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^{13}C and ^{61}Ni ISOTOPE SUBSTITUTIONS CONFIRM THE PRESENCE OF A NICKEL(III)-CARBON SPECIES IN ACETOGENIC CO DEHYDROGENASES

Steve W. Ragsdale, Lars G. Ljungdahl, and
Daniel V. DerVartanian

Department of Biochemistry
University of Georgia
Athens, Georgia 30602

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SUMMARY: The nickel-containing CO dehydrogenases from Acetobacterium woodii and Clostridium thermoaceticum were studied by EPR spectroscopy in order to define the components involved in the EPR spectrum obtained by reaction of the enzymes with the substrate, CO. Using isotopic substitution techniques, these experiments unequivocally establish that a nickel-carbon species is involved in the g=2.08, 2.02 EPR signal. Comparing the ^{61}Ni- and ^{59}Ni-substituted enzymes, the g=2.08 component of the resonance was found to be mainly due to nickel with a smaller contribution by the carbon species. Reaction of the CO dehydrogenase with [^{13}C]CO versus [^{12}C]CO showed that a carbon species, formed from CO, was the major contributor to the g=2.02 EPR signal. In addition, the oxidized CO dehydrogenase was found to exhibit a Ni(III) EPR signal analogous to that of the hydrogenases from the methanogenic and sulfate-reducing bacteria.

Carbon monoxide dehydrogenase (CO dehydrogenase) carries out the reaction: $CO + H_2O \longrightarrow CO_2 + 2\ e^- + 2\ H^+$. CO can serve as sole carbon and energy source for the aerobic carboxydobacteria (1) and is utilized as a substrate for growth by several anaerobic acetogenic bacteria (2,3). Wood et al, (4) have suggested that CO dehydrogenase plays an essential role in the growth of acetogens on H_2/CO_2, CO, and hexoses.

Nickel has been shown to be a component of the CO dehydrogenases from acetogenic bacteria by nutritional studies (5,6), by electrophoresis of a radioactive ^{63}Ni-substituted enzyme (7), and by purification to homogeneity of the CO dehydrogenases from Acetobacterium woodii (8) and Clostridium thermoaceticum (9). Approximately 5-6 mol Ni and 6-9 (4Fe-4S) clusters per mol protein are present in the acetogenic CO dehydrogenases (8,9).

Treatment of CO dehydrogenase with CO results in reduction of the iron-sulfur centers (8,9) and the appearance of an unusual EPR spectrum with g-

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values at 2.08 and 2.02 (8,10). This EPR-detectible species was postulated to result from a Ni(III)-carbon species (10). However, evidence for this assignment was indirect, and isotopic substitution of Ni and CO with nuclei containing magnetic moments is necessary to unambiguously identify the EPR-detectible species. In the experiments reported here, the CO dehydrogenase from C. thermoaceticum was substituted with ^{61}Ni (I = 3/2) and the EPR spectrum of the CO-treated enzyme was compared to the CO-treated ^{59}Ni enzyme. Both the ^{61}Ni- and ^{59}Ni-CO dehydrogenase of C. thermoaceticum and the natural ^{59}Ni enzyme from A. woodii were treated with [^{12}C]CO and [^{13}C]CO and the spectra were compared. In this paper, we offer direct proof that the nickel atom in CO dehydrogenase reacts with CO and thereby forms a Ni(III)-carbon species. The possible physiological importance of this EPR-detectible species is discussed.

