Azide Binding to Carbon Monoxide Dehydrogenase from *Clostridium thermoaceticum*

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Carbon monoxide dehydrogenase (CODH) plays a central role in a recently discovered pathway of anaerobic CO and CO_2 fixation.[1] The enzyme has been shown to contain 12–14 Fe, 2 Ni, Zn, and 13 S^{2-} per 150 kDa dimer.[2] These metals are organized into three distinct centers that have been characterized[3–9] and are called centers A, B, and C. A possible fourth metal site, "ferrous component II", was detected by Mössbauer[8] spectroscopy and may be a separate metal center or a component of one of the other centers. Center A is the site of acetyl-CoA synthesis. Based on EPR,[4,7] Mössbauer,[8] and ENDOR[9] studies, center A has been described as a [Ni-X-Fe_{3-4}-S_4] cluster, where X is an unknown bridging ligand between Ni and Fe. Center B is a typical $[4Fe\text{-}4S]^{2+/+}$ cluster that functions in electron transfer. Center C is an iron-containing cluster whose structure remains undefined. The spectroscopic properties of this center are similar to those of the metal site in CODH from *Rhodospirillum rubrum* that catalyzes CO oxidation. Nickel was proposed to be a constituent of this *R. rubrum* center as a result of EPR studies that show a broadening of the low field spectral feature upon ^{61}Ni substitution.[10] Ni-EXAFS results have demonstrated that nickel is not part of a cubane center,[11] indicating that like center A, Ni and Fe in center C must be bridged by a common external ligand. In this Communication, the EPR properties of a paramagnetic species that results from azide treatment of reduced CODH are explored. Azide, an inhibitor of CO oxidation, was found to dramatically alter the EPR spectrum of center C that is normally observed for untreated samples under reducing conditions. Electron spin echo envelope modulation (ESEEM) measurements show that azide is bound to the new paramagnetic species.

Several anions, including azide, thiocyanate and cyanate, have been shown to bind to center C and to inhibit CO oxidation.[12] For CODH isolated from *R. rubrum*[13] and *Clostridium thermoaceticum*,[14] cyanide has been shown to be a slow binding inhibitor. Studies on the *C. thermoaceticum* enzyme show that inhibition of CO oxidation by cyanide has little effect on acetyl-CoA synthesis.[2,15,16] Electron nuclear double resonance studies of this CODH demonstrated that cyanide binds to center C.[17] In the *R. rubrum* CODH, CO and cyanide have been proposed to bind to a nickel site.[13,18]

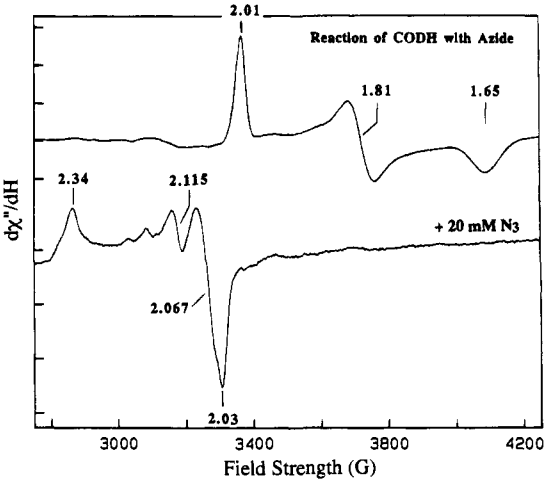

Figure 1. Continuous wave EPR spectra of reduced CODH (top) and reduced CODH treated with 20 mM azide (bottom). Spectra were collected on a Bruker ESP-300E spectrometer under the following set of conditions: microwave frequency, 9.4412 GHz; microwave power, 40 mW; magnetic field modulation frequency, 100 kHz; modulation amplitude, 28.6 G; sample temperature, 10 K. Enzyme was 1 mM in concentration suspended in 50 mM Tris buffer at pH = 6.02.

The effects of azide treatment on the continuous wave (cw)-EPR spectrum of reduced *C. thermoaceticum* CODH are shown in Figure 1. The enzyme was isolated using published procedures,[15] and dithionite was removed using a Penefsky column.[19] Under these conditions, the only EPR spectrum observed is from center C, with peaks at $g = 2.01$, 1.81, and 1.65 (Figure 1). Treatment of reduced enzyme with 20 mM azide results in the EPR spectrum shown in the lower trace of Figure 1. Analysis of this spectrum by numerical simulation of the line shape shows that it represents a composite of two different paramagnetic species. One is characterized by a nearly axial g-tensor, showing g-values of 2.34, 2.067, and 2.03; the second is a more minor species with g-values of approximately 2.34, 2.11, and 2.04. The minor components observed at 2.21 and 2.17 for azide-treated enzyme are currently unassigned. These spectral changes are indicative of a substantial change in magnetic coupling between the components of the center C metal cluster upon the binding of azide. Parallel studies on the inhibition of CO oxidation by thiocyanate and cyanate gave rise to nearly identical changes in the EPR spectrum of center C.[29] Because these three anions share a common coordination

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(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) Shin, W.; Stafford, P. R.; Lindahl, P. A. *Biochemistry* **1992**, *31*, 6003–6011.
(4) Ragsdale, S. W.; Wood, H. G.; Antholine, W. E. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 6811–6814.
(5) Ragsdale, S. W.; Ljungdahl, L. G.; DerVartanian, D. V. *Biochem. Biophys. Res. Commun.* **1983**, *115*, 658–665.
(6) Ragsdale, S. W.; Ljungdahl, L. G.; DerVartanian, D. V. *Biochem. Biophys. Res. Commun.* **1982**, *108*, 658–663.
(7) Lindahl, P. A.; Münck, E.; Ragsdale, S. W. *J. Biol. Chem.* **1990**, *265*, 3873–3879.
(8) Lindahl, P. A.; Ragsdale, S. W.; Münck, E. *J. Biol. Chem.* **1990**, *265*, 3880–3888.
(9) Fan, C.; Gorst, C. M.; Ragsdale, S. W.; Hoffman, B. M. *Biochemistry* **1991**, *30*, 431–435.
(10) Stephens, P. J.; McKenna, M.-C.; Ensign, S. A.; Bonam, D.; Ludden, P. W. *J. Biol. Chem.* **1989**, *264*, 16347–16350.
(11) Tan, G. O.; Ensign, S. A.; Ciurli, S.; Scott, M. J.; Hedman, B.; Holm, R. H.; Luden, P. W.; Korszun, Z. R.; Stephens, P. J.; Hodgson, K. O. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 4427–4431.
(12) Kumar, M.; Seravalli, J.; Lu, W.-P.; Ragsdale, S. W. *J. Biol. Chem.* submitted.
(13) Ensign, S. A.; Hyman, M. R.; Ludden, P. W. *Biochemistry* **1989**, *28*, 4973–4979.
(14) Morton, T. A. Ph.D. Thesis, University of Georgia, Athens, GA, 1991.
(15) Ragsdale, S. W.; Wood, H. G. *J. Biol. Chem.* **1985**, *260*, 3970–3977.
(16) Raybuck, S. A.; Bastian, N. R.; Orne-Johnson, W. H.; Walsh, C. T. *Biochemistry* **1988**, *27*, 7698–7702.
(17) Anderson, M. E.; DeRose, V. J.; Hoffman, B. M.; Lindahl, P. A. *J. Am. Chem. Soc.* **1993**, *115*, 12204–12205.
(18) Ensign, S. A.; Bonam, D.; Ludden, P. W. *Biochemistry* **1989**, *28*, 4968.
(19) Penefsky, H. S. *J. Biol. Chem.* **1977**, *252*, 2891–2899.
(20) Kruger, H-J.; Holm, R. H. *Inorg. Chem.* **1989**, *28*, 1148–1155.
(21) Latos-Grazynski, L.; Olmstead, M. M.; Balch, A. L. *Inorg. Chem.* **1989**, *28*, 4065–4066.
(22) (a) Pappenhagen, T. L.; Kennedy, W. R.; Bowers, C. P.; Margerum, D. W. *Inorg. Chem.* **1985**, *24*, 4356–4362. (b) Wang, Y. L.; Beach, M. W.; Pappenhagen, T. L.; Margerum, D. W. *Inorg. Chem.* **1988**, *27*, 4464–4472.

