Paramagnetic Centers of Carbon Monoxide Dehydrogenase from Aceticlastic Methanosarcina barkeri*

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Carbon monoxide dehydrogenase from Methanosarcina barkeri, purified to 95% homogeneity, contains 30 Fe, 2 Ni, 1 Zn, and 1 Cu (per αβ2 enzyme). Core extrusion experiments indicate 6 [4Fe-4S] clusters/tetramer, and electron paramagnetic resonance (epr) spectroscopy detects at least one of these clusters, in the reduced form, with apparent g values of 2.05, 1.94, and 1.90, and $E_{max} = -390$ mV. A second epr signal, also seen in the reduced enzyme, has apparent g values of 2.005, 1.91, and 1.76, and $E_{max} = -35$ mV. Two signals were seen in thionin-oxidized enzyme, one with a line shape suggestive of Cu(II), and the other resembling that of a [3Fe-4S] cluster. The enzymes nonsynphysiological substrate, CO, caused several spectral changes to the reduced enzyme, most notably a shift of the g = 1.76 feature to g = 1.73.

Carbon monoxide dehydrogenase has been implicated in one- and two-carbon metabolism in methanogenic (1, 2), acetogenic (3, 4), and sulfate-reducing (5) bacteria; all these phenotypic groups include species where the enzyme catalyzes the formation of acetyl-CoA from a methyl donor and an enzyme-bound carbonyl moiety, the latter apparently generated from the reduction of CO2 by the enzyme (3).

On the other hand, carbon monoxide dehydrogenase has been suggested to play the pivotal role in the cleavage of the C-C bond of acetate in those methanogens capable of forming CH4 and CO2 from acetate. The enzyme makes up a large percentage of the soluble cell protein of these organisms (6-8), and is present at up to 10 times the specific activity found in methanogens which cannot utilize acetate as a source of methane, but which still synthesize acetyl-CoA as an early anabolic intermediate (9-12).

Carbon monoxide dehydrogenase has been isolated from four acetotrophs (6, 8, 34) and the autotroph (13), the enzyme has an αβ2 structure, with subunits of approximately 90,000 and 18,000 daltons. The fourth acetotroph has a 5-subunit enzyme, but still possesses subunits with molecular mass near 90,000 and 18,000 daltons (7). In contrast, the enzyme isolated from acetogenic bacteria has subunits of 78,000 and 71,000 daltons, and exists as an α3β3 complex (14, 15).

Despite these differences, all the enzymes which have been examined contain nickel and iron. Electron paramagnetic resonance spectroscopy of the enzyme from the acetogen has detected a signal typical of a [4Fe-4S] cluster, together with a novel signal with apparent g values of 2.01, 1.86, and 1.75 (16). In the presence of CO, a new epr signal is induced that arises from a center with detectable hyperfine interaction from iron, carbon, and nickel when these nuclei are present as stable magnetic isotopes (17).

Here we report on the paramagnetic centers of a carbon monoxide dehydrogenase isolated from the methanogen Methanosarcina barkeri, established the oxidation-reduction midpoint potentials of the two major signals, and report on the effect of CO on the paramagnetic centers.

Materials and Methods

M. barkeri strain MS (DSM 800) was cultured in 451 carboys in the phosphate-buffered medium described by Kenealy and Zeikus (18), supplemented with 75 mM acetate, under an N2 atmosphere. Purity of the culture was routinely ascertained by phase-contrast microscopy. Cells were harvested anaerobically after 19 days growth at 37°C, washed twice in 50 mM Tris, 1 mM dithiothreitol, 1 mM dithionite, pH 8.0, and frozen in liquid N2. They were stored under N2 at -70°C until use.

