, B.; Holm, R. H.; Ludden, P. W.; Korszun, Z. R.; Stephens, P. J.; Hodgson, K. O. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 4427–4431.

(23) (a) Krzycki, J. A.; Mortenson, L. E.; Prince, R. C. *J. Biol. Chem.* **1989**, *264*, 7217–7221. (b) Krzycki, J. A.; Prince, R. C. *Biochim. Biophys. Acta* **1990**, *1015*, 53–60. (c) Jetten, M. S. M.; Pierik, A. J.; Hagen, W. R. *Eur. J. Biochem.* **1991**, *202*, 1291–1297. (d) Jetten, M. S. M.; Hagen, W. R.; Pierik, A. J.; Stams, A. J. M.; Zehnder, A. J. B. *Eur. J. Biochem.* **1991**, *195*, 385–391.

(24) (a) Belford, R. L.; Nilges, M. J. *Computer Simulation of Powder Spectra EPR Symposium*, 21st Rocky Mountain Conference, Denver, CO; August, 1979. (b) Nilges, M. J. Ph.D. Thesis, University of Illinois, 1981. (c) Atman, T. E. Ph.D. Thesis, University of Illinois, 1981. (d) Maurice, A. M. Ph.D. Thesis, University of Illinois, 1982. (e) Duliba, E. P. Ph.D. Thesis, University of Illinois, 1983.