0002-7863/95/1517-2939$09.00/0

chemistry, it is not surprising that they inhibit CO oxidation by binding to CODH in a similar fashion. Experimental observations that point to center C as the azide binding site include (a) a concomitant decrease in the EPR signal of center C with the rise in intensity of the signal attributed to the azide adduct, (b) the finding of similar EPR relaxation properties for center C and the signal obtained upon treatment with azide, along with the observation that they are both very different from those of centers A and B, and (c) steady state kinetic measurements that yield a dissociation constant for thiocyanate binding to CODH that is similar to the inhibition constant for the CO oxidation reaction.[29]

The spectral changes detailed in Figure 1 are much different from those observed upon cyanide binding to center C and are most consistent with a different mode of inhibitor binding to the enzyme. The EPR spectrum obtained upon azide treatment of reduced CODH could result from the conversion of center C, a paramagnetic center that consists of a strongly coupled metal cluster, to one where the unpaired spin resides predominantly on a single, low-spin metal ion. Although the g-values observed for the azide-treated sample are consistent with those found typically for Ni(I) or Ni(III) model complexes,[20-24] substitution of ^{61}Ni into CODH does not broaden the EPR spectrum of the azide adduct. However, broadening of the EPR spectrum that results from azide treatment has been observed for ^{57}Fe-substituted enzyme.[12]

ESEEM studies were carried out on azide-treated samples to determine if the inhibitor was bound to the new paramagnetic center. The pulsed EPR instrument used for these studies has been described previously.[25] Figure 2a shows 3-pulse or stimulated echo ($90°-\tau-90°-T-90°$) ESEEM data collected for CODH treated with $^{14}N_3^-$ at a magnetic field position corresponding to $g = 2.22$. The Fourier transform of these data was obtained after dead-time reconstruction[26] and is shown in Figure 3(top trace). A narrow peak at 1.7 MHz and a broad peak centered at 3.2 MHz are resolved. Figure 2b shows ESEEM data obtained under conditions identical to those used for Figure 2a except that $^{15}N_3^-$-treated enzyme was used. The Fourier transform of these data, Figure 3 (lower trace), shows no evidence for the 1.7 or 3.2 MHz components. ESEEM experiments performed at $g = 2.15$ gave similar results in that low-frequency modulations at 0.75, 1.6, and 3.05 MHz were observed only for the $^{14}N_3^-$-treated sample. Some of the samples used for our studies showed spectral contributions from unreacted center C. ESEEM experiments performed at $g =$ 1.98, 1.81, and 1.72 on these samples provided no evidence for azide binding.

The modulation depths, rapid damping, and frequency spectra found for the azide-derived ^{14}N-ESEEM data are characteristic of coupling schemes characterized by large anisotropic and, possibly, isotropic hyperfine couplings. ESEEM studies of methylamine bound to the Cu(I)−topa semiquinone intermediate of amine oxidases gave rise to similar ESEEM observations for substrate-derived nitrogen.[27] Simple, relatively broad spectral features were observed at low frequencies for enzyme where the radical had been generated with ^{14}N-methylamine, but not with ^{15}N-methylamine. Analyses of these data along with the results of recent ^{2}H-ESEEM studies[28] have shown that

