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EPR EVIDENCE FOR NICKEL-SUBSTRATE INTERACTION IN CARBON MONOXIDE DEHYDROGENASE FROM CLOSTRIDIUM THERMOACETICUM

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SUMMARY, Carbon monoxide dehydrogenase from Clostridium thermoaceticum contains two different Fe_4S_4 rhombic-type EPR resonances with g-values at 2.04, 1.94, 1.90 and 2.01, 1.86, 1.75, respectively. The enzyme after reacting with CO or HCO_3^-/CO_2 also reveals in EPR signal at g = 2.07 and 2.02. This signal, readily observed at 95K, is attributed to a Ni(III) interaction with a radical species formed from CO or HCO_3^-/CO_2.

INTRODUCTION

Nutritional studies have implied, that nickel is a constituent of carbon monoxide dehydrogenase (CO dehydrogenase) of the anaerobic bacteria Clostridium pasteurianum (1) Clostridium formicoaceticum and Clostridium thermoaceticum (2). With the use of ^{63}Ni it has been demonstrated, that nickel is incorporated into a partially purified CO dehydrogenase from C. thermoaceticum (3) and that a protein band obtained by polyacrylamide gel electrophoresis of C. pasteurianum extracts contains nickel and CO dehydrogenase (4).

The CO dehydrogenase from C. thermoaceticum has now been purified to apparent homogeneity (5). The enzyme with a molecular weight of about 440,000 is hexamerous and it consists of three each of two different subunits giving the structure $(\alpha\beta)_3$. The dimer, $\alpha\beta$, apparently contains per mol 2 nickel, 3 zinc, 12 iron and 14 acid-labile sulfur. However, the metal content may vary between different preparations of the enzyme.

Under anaerobic conditions in the presence of the substrate, CO, the enzyme has a porphyrin-type absorption spectrum, with peaks at 600, 393,

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380, 310 and 277 nm and shoulders at 670 and 550 nm. Oxygen destroys the enzyme activity, and converts the spectrum of the enzyme to that of a typical iron-sulfur protein. Thus, the enzyme appears to contain iron-sulfur centers as well as an additional chromphore which seems to react with CO. In this communication we report on a EPR-study of the iron sulfur centers and a possible nickel chromophore of the CO dehydrogenase from C. thermoaceticum.

EXPERIMENTAL

C. thermoaceticum (ATCC 39073) was grown at 58°C in a 400-liter fermenter in a previously described medium (6) modified by the addition of 10^{-6}M $NiCl_3$. The enzyme from 180 g of wet weight cells was purified to an activity of 450 (about 40-fold) µmol of CO oxidized min^{-1} mg^{-1} when assayed at 50°C with 10 mM MV as electron acceptor (7). The purification, which is described in detail elsewhere (5) was performed inside an anerobic chamber in an atmosphere of N_2:H_2(95:5) using as buffer 50 mM Tris/HCl, pH 7.4 containing 2mM sodium dithionite. The purification steps included preparation of cell extract using a French pressure cell, heat treatment at 67°C for 30 min, ammonium sulfate fractionation, (45-60% saturation), chromatography on DE32-cellulose, Bio-Gel HTP and DEAE Sephacel, and gel filtration with Bio-Gel A.1.5 M and Ultrogel AcA22.