EXPERIMENTAL

Clostridium thermoaceticum, DSM 521, was grown at 58°C in a 400 ℓ fermenter in a previously described medium (11) with the addition of 10^{-6} M $NiCl_2 \cdot 6\ H_2O$. For substitution of ^{61}Ni in the enzyme, the growth medium was modified, since the medium described above without addition of supplementary nickel was found by plasma emission spectroscopy to contain 480 nM nickel. We were able to reduce this to 260 nM by dissolving the yeast extract (Difco) in water and passing it through a Chelex 100 (BioRad) column (4g Chelex/g yeast extract). A mineral solution (12), added in the amount described for growth of A. woodii (13) was necessary to supplement the medium since the Chelex treatment severely reduced the level of most trace metals. All other solutions, except nickel, were exactly as described in reference 11 including the addition of Co, Mo, Se, W, and Fe. ^{61}Ni, obtained from Oak Ridge National Laboratories (88.84% isotopic purity) was dissolved in a minimum volume of concentrated nitric acid and added to the growth medium to 7 μM final concentration. Cultures were grown in 1 liter glass Ehrlenmeyer flasks for three transfers before transferring to 20 ℓ glass carboys. The doubling time of the culture was 6-7 hours and acetate production reached 100 μM, with no sign of other volatile products. Microscopic examination also indicated that the culture was uncontaminated and that the new medium was suitable for healthy growth of C. thermoaceticum. The calculated isotopic purity of the resulting ^{61}Ni protein should be 86% ($88.84 \times \frac{7.00}{7.26}$). The natural abundance of ^{61}Ni is 1.19%. [^{13}C]CO was obtained from Merck and Co. (99% isotope purity) and [^{12}C]CO was from Matheson (99.99%, Matheson grade).

Acetobacterium woodii (ATCC 29683) was cultured by the method of Balch et al, (13) on fructose using a N_2/CO_2 (66/33) atmosphere. Other details of growth of 400 ℓ of the heterotrophic cells are given in reference 8.

The C. thermoaceticum (9) and A. woodii (8) CO dehydrogenases were isolated as described previously. Both the C. thermoaceticum and A. woodii CO dehydrogenases had a final specific activity of 500 units mg^{-1} using the

assays described in reference 8. Both enzymes were homogeneous by SDS and alkaline gel electrophoresis.

The purified CO dehydrogenases were concentrated to 1 ml and gel filtrated on a 1 x 25 cm Sephadex G-25 Superfine column equilibrated with anaerobic 50 mM Tris/HCl buffer, pH 7.6 (no reducing agent). The resulting brown protein, which still had at least 90% of the original activity, was concentrated using an Amicon XM-100 ultrafiltration membrane and placed in calibrated 4.00 mm (outer diameter) quartz EPR tubes. Other details of sample preparation are given in the FIGURE LEGENDS.

EPR spectroscopy was performed with a Varian E-109 spectrometer interfaced with a Hewlett-Packard HP-85 microcomputer. Measurements in the liquid-nitrogen and liquid-helium temperature ranges were performed with an Air Products APD-E automatic temperature controller. Other EPR experimental conditions are found in FIGURE LEGENDS.

RESULTS AND DISCUSSION

The acetogenic CO dehydrogenases, when chromatographed on Sephadex G-25 to completely remove dithionite, exhibit a light absorption spectrum characteristic of an oxidized Fe-S protein (8,9). Under these conditions, an EPR spectrum with g-values at 2.21, 2.11, and 2.02 is obtained (Fig. 1). These g-values and temperature and microwave power characteristics are similar to the Ni(III) signal found with purified hydrogenases from the sulfate-reducing and methanogenic bacteria (14-17). However, the EPR quantitation for the Ni(III) EPR signal demonstrates a spin recovery of only 10% of the total nickel, in contrast to the values of 50-60% reported for the various hydrogenases (14-17). Reaction of CO_2 as an oxidant or of potassium ferricyanide with reduced or oxidized enzyme did not result in the appearance of the above Ni(III) EPR signal. These results may

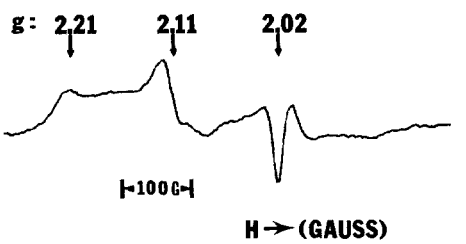

Figure 1. EPR spectrum of C. thermoaceticum CO dehydrogenase after gel filtration (88 μM in protein, in 50 mM Tris/HCl buffer, pH 7.6). EPR conditions: microwave power, 10 mW; temperature, 77 K; frequency, 9.161 GHz; modulation amplitude, 10 G; scan range, 1000 G; scanning rate, 400 G per min; time constant, 0.1 sec.

reflect the difficulty of trapping the Ni signal of CO dehydrogenase in the oxidation state of (III).

CO dehydrogenase, when reacted with CO or CO_2/HCO_3^-, shows an EPR signal at g=2.08 and 2.02, which is observable at temperatures near liquid N_2(10). This signal has been postulated to be due to an interaction between Ni(III) and a carbon species formed from CO or HCO_3^-/CO_2 (10) based on the analogy with certain EPR characteristics of the Co(II)-carbon intermediate reported by Babior et al, (18). In order to establish that a carbon species is involved in the resonance, CO dehydrogenases from A. woodii and C. thermoaceticum were treated with [^{13}C]CO (I=1/2) and [^{12}C]CO (I=0). Fig. 2 compares the EPR spectra when [^{12}C]CO or [^{13}C]CO was added to CO dehydrogenase. With [^{13}C]CO, a significant broadening was observed in the signal component at g=2.02, with a half-minimum width broadening of 9 gauss relative to [^{12}C]CO. A smaller degree of broadening was noted in the signal component at g=2.08 with a half-maximum width broadening of approximately 2 gauss. The EPR changes shown in Fig. 2 are with the CO dehydrogenase purified from A. woodii. However, identical results were obtained with the CO dehydrogenase from C. thermoaceticum. The results confirm that the EPR signals at g=2.08, 2.02 are in part due to a carbon species formed from

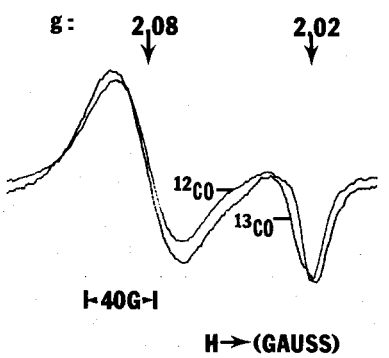

Figure 2. EPR spectra of CO dehydrogenase from A. woodii (30 μM in protein, in 50 mM Tris/HCl buffer, pH 7.6) reacted with [^{12}C]CO or [^{13}C]CO for 13 min under anaerobic conditions. EPR conditions are as in Figure 1 except that the temperature was 12 K; modulation amplitude, 5 G; scan range, 400 G; scan rate, 250 G per min; microwave power, 0.1 mW.

CO. CO_2-treatment also shows the g=2.08, g=2.02 EPR signal, but only with reduced enzyme.

The CO dehydrogenase from C. thermoaceticum was isotopically substituted with ^{61}Ni(I=3/2) in order to demonstrate that nickel is involved in the g=2.08, 2.02 resonance. C. thermoaceticum was grown on the nuclear isotope ^{61}Ni and the ^{61}Ni-enriched CO dehydrogenase purified to homogeneity. If nickel is involved in the g=2.08, 2.02 signal, a significant broadening or induced hyperfine interaction should be observed in the EPR signal on the addition of CO to the ^{61}Ni-enriched CO dehydrogenase when compared to the natural ^{59}Ni-containing enzyme. As seen in Fig. 3A versus 3B, significant broadening but no induced hyperfine pattern was detected. In this case, the greatest degree of broadening was found in the g=2.08 region

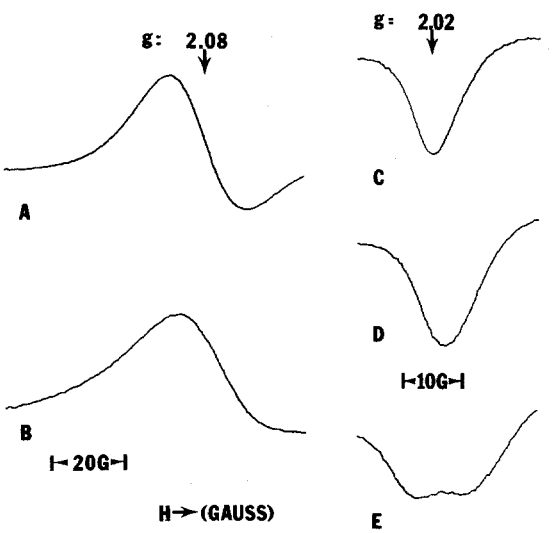

Figure 3. EPR spectra of C. thermoaceticum CO dehydrogenase reacted with CO under anaerobic conditions. EPR conditions are as in Figure 1, except that the modulation amplitude was 2 G. (A) unenriched ^{59}Ni-enzyme (130 μM in protein, in 50 mM Tris/HCl buffer, pH 7.6) reacted for 13 min with [^{12}C]CO under anaerobic conditions; scan range 160 G; scan rate, 60 G per min (g=2.08 region). (B) ^{61}Ni-enriched enzyme (75 μM in protein in the same buffer) reacted with [^{12}C]CO for 13 min; scan range and rate as in (A) (g=2.08 region). (C) protein and conditions are as in (A), but scan range of 100 G and scan rate of 40 G per min (g=2.02 region). (D) protein and conditions as in (B), except scan range of 100 G and scan rate of 40 G per min (g=2.02 region). (E) ^{61}Ni-enriched enzyme (75 μM in protein and same buffer) reacted for 13 min with [^{13}C]CO; scan range and rate as in (D).

where a half-maximum broadening of 11 gauss was detected. A smaller degree of broadening (about 2 gauss) was noted in the g=2.02 region (Fig.3C versus 3D). The extent of broadening noted for the substitution of ^{61}Ni for the non-nuclear natural isotope of ^{59}Ni is in accord with a similar magnitude of broadening reported in ^{61}Ni-enriched hydrogenase from the sulfate-reducing bacterium, D. gigas (17). The results presented in Figures 3A, 3B and 3C, 3D unequivocally confirm that both nickel and a carbon species are involved in the g=2.08, 2.02 EPR signal. The extent of broadening with [^{13}C]CO or ^{61}Ni suggests that a greater involvement of nickel occurs at the g=2.08 component. Conversely, the isotopic substitution studies indicate that the carbon species has greater participation in the component at g=2.02. The microwave power dependence on the intensity of the g=2.08 and g=2.02 components are identical, which indicates that the signals arise from the same species. Figure 3E illustrates that when [^{13}C]CO is reacted with ^{61}Ni-enriched CO dehydrogenase, the degree of broadening observed for each nuclear isotope substituted appears to be additive. For example in the g=2.02 region the contribution of broadening from ^{61}Ni-substitution (2 gauss) and from [^{13}C]CO substitution (9 gauss) adds up to a total of 11 gauss which is close to what is observed, 13 gauss. Also observed is a distinct induced hyperfine pattern of 2 resolved lines due to the nuclear spin of [^{13}C]CO. There appears to be no additional significant magnetic interaction between the ^{13}C and ^{61}Ni nuclei.

The question then arises as to the role of this stable, high-yield Ni(III)-carbon species in the mechanism of action of CO dehydrogenase and in the overall pathway as an intermediate in acetate biosynthesis. Since the concentration of the EPR-detectible species relative to a Cu^{+2} standard, accounts for a substantial fraction of the total nickel present, (approximately 40%) (10), the Ni-carbon species apparently is a viable intermediate in the catalytic conversion of CO to CO_2. CO dehydrogenase has also been implicated as a carrier of the C-1 unit which reacts with 5-methyltetrahydrofolate and another enzyme component(s) in the formation

of acetyl CoA (4,19). This enzyme-bound intermediate is proposed to be formed from CO_2, CO, or the pyruvate carboxyl as well as from acetyl CoA during the exchange reaction with CO (4). If, indeed, CO dehydrogenase does link pyruvate, CO, CO_2, and acetyl CoA as C-1 donors, then it is plausible that the EPR-detectible Ni-carbon species could be the enzyme-bound C-1 intermediate. The described properties of the nickel-carbon species suggest an important role which will be explored in further studies.

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