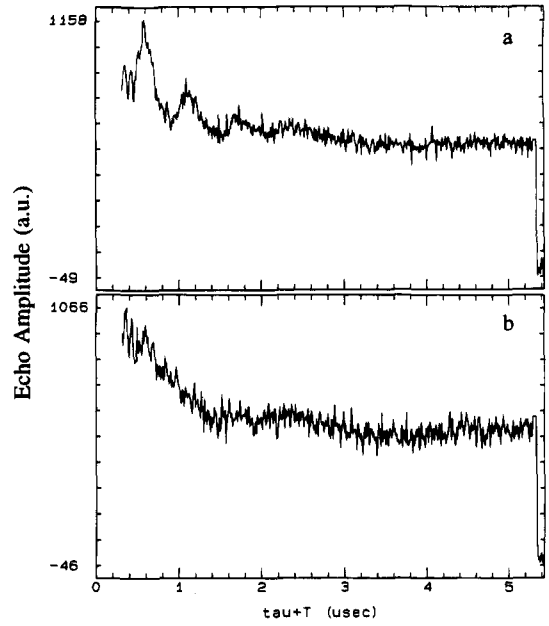

Figure 2. Time domain ESEEM data from azide-treated CODH samples. (a) Sample treated with $^{14}N_3^-$; (b) sample treated with $^{15}N_3^-$. Conditions common to the two measurements were the following: microwave frequency, 9.030 GHz; magnetic field strength, 2900 G; microwave pulse power, 30 W; sample temperature, 4.2 K; τ value, 240 ns; sequence repetition rate, 100 Hz. Each point represents the average of 36 events; fourscans were added to obtain the data shown. Data collection was initiated at $\tau + T = 280$ ns.

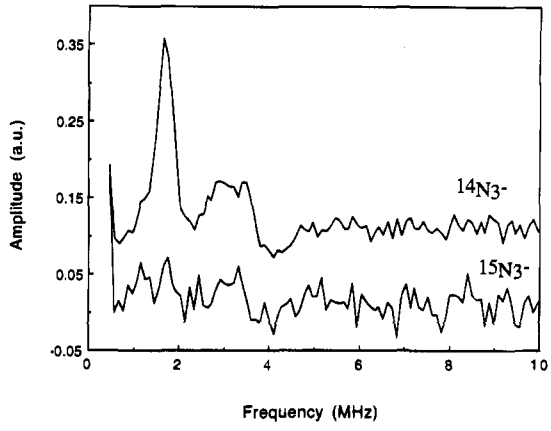

Figure 3. ESEEM spectra derived from Fourier transformation of the time domain data shown in Figure 2. The upper trace was obtained for the $^{14}N_3^-$-treated sample and is offset from the lower trace ($^{15}N_3^-$-treated sample) for display purposes.

such spectra arise when the anisotropy in the ^{14}N hyperfine coupling is so large that only the perpendicular singularities of the line shapes can be resolved. A detailed analysis of the $^{14}N_3^-$ ligand hyperfine coupling will be undertaken when multifrequency ESEEM data are available. The results presented here serve to establish that azide is bound to the paramagnetic species that results from the treatment of reduced CODH with the inhibitor. Future cw- and pulsed EPR experiments will be aimed at determining the structure of this modified redox center.

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(23) (a) Grove, D. M.; van Koten, G.; Zoet, R.; Murrall, N. W.; Welch, A. J. *J. Am. Chem. Soc.* **1983**, *105*, 1379−1380. (b) Kruger, H-J.; Peng, G.; Holm, R. H. *Inorg. Chem.* **1991**, *30*, 734−742.

(24) Kumar, M.; Day, R. O.; Colpas, G. J.; Maroney, M. J. *J. Am. Chem. Soc.* **1989**, *111*, 5974−5976.

(25) McCracken, J.; Shin, D-H; Dye, J. L. *Appl. Magn. Reson.* **1992**, *3*, 305−316.

(26) Mims, W. B. *J. Magn. Reson.* **1984**, *59*, 291−306.

(27) McCracken, J.; Peisach, J.; Cote, C. E.; McGuirl, M. A.; Dooley, D. M. *J. Am. Chem. Soc.* **1992** , *114*, 3715−3720.

(28) Warncke, K.; Babcock, G. T.; Dooley, D. M.; McGuirl, M. A.; McCracken, J. *J. Am. Chem. Soc.* **1994**, *116*, 4028−4037.

(29) Saravalli, J.; Kumar, M.; Lu, W.-P.; Ragsdale, S. W. *Biochemistry*, submitted.