# Freeze–Quench Resonance Raman Spectroscopic Evidence for an Fe–CO Adduct during Acetyl-CoA Synthesis and Ni Involvement in CO Oxidation by Carbon Monoxide Dehydrogenase from *Clostridium thermoaceticum*

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Carbon monoxide dehydrogenase (CODH) from *Clostridium thermoaceticum* is a Ni–Fe metalloenzyme that has two separate but linked activities of CO metabolism. One molecule of CO is oxidized to $CO_2$ at an EPR-detectable site called center C, while another molecule of CO is combined with a methyl group and coenzyme A to form acetyl-CoA at a separate EPR-detectable site called center A. In the work reported here, we have adapted the freeze-quench technique[1] for resonance Raman (RR) spectroscopy. We report that Ni is involved in CO oxidation at center C. We also confirm our previous inference[2] that CO binds to center A at an Fe atom, and not at Ni.

The CODH from acetogenic[3,4] bacteria contains two Ni atoms, as well as 11–14 Fe atoms. One Ni is associated with an Fe–S cluster at center A.[5] The location of the second Ni atom has been uncertain. Center C has been suspected of harboring the second Ni, by analogy with the CO oxidation site of the *R. rubrum* CODH. This enzyme lacks acetyl-CoA synthesis activity and has a single Ni atom.[6] Its EPR spectrum shows a slight increase in its EPR line width upon $^{61}$Ni substitution, indicating that nickel is a component of the EPR-detectable center.[7] EXAFS studies of the *R. rubrum* enzyme indicate that the Ni–Fe distance is greater than 3.4 Å, indicating that this center is not a $NiFe_3S_4$ cubane cluster.[8]

Freeze–quench EPR spectroscopic studies of the *C. thermoaceticum* CODH have demonstrated that CO oxidation and acetyl-CoA synthesis occur at separate sites and that these two reactions occur on vastly different time scales.[9] CO interacts with center C within 5 ms, converting it to the C′ state and altering its EPR signal.[9] It has been proposed that conversion of C to C′ is a two-electron reduction.[10] Subsequently, an $Fe_4S_4$ cluster, called center B, is reduced with a rate constant of ~60 $s^{-1}$.[9] Finally, the EPR spectrum of the CO adduct with center A develops with a rate constant of 0.16 $s^{-1}$.[9] Thus, apparently, electrons extracted during the oxidation of the first CO are passed via center B to center A, which is then able to bind the second CO and promote its incorporation into the acetyl group.

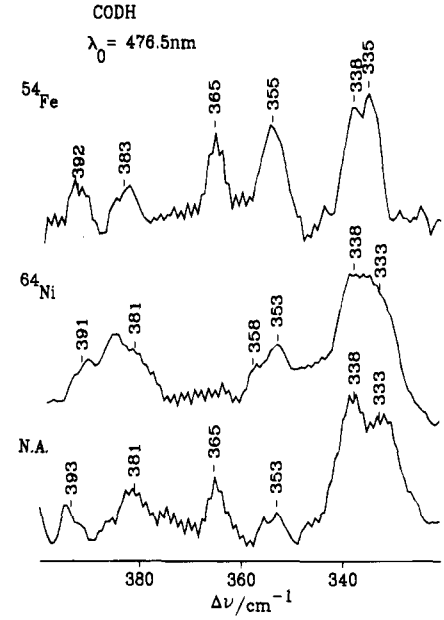


**Figure 1.** 476.5 nm excited RR spectra of natural abundance (NA) CODH and protein isolated from bacteria grown on $^{54}$Fe and $^{64}$Ni.[14] The spectra were collected with a SPEX 1877 triplemate spectrograph equipped with a 2400 grooves/mm grating and an intensified diode array detector (Princeton Instruments). The $Ar^+$ laser power was ca. 90 mW at the sample. The sample was kept at liquid nitrogen temperature throughout the experiment. The slit width was 3 $cm^{-1}$. Four scans were performed at 30 min/scan. The spectra were smoothed with a Savitsky-Golay routine, and the background was corrected using a polynomial line fit. A weak 358 $cm^{-1}$ band of the Tris buffer was subtracted digitally.

Figure 1 shows the RR spectra in the 300–400 $cm^{-1}$ region, where metal–ligand stretching vibrations are expected. The assignment of the vibrational bands to the distinct centers on CODH was the focus of freeze–quench RR (FQ-RR) studies. In the resting enzyme, all three centers are oxidized, and the bands arise from Fe–S and Ni–S vibrations. The 365 $cm^{-1}$ band of the resting enzyme is associated with Ni, as revealed by isotopic substitution. The band has moved to 358 $cm^{-1}$ in the $^{64}$Ni-substituted protein. In addition, the 333 $cm^{-1}$ band is weaker and broader in the $^{64}$Ni spectrum, indicating that a component of this band is also associated with Ni. The other component of the 333 $cm^{-1}$ band is associated with Fe, since the band becomes more prominent and shifts up by 2 $cm^{-1}$ upon $^{54}$Fe substitution. Likewise, the 353 $cm^{-1}$ band strengthens and shifts up slightly in the $^{54}$Fe spectrum.

CODH was reacted with CO for varying times using a freeze–quench unit (Update Instruments, Madison, WI), and the EPR sample cell was subjected to RR spectroscopy (Figure 2). Figure 3 shows a diagram of the sample cell that was modified for FQ-RR. there are notable early changes relative to the resting enzyme. At 10 ms, the band at 333 $cm^{-1}$ weakens, and the bands at 353 and 365 $cm^{-1}$ *disappear*. These bands are therefore associated with center C, the only center to respond on this short time scale. The FQ-RR spectrum of the *C. thermoaceticum* CODH clearly implicates Ni in center C. Disappearance of the 353 and 365 $cm^{-1}$ bands within 10 ms after CO reacts with center C implies that Ni is directly involved in the chromophore electronic system, probably through a bridge to the Fe atom(s). Thus, center C contains Ni and at least one Fe atom; the frequencies of the bands suggest assignment to metal–sulfur stretching vibrations of an Fe–S cluster.

The bands at 339, 381, and 393 $cm^{-1}$, also seen in the resting

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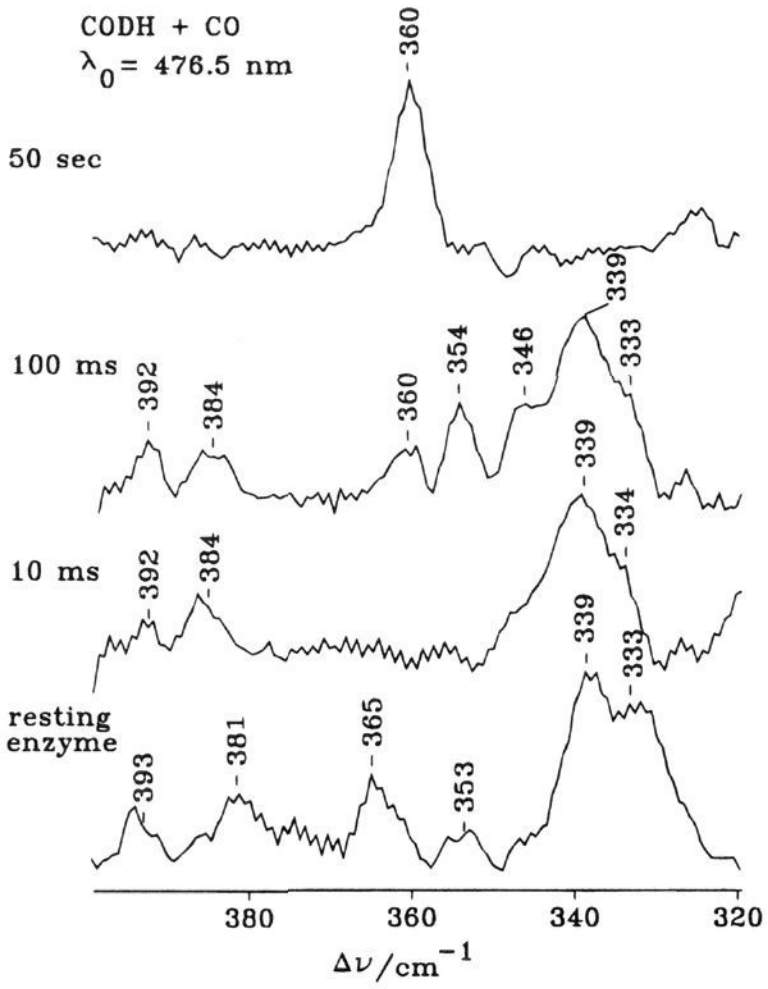


**Figure 2.** Freeze–quench RR spectra of CODH reacted with CO. The reaction was quenched at 10 ms, 100 ms, and 50 s. Conditions and data collection and smoothing were as in Figure 1. The 358 cm$^{-1}$ buffer band was subtracted digitally from the spectra.

protein, are retained in the spectra obtained from samples quenched after 10 and 100 ms. These are assigned to an $Fe_4S_4$ component of center A, since they coincide in frequency and relative intensity with modes of other $Fe_4S_4$ proteins and models,[11] and since they disappear only in the last stage of the reaction when center A is reduced. Also, in the 100 ms spectrum, new bands arise at 360, 354, and 347 cm$^{-1}$. These bands are unlikely to arise from center B since it becomes reduced on this time scale. Since it is a classical $Fe_4S_4$ center, center B would not be expected to show vibrational bands at these frequencies. They are unlikely to arise from center A since FQ-EPR data indicate that it does not appear to undergo redox chemistry on this time scale.[9] Possibly, these bands could arise from center C if it becomes reoxidized as electrons are transferred to center B. However, the FQ-EPR results do not indicate conversion of center C to its resting form on this time scale. Assignment of these bands awaits further isotopic studies.

Fifty seconds after reaction with CO, the RR spectrum shows only a single band, at 360 cm$^{-1}$. The absence of bands due to metal–sulfur stretching vibrations in the 50 s spectrum is consistent with all three centers being reduced, since Fe–S clusters are known to be much weaker Raman scatterers when reduced than when oxidized.[11] The slow rate of development of the 360 cm$^{-1}$ band is consistent with the time course observed for formation of the CO adduct with center A.[9] The 360 cm$^{-1}$ band was observed earlier by RR spectroscopy.[2] It was found to be sensitive to isotope substitution with $^{13}C$ and $^{54}Fe$ and insensitive to substitution with $^{64}Ni$. Therefore, this band was assigned as an Fe–CO stretching vibration.[2] Evidence that the 360 cm$^{-1}$ band originates from center A and not center C was obtained by demonstrating that the carbonyl group in the Fe–CO adduct underwent an isotopic exchange reaction with the carbonyl group of acetyl-CoA, a diagnostic reaction of center A.[2] However, since CO interacts rapidly with center C, it remained possible that CO could migrate from center A to center C during the experiment. In that case, it could be argued that the Fe–CO band arises from the CO adduct with center C.

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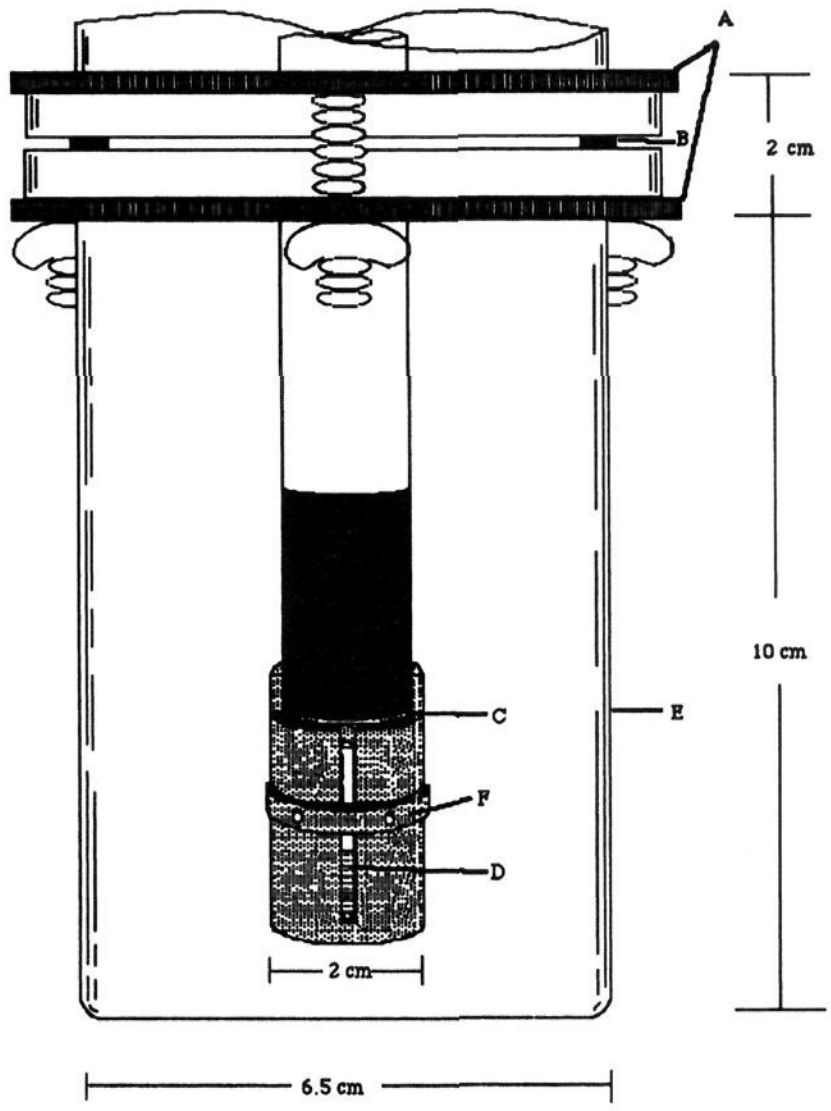


**Figure 3.** Lower half of the apparatus for the FQ-RR measurement, modified from the device described earlier.[16] Samples were prepared in a quartz EPR tube and characterized by EPR spectroscopy. The frozen samples were then mounted on the brass cold finger, and the apparatus was immediately evacuated to prevent frost formation. The temperature was monitored with a thermocouple during loading and did not exceed 135 K. A, McCarter clamp; B, O-ring; C, cold finger; D, sample in the tube; E, shroud; F, clamp to hold the tube in place.

The FQ-RR experiment clearly demonstrates that the 360 cm$^{-1}$ band arises from a Fe–CO adduct at center A, not center C, since carbonylation of center C has been shown to occur within the first 5 ms of reaction with CO and carbonylation of center A to require over 20 s at room temperature.[9] These results support our previous proposal of a bimetallic mechanism for acetyl-CoA synthesis. In this mechanism, we propose that Fe–CO and methyl–Ni adducts are formed at center A, which serve as the precursors of the carbonyl and methyl groups of acetyl-CoA, respectively. Acetