Kinetic Evidence that Carbon Monoxide Dehydrogenase Catalyzes the Oxidation of Carbon Monoxide and the Synthesis of Acetyl-CoA at Separate Metal Centers†

Manoj Kumar, Wei-Ping Lu, Lifei Liu, and Stephen W. Ragsdale*

Department of Biochemistry, East Campus University of Nebraska, Lincoln, Nebraska 68583-0718

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This communication describes pre-steady-state kinetic studies of the reaction of CO with carbon monoxide dehydrogenase (CODH). It is shown that CO rapidly binds to center C. The [center C]–CO complex is oxidized to CO_2 as electron transfer occurs to center B. A second mole of CO binds to center A of CODH, resulting in the formation of a [Ni–Fe–S]–CO complex. This species is kinetically competent as an intermediate in the synthesis of acetyl-CoA. However, it is formed much too slowly to implicate it as an intermediate in the oxidation of CO to CO_2.

Many strict anaerobes perform CO_2 fixation by the reductive acetyl-CoA or the Wood/Ljungdahl pathway which involves the condensation of two one-carbon units to form acetate.[1,2] Carbon monoxide dehydrogenase (CODH) catalyzes the reversible oxidation of CO to CO_2 and also catalyzes the assembly of acetyl-CoA from a methyl group, CO, and CoA. An unresolved question is whether CO oxidation and acetyl-CoA assembly occur on the same metal center or different metal centers.

CODH from *Clostridium thermoaceticum* contains 2 Ni, 11–14 Fe, ~14 inorganic sulfides, and variable amounts of zinc.[3] These metals are organized into at least three distinct iron-containing centers that we will call centers A, B, and C. Center A is a Ni/Fe–S cluster that elicites an EPR spectrum with g values of 2.08, 2.07, and 2.03 when CODH is reacted with CO.[4] The minimal working model of the structure of this CODH–CO complex is [Ni–X–Fe_4S_4]–CO, where X is an unknown bridge between the Ni site and the [4Fe–4S] cluster.[4–7] Center B is a [4Fe–4S]$^{2+/1+}$ cluster with g values at 2.05, 1.94, and 1.89.[5,6,8] Center C, for reasons described below, is thought to exist in two EPR distinguishable states: C with g values at 2.01, 1.81, and 1.65 and C′ with g values at 1.97, 1.86, and 1.75. Elucidation of the functions of these metal centers is important in understanding the mechanisms of acetyl-CoA synthesis and CO oxidation.

Several results indicate that CO oxidation and acetyl-CoA synthesis occur at different sites. (i) Center A has been shown to be important in the synthesis of acetyl-CoA.[4,9–11] When CODH is reacted with CO, center A forms an adduct in which the bound CO serves as the precursor of the carbonyl group of acetyl-CoA.[9] (ii) Center C has been implicated in CO oxidation since cyanide, which strongly inhibits the oxidation of CO to CO_2 and only affects acetyl-CoA synthesis at relatively high concentrations,[3,12] binds to center C.[13] (iii) Removal of ~15% of the nickel from CODH causes loss of acetyl-CoA synthesis activity and Ni–Fe–C EPR signal, but has no effect on the CO oxidation activity.[10] (iv) Bacteria that perform CO oxidation contain iron–sulfur clusters similar to centers B and C, whereas only those which can synthesize or catabolize acetyl-CoA share center A.

Kinetic methods are valuable in observing and ruling out putative intermediates in a reaction scheme. Freeze quench EPR and stopped flow kinetic studies of the reaction of CODH with CO have been performed to observe the time course of formation of intermediates in CO oxidation and acetyl-CoA synthesis. Before reaction with CO, CODH[19] shows the EPR spectrum[20] from center C and a trace amount of the signal from center B. When such samples are reacted at 5 °C with a buffer solution containing 20% (180 μM) CO,[21] the decay of this EPR signal coincides with formation of the spectrum of center C′ (k_{obs} ~ 400 s^{-1}) (Figure 1).[22] This corresponds to approximately 17 200 s^{-1} at 55 °C.[23] Both rates increase as the concentration of CO increases;[23] therefore, they correspond to second-order reactions that appear to reflect the binding of CO to center C to form C′. CO_2 causes an apparent increase in the reduction potential of C′,[5] also indicating that CO_2 or CO can bind to the reduced form of this center. The second-order rate constant can be estimated at 4.4

* For communications regarding this manuscript, please contact: Dr. Stephen W. Ragsdale, Department of Biochemistry, East Campus, University of Nebraska, Lincoln, Nebraska 68583-0718. Phone: 402-472-2943. Facsimile: 402-472-7842.

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(19) CODH Purification. *C. thermoaceticum* strain ATCC 39073 as previously described.[17] Isolation of CODH was performed[12] under strictly anaerobic conditions in a Vacuum Atmospheres anaerobic chamber at 16 °C at an oxygen level below 1 ppm. CODH was stored in liquid nitrogen until use. After addition of the oxidant, thionin (0.5 mM, final), CODH was washed three times with 50 mM Tris-HCl, pH 7.6, containing 2 mM dithiothreitol in an Amicon PM30 ultrafiltration membrane. CO oxidation assays were performed as previously described.[3] The average specific activity after thionin treatment and desalting was 260 μmol min^{-1} mg^{-1} in the CO oxidation reaction and 140 nmol min^{-1} mg^{-1} in the exchange of [1-^{14}C]acetyl-CoA with CO. The molar concentration of CODH is expressed as the α,β dimeric form (M_r = 149 000).

(20) EPR spectra were recorded on a Bruker ESP 300E spectrometer equipped with an Oxford ITC4 temperature controller, automatic frequency counter (Hewlett-Packard, Model 5340A), and gaussmeter (Bruker). Spin concentrations were measured by comparing the double integrals (using supplied Bruker software) of the spectra with those of a 1 mM copper perchlorate standard. Spectroscopic parameters are given in the figure captions.

(21) Freeze quenching was performed basically as described[18] using an Update Instruments chemical/freeze quench instrument with a computer-operated Model 745 controller. The temperature of the isopentane bath was maintained at −140 °C. Quenching times were determined by measurement of the EPR signal formed when metmyoglobin is reacted with azide.[18] Rate constants were determined by a nonlinear least squares fit of the data to a single exponential. Stopped flow experiments were recorded on an Applied Photophysics spectrofluorimeter. Protein and CO solutions were made in the anaerobic chamber before transfer into tonometers which served as reservoirs for the drive syringes. The temperature was maintained at 25 °C with a bath of nitrogen-bubbled water from a circulating pump. Data were fit with software purchased from Applied Photophysics.

(22) To obtain quenching times in the 1–5-ms time scale, it was necessary to use extremely high linear velocities (8 cm/s).

(23) The rate constant determined for the decay of C and formation of C′ is 950 ± 160 s^{-1} when CODH is reacted with 50% CO at 5 °C. We are too near the limit of the freeze quench experiment to obtain an accurate temperature dependence for the rates of formation and decay of the signals from center C. The activation energy used in calculating the rate constants for formation/decay of the signals from centers B and C is 57 ± 4 kJ/mol, which is the value we determined for the oxidation of CO to CO_2.

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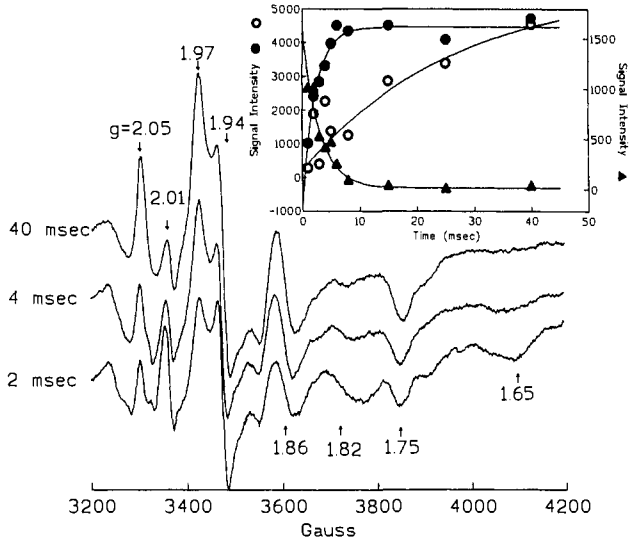

Figure 1. Freeze quench and low-temperature EPR spectra of the reaction of CODH with CO. CODH (30 μM) in 50 mM Tris-HCl, pH 7.6, 2 mM dithiothreitol was mixed at 5 °C with a solution of the same buffer which had been equilibrated with a CO/N_2 (20%/80%) gas mixture. Selected EPR spectra at the delay times shown. EPR conditions: microwave power, 10 mW; sample temperature, 10 K; microwave frequency, 9.4436 GHz. (Inset) Plot of signal intensities of the $g = 1.86$ (●), $g = 1.65$ (▲), and $g = 2.05$ (○) resonances versus time. The first-order best fit lines are drawn through the data points. The calculated rate constants are 440 ± 170 s^{-1} (formation of the signal from C'), 340 ± 120 s^{-1} (decay of the signal from C), and 64 ± 150 s^{-1} (formation of the signal from center B).

× 10^6 M^{-1} s^{-1} (5 °C) or ~2 × 10^8 M^{-1} s^{-1} (55 °C),[23] which is greater than the k_{cat}/K_m^{CO} for CO oxidation (2 × 10^7 M^{-1} s^{-1} at 55 °C). No changes in the UV–visible spectrum were observed in this time scale, indicating that this process does not involve redox chemistry.

The EPR signal from center B develops with a k_{obs} of ~60 s^{-1} at 5 °C with 20% CO (Figure 1). Reduction of center B was also observed by stopped flow where a k_{obs} value of 90 ± 20 s^{-1} at 5 °C with 100% CO was obtained. The k_{cat} for CO oxidation at 5 °C was determined to be 47 s^{-1}.[23] One can estimate[23] the rate constant for reduction of this center at 55 °C to be 2600–3900 s^{-1}, which is greater than the k_{cat} for CO oxidation (2000 s^{-1} at 55 °C).[3]

The EPR signal arising from the CO complex with center A (the NiFeC species) develops with a k_{obs} of 0.16 s^{-1} at 25 °C (Figure 2). Assuming an activation energy of 52.2 kJ/mol,[9] the

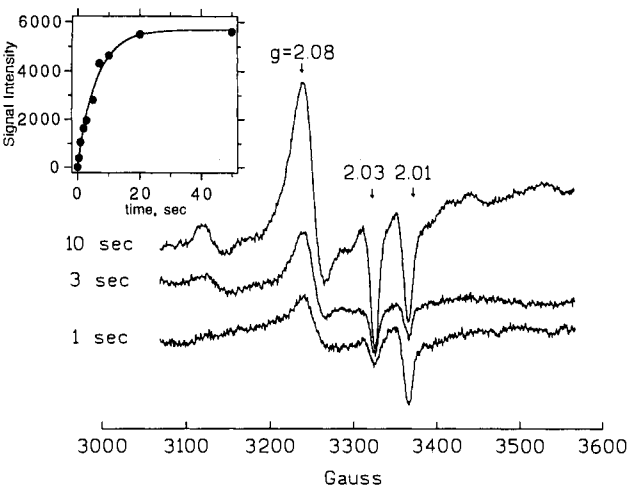

Figure 2. Freeze quench and high-temperature EPR spectra of the reaction of CODH with CO. The experimental conditions were the same as described in the caption to Figure 1 except that the reaction was performed at 25 °C and CODH was reacted with buffer saturated with 1 atm of CO. Selected EPR spectra at the delay times shown. EPR conditions: microwave power, 40 mW; sample temperature, 90 K; microwave frequency, 9.4458 GHz. (Inset) Plot of signal intensity of the $g = 2.08$ (●) resonance versus time. The first-order best fit line is drawn through the data points. The calculated rate constant is 0.16 ± 0.02 s^{-1}.

rate constant at 55 °C would be 1.1 s^{-1}, which approximates both the k_{cat} for the exchange between CO and acetyl-CoA[12,14] and that for the synthesis of acetyl-CoA from methyl tetrahydrofolate, CO, and CoA.[15] Thus, the NiFeC species is kinetically competent to serve as an intermediate in the synthesis of acetyl-CoA. There is evidence that CO binds to the reduced state of center A.[16] The standard reduction potential of the $NiFeC^{ox/red}$ couple is −540 mV.[9] Thus, the CO oxidation activity of CODH allows reductive activation of center A so that it can bind CO for the succeeding steps of carbon–carbon and carbon–sulfur formation. However, the NiFeC species clearly *cannot be an intermediate in the oxidation of CO* to CO_2 since it is formed at 1.1 s^{-1}, approximately 1800-fold more slowly than the k_{cat} for this reaction (~2000 s^{-1}).

Our results from kinetic studies demonstrate that CO oxidation and acetyl-CoA synthesis occur at separate sites on CODH. It appears that center C binds CO and electrons are transferred to centers A and B as CO_2 is formed. Reductive activation of center C allows it to form a stable complex with CO. The NiFeC complex then serves as the precursor of the carbonyl group of acetyl-CoA.

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