The cells were thawed, diluted to 1 g, wet weight/2 ml of the buffer described above, and passed through a H2-flushed French pressure cell at 20,000 psi. The lysate was collected under H2, and centrifuged at 30,000 g in polycarbonate bottles. The supernatant was then centrifuged at 150,000 g to remove membrane fragments. Carbon monoxide dehydrogenase was assayed by following methyl viologen reduction in 1.4-m1 stoppered cuvettes containing 1 ml of 60 mM phosphate buffer, pH 7.0, and 5 mM methyl viologen under 1 atm CO. All purification steps were carried out anaerobically, with all columns equilibrated with 1 mM dithiothreitol and dithionite, all buffer reservoirs and fraction collection vials continually gassed with Ar. In a typical purification, 570 ml of a 15 mg/ml extract (5 μmol of CO oxidized per min/mg protein) was loaded onto a 10 x 16-cm column of DE52 cellulose (Whatman), and eluted with a 6-liter 0.1-0.4 M NaCl gradient in 365 ml, and was loaded after a 3-fold dilution onto a 7.5 x 25-cm DEAE-Sepharose 6B column (Pharmacia LKB Biotechnology Inc), and this column was eluted with a 5-liter 0.1-0.5 M NaCl, 50 mM Tris, pH 8.0, gradient. Carbon monoxide dehydrogenase activity eluted at approximately 0.25 M NaCl in 365 ml, and was loaded after a 3-fold dilution onto a 7.5 x 25-cm DEAE-Sepharose 6B column (Pharmacia LKB Biotechnology Inc), and this column was eluted with a 5-liter 0.1-0.4 M NaCl gradient in 50 mM Tris, pH 7.5. The pooled enzyme again eluted near 0.2 M NaCl in a volume of 335 ml, was diluted 3-fold and applied to a 5 x 15-cm DEAE-Sephaloc column (Pharmacia), and eluted with 0.1-0.3 M NaCl in 50 mM Tris, pH 7.5. Fractions containing enzyme were pooled, diluted, and applied to a 7.5 x 14-cm DEAE-Sephadex column (Pharmacia), and finally eluted with a 5-liter 0.05-0.6 M Tris-Cl, pH 7.5, gradient. The enzyme eluted at 0.3 M Tris, and its final specific activity was 135 μmol of CO oxidized min-1 mg-1, and yield varied from 10 to 15%. Purity was assayed using sodium dodecyl sulfate electrophoresis (19), and scanning gel densitometry indicated that the 92,000- and 18,000-dalton subunits comprised >95% of the protein in the sample.

Metals in the enzyme were assayed using plasma emission spectroscopy by the Exxon Corporate Research Analytical Laboratory. Protein was routinely assayed by the method of Bradford (20), using bovine serum albumin as standard. No significant differences were found for the purified enzyme when the method of Lowry et al. (21)
was used instead. Core extrusion followed the method of Gillum et al. (22); the enzyme in 50 mM Tris, pH 8.0, was mixed with 4 parts hexamethylenophosphoramide under N₂, and the formation of the peak at 458 nm (Fig. 2), due to the formation of the thiophenol adduct of [4Fe-4S] clusters, was monitored after the addition of 1 μl of thiophenol. The adduct was quantified using a molar extinction coefficient of 17,200 M⁻¹ cm⁻¹ (22).

Electron paramagnetic resonance spectra were obtained with a Varian E-109 spectrometer equipped with an Oxford Instrument helium flow cryostat and an EIP 548A microwave frequency counter. Spin quantitation used 1 mM CuSO₄, 10 mM EDTA as a standard. Redox potentiometry followed the method of Dutton (23), and used 40 μM of each of the following redox mediators: benzyl and methyl viologens, neutral red, 2-hydroxy-1,4-naphtho- and anthraquinones, phenazine, indigo di- and trisulfonates, duroquinone and 1,4-naphthoquinone. Prior to titration, dithionite and dithiothreitol were removed from the enzyme by passage through an anaerobic Sephadex G-25 (Pharmacia) column equilibrated with 50 mM glycine, pH 9.2. The enzyme was diluted to 1 mg of protein ml⁻¹, and the E₅₅₀ adjusted with dithionite to −600 mV. Oxidizing titrations used thionin or potassium ferricyanide as titrant; samples withdrawn during the titration showed that enzyme activity remained constant between E₅₅₀ −550 and +350 mV.

RESULTS

Plasma emission spectroscopy confirmed earlier experiments (6) that suggested that carbon monoxide dehydrogenase is a nickel-iron enzyme, and revealed that copper and zinc were also present in approximately stoichiometric amounts. The average (±S.D.) of determinations on five different preparations was 29.7 ± 0.8 Ni atoms, 1.5 ± 0.3 Cu atoms, 0.94 ± 0.20 Zn atoms, and 0.85 ± 0.1 Fe atoms/mol tetramer. No other metals were present at above background levels.

Core extrusion experiments with thiophenol (Fig. 1) indicated that the majority of the Fe was extrudable as the thiophenol adduct of [4Fe-4S] clusters, with 6.1 ± 0.7 Fe atoms, 1.5 ± 0.3 Ni atoms, 0.94 ± 0.20 Cu atoms, and 0.85 ± 0.1 Zn atoms/mol tetramer. No other metals were present at above background levels.

Core extrusion experiments with thiophenol (Fig. 1) indicated that the majority of the Fe was extrudable as the thiophenol adduct of [4Fe-4S] clusters, with 6.1 ± 0.78 cores/protein tetramer (average of three separate preparations, ±S.D.). While no [2Fe-2S] clusters were detected, a small contribution, perhaps one or two per enzyme, cannot be ruled out. Core extrusion of oxidized enzyme, stripped of dithionite but under nitrogen, resulted in the formation of a purple complex (optical absorption maxima at 288, 332, 392, and 560 nm), which, when reduced with dithionite, gave a much reduced absorbance maximum at 458 nm.