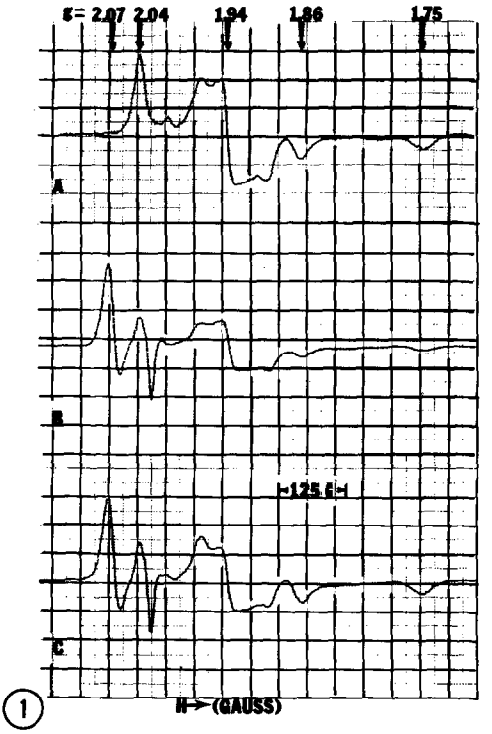
The purified enzyme was prepared for EPR studies by applying a solution containing 40 mg to a DE32-cellulose column (1.5 x 5 cm), previously equilibrated with 50 mM Tris/HCl, pH 7.6 and 2 mM sodium dithionite. The column was washed with 5 volumes of oxygen-free 50 mM Tris/HCl, pH 7.6, without the dithionite. The enzyme was then eluted in 6 ml with 0.5 M Tris/HCl, pH 7.6, and concentrated to 2 ml using a PM 30 ultrafiltration membrane and an Amicon ultrafiltration cell (Amicon Lexington, Mass.). The final protein concentration was 20 mg ml^{-1}.

Samples for EPR were prepared anaerobically. Each EPR tube contained in 260 µl 0.5 M Tris/HCl, pH 7.6, and 4.8 mg of CO dehydrogenase. Additions to individual tubes were as follows: none, sodium dithionite, 2 mM; potassium ferricyanide, 0.5, 1 or 2 mM; and sodium bicarbonate, 80 mM. Tubes were also prepared with 100% CO (99.99% pure, Matheson, Morrow, GA.) or air as gas phase. Prepared tubes were stored in liquid nitrogen until analyzed with EPR spectroscopy.

EPR spectroscopy was performed with a Varian E-109 spectrometer interfaced with a Hewlett-Packard HP-85 microcomputer. Measurements in the liquid-helium temperature range were performed with an Air Products APD-E automatic temperature controller. Other EPR experimental conditions are found in Figure Legends. Nickel and iron were determined using plasma emission spectroscopy (8).

RESULTS AND DISCUSSION

The EPR spectrum at 10K of C. thermoaceticum CO dehydrogenase as isolated under anaerobic conditions and without any addition is shown in Fig. 1A. The spectrum indicates reduced multiple iron-sulfur centers. These signals



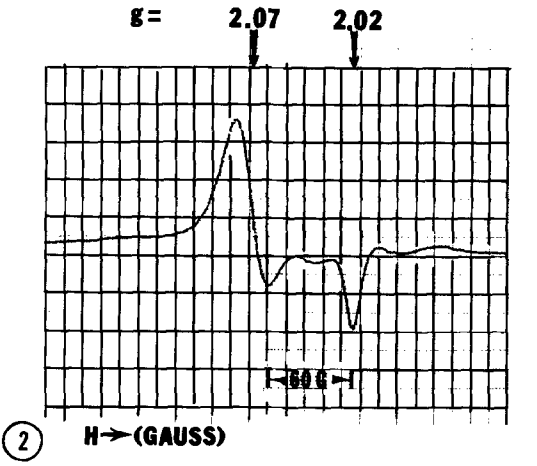


Figure 1. EPR Spectra of Carbon Monoxide Dehydrogenase Purified from Clostridium thermoaceticum. A.) Purified CO dehydrogenase (42 μM in protein in 500 mM Tris buffer, pH 7.6) maintained under anaerobic conditions (argon). EPR conditions: microwave power, 20 μW; microwave frequency, 9.162 GHz; modulation amplitude, 10 G; temperature, 10 K; scanning rate, 500 G per min; time constant, 0.1 sec; Gain = 1.25×10^4. B.) Purified CO dehydrogenase as in (A) but reacted under anaerobic conditions with carbon monoxide for 1 hour. EPR conditions as in (A) except that Gain = 5×10^3. C.) Purified CO dehydrogenase as in (A) but reacted under anaerobic conditions with 80 mM sodium bicarbonate for 1 hour. EPR conditions as in (A) except that Gain = 8×10^3.

Figure 2. EPR Spectum of Carbon Monoxide Dehydrogenase at the Redox State of Figure 1 B. EPR conditions as in Fig. 1 B except that the scan range was two-fold expanded; microwave power, 10 mW; microwave frequency, 9.161 GHz; temperature, 95 K; scanning rate, 250 G per min and Gain = 5×10^3.

are not observed when EPR measurements are made at 95K. There appear to be two Fe_4S_4 rhombic-type EPR resonances; one exhibits g-values at 2.04, 1.94 and 1.90, while the other has g-values of 2.01, 1.86 and 1.75. Quantitation by double-integration of this EPR spectrum under non-saturating conditions using cupric-EDTA as the standard and by correcting for g-value dependence on transition probability by the procedure of Aasa and Vanngard (9) yields 1.8 electrons per mol of dimeric enzyme. This is consistent with the appearance of two Fe_4S_4 centers. Titration of the enzyme with ferricyanide using EPR spectroscopy results in diminishment of the signal

intensities of the iron-sulfur centers (not shown). A maximum decrease (based on double-integration) of approximately 70% occurs with the highest ferricyanide concentration (2 mM), that was used. Enzyme inactivated by oxygen is essentially EPR silent.

The exposure of the anaerobic enzyme to CO at 10K yields a slight increase (12%) in the intensity of the signals of the reduced iron-sulfur centers. However, more interestingly, a new intense signal appears with g-values at 2.07 and 2.02 (Fig. 1B). This EPR signal is readily observed at 95K, when the interfering signals of the iron-sulfur centers are absent (Fig. 2). Addition of HCO_3^-/CO_2 to the anaerobic enzymes (Fig. 1C) has an effect very similar to that of exposing the enzyme to CO, except that the new signal at g = 2.07 and 2.02 is less intense and that the increase of signals of the iron-sulfur centers is only 5%.

The enzyme species represented by the new signal at g = 2.07 and 2.02 is of obvious biological significance. It is only formed under anaerobic conditions by interaction of the enzyme with the substrate, CO, or the product, HCO_3^-/CO_2. The signal is not observed when the enzyme is reduced with sodium dithionite and then reacted with CO or HCO_3^-/CO_2. Neither is it formed when the enzyme is titrated over a wide range with ferricyanide nor in the presence of oxygen (the latter inactivates the enzyme). The microwave power saturation behavior and temperature sensitivity strongly suggests that the new signal comes from a transition metal complex. This new signal does not saturate at a microwave power of 30 mW when measured at 95K. Since nickel is the only transition metal present (besides iron, which is in the form of the Fe_4S_4 centers), it is probable that a form of nickel is involved in the new signal. Double-integration of this proposed nickel signal accounts for 0.4 electrons per mol which indicates that a substantial amount of nickel is in this paramagentic form (20%). A number of hydrogenases have now been shown to have a Ni(III) EPR signal with g-values at 2.3, 2.2 and 2.0 (10,11). The EPR signal in Fig. 2 is obviously quite different from any Ni(III) EPR signal obtained with hydrogenase. In

fact the new signal resembles strongly in EPR properties the radical doublet intermediate reported by Babior et al (12) for ethanolamine ammonia lyase and adenosylcobalamin in the presence of amino-2,1 propanol. Their signal was attributed to an interaction between Co(II) in the low-spin state and a radical species. Since Ni(III) is isoelectronic with Co(II) and the possibility of a radical species involving CO or HCO_3 is quite likely, we would like to propose that the new EPR signal with g-values at 2.07 and 2.02 is a Ni(III) species interacting with a radical species formed from CO or HCO_3^-/CO_2. The signal shown for CO dehydrogenase is not centered at g = 2 nor does it exhibit a broad line near g = 2.3 as does the signal described by Babior et al (12); these differences may be due to a unique nickel (III)-radical species which is low-field shifted.

Hu et al (13) have demonstrated that acetate is synthesized from CO and 5-methyltetrahydrofolate by an enzyme system obtained from C. thermoaceticum CO dehydrogenase is a part of this enzyme system and they have proposed that the enzyme may contain an C-1 species on the oxidation level of formate. Our results appear to indicate that the C-1 species is in the form of a radical, which is associated with nickel in turn possibly associated with a porphyrin-like factor. This factor appears not to be identical to the nickel-containing porphinoid factor F_{430} from Methanobacterium thermoautotrophicum (14) since the native enzyme lacks an absorption at 430 nm (5). The structure of the porphinoid system of Factor F_{430} has recently been elucidated (15).

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