The reduced enzyme exhibits complex epr spectra at low temperatures (Fig. 2), and two major signals are apparent. One, with apparent g values of 2.05, 1.94, and 1.90 (gₑᵥ = 1.96), is seen in isolation at 22 K (Fig. 2), and remains detectable at 35 K, consistent with it arising from one or more [4Fe-4S] clusters. The second signal is recognizable at lower temperature from the feature with an apparent g value of 1.76. This species is seen in Fig. 3 in the absence of the gₑᵥ = 1.96 signal; it has apparent g values of 2.01, 1.91, and 1.76 (gₑᵥ = 1.89). The very broad feature at 310 mT may represent an additional epr-active site, although such broad features are difficult to quantify. Integration of spectra of the fully reduced enzyme under conditions where neither epr signal was saturable (10 K, 1 mW) indicated approximately 2.2 spins/αβ₂ tetramer, but the spectra are sufficiently broad that this should be considered only a very approximate estimate.

Redox titrations (Figs. 4 and 5) further corroborated the identification of two spectral contributions to the spectra of Fig. 2. The cluster with g values of 2.05, 1.94, and 1.90 had Eₑᵥ of −390 mV, while the cluster with g values of 2.01, 1.91, and 1.76 had Eₑᵥ of −35 mV.

Thionin-oxidized enzyme displays two epr signals (Fig. 6). One, at g = 2.06, was detectable at 77 K and might be attributable to Cu(II). The other, at g = 2.016, was not detectable above 20 K, and is consistent with an origin from

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**Fig. 1.** Extrusion of [4Fe-4S] cores from carbon monoxide dehydrogenase. A, the absorption spectrum of the enzyme (2.2 mg/ml) in 80% hexamethylenophosphoramide, 20% 50 mM Tris, pH 8.0. B, the spectra 15 min (dotted) and 35 and 120 min (solid line) after the addition of thiophenol. The A₄₅₈₅₋₄₅₈ nm ratio averaged 2.03 over five determinations, which is close to that expected for the tetranuclear iron-thiolate complex (22).

**Fig. 2.** Electron paramagnetic resonance spectra of reduced carbon monoxide dehydrogenase. Enzyme (4.6 mg/ml) in 50 mM Tris, 1 mM dithionite, 1 mM dithiothreitol, pH 8.0. The microwave power was 10 mW and modulation amplitude 1.0 mT. The spectrum at 9.5 K is displayed at 81% of the amplification of the others.
EPR of M. barkeri CO Dehydrogenase

FIG. 3. Electron paramagnetic resonance spectrum of partially reduced carbon monoxide dehydrogenase. Enzyme (3 mg/ml) was exchanged into 50 mM Tris, 5 mM dithiothreitol, pH 8.0, and incubated at 4 °C for 48 h. Spectrometer conditions: 10 mW microwave power, 1.0 mT modulation amplitude, 10 K.

Fig. 7 shows the effect of adding the substrate CO to dithionite-reduced enzyme. It reveals a substantial broadening of the spectrum, together with substantial shifts in apparent g value, most notably of the g = 1.76 feature to g = 1.73. The g = 1.90 feature shifted to g = 1.89, and a new feature, which could not be detected above 10 K, appeared at g = 1.86. We were unable to attribute any features of reduced or oxidized enzyme treated with CO to the iron-nickel-carbon center detected by Ragsdale et al. (17) in the enzyme from Clostridium thermoaceticum.

DISCUSSION

Enzymes that oxidize CO to CO₂ form a remarkably diverse group. The aerobic carboxydotrophic bacteria, including several Pseudomonads, consume CO as their sole source of carbon and reducing potential; their CO oxidases are typically molybdo-flavo enzymes with two [2Fe-2S] clusters (27). These enzymes are quite stable in air. In contrast, many anaerobic bacteria that can manipulate CO do this only as a secondary reaction, and the enzymes are known as CO dehydrogenases. In the acetogen C. thermoaceticum and the sulfate-reducer...
Desulfovibrio baarsii, the enzyme is proposed to function in acetyl-CoA synthesis (1, 2, 5), while in acetate-consuming methanogens it functions in acetate cleavage (6–8). These anaerobic enzymes typically contain nickel and iron and are very oxygen-sensitive.

In addition to Fe and Ni, carbon monoxide dehydrogenase from M. barkeri contains significant amounts of Cu and Zn, although the levels of the latter two elements were only approximately 0.5/a8 enzyme. Copper has not previously been reported in a carbon monoxide dehydrogenase, and it is a common adventitious metal in isolated proteins. Nevertheless, it has been found in a hydrogenase from Methanobacterium formicicum (25), where it was epr active, and as discussed above, we tentatively ascribe the g = 2.06 signal of the oxidized carbon monoxide dehydrogenase (Fig. 6) to Cu(II). Zinc is also a common adventitious metal, but it has been associated with all the carbon monoxide dehydrogenases isolated to date; from two acetogens (15, 26), three methanogens (7, 8, 13), and a photosynthetic bacterium (28).

The major paramagnetic species observable in reduced carbon monoxide dehydrogenase from M. barkeri are very similar to those reported for the enzyme isolated from C. thermoaceticum (16, 17). Both enzymes display signals attributable to a [4Fe-4S]1+/2– cluster, with g values of approximately 2.04, 1.94, and 1.90, and a second species with g values of approximately 2.01, 1.91, and 1.76. This despite the fact that C. thermoaceticum is a eubacterial acetogen rather than an archaebacterial methanogen, and the fact that the C. thermoaceticum enzyme exists as either an αα or αβ complex, with subunits of 78,000 and 72,000 daltons (14, 15), while the M. barkeri enzyme is an αβ complex with subunits of 92,000 and 18,000 daltons (6). Spin quantification of the signals in the M. barkeri enzyme suggest less than a single epr-detectable cluster of each type per αβ enzyme, although it should be emphasized that the integrations are over a wide range of magnetic field, and are therefore subject to considerable uncertainty. The core-extrusion experiments (Fig. 1) indicate three [4Fe-4S] clusters per αβ enzyme, and although the gsv = 1.88 has rather low g values for a [4Fe-4S] cluster, our epr data are most simply interpreted as two different [4Fe-4S] clusters detectable in the reduced form, together with the [3Fe-4S] cluster tentatively identified in Fig. 6. Taken at face value, this leaves 4 ± 3 Fe atoms, detected by plasma emission spectroscopy, unaccounted for.

The enzyme from Rhodospiroplum rubrum, which is a monomer (M. 61,800), also displays epr signals similar to those seen here (28). Under the conditions used here the gsv = 1.96 signal is rather broader than in the M. barkeri enzyme, but the most notable difference is that the signal apparently analogous to the gsv = 1.89 signal is not seen in the fully reduced enzyme, but appears on oxidation with FMN.

The E′ of the CO/CO2 couple is −520 mV (29), and the low potential of the cluster(s) giving rise to the gsv = 1.96 signal (E′,m ≈ −390 mV) is suggestive that this cluster accepts electrons from the oxidation of CO. These electrons have been suggested to pass on to a membrane-bound electron transfer system (1, 30) to eventually reduce methyl-CoM to methane.

The function of the higher potential center (E′′,m ≈ −35 mV) is less clear, since its potential renders it an unlikely participant in CO oxidation. Nevertheless, it is this center which shows the most pronounced shift in g value upon the addition of CO. One possibility is that the cluster plays a non-redox catalytic role. The shift in g value upon substrate binding is reminiscent of the changes seen in aconitase upon addition of citrate (31), where the shift has been attributed to the binding of H2O induced by the substrate. This suggests some interesting avenues for future work with carbon monoxide dehydrogenase, especially since one oxygen of the CO2 produced from the oxidation of CO almost certainly comes from H2O. Furthermore, the shift in g value is seen in whole cells actively producing methane from acetate. This suggests that carbon monoxide dehydrogenase does indeed interact with a species similar to CO during physiological turnover, and will be the subject of future work.

We have been unable to detect any epr signal which we could attribute to nickel, in the presence or absence of dithionite or carbon monoxide. This is in contrast to work with the clostridial enzyme, where carbon monoxide induced a signal with hyperfine interactions from nickel, iron, and carbon (16, 17). Similar signals were seen in the carbon monoxide dehydrogenase-corrinoid complex isolated from Methanoarcinina thermophila (32), but not in the enzyme isolated from Methanococcus vannielii (13). One explanation is that the nickel-iron-carbon signal arises from a part of the enzyme responsible for CO2/acetyl-CoA exchange; dithionite is reported to inhibit this exchange (33), and our enzyme is prepared in the presence of dithionite. Alternative explanations include the possibility that the nickel, as Ni(II), does not undergo redox reactions in the M. barkeri enzyme, or that, as Ni(III), it is spin-coupled in some way that it becomes undetectable.

REFERENCES


Fig. 7. The effect of CO on the electron paramagnetic resonance spectrum of reduced carbon monoxide dehydrogenase. Enzyme (8 mg/ml) reduced with dithionite and dithiothreitol was incubated under O2-free Ar (A) or CO (B) for 5 min and then frozen. Spectrometer conditions: 0.7 mW applied power, 0.2 mT modulation amplitude, 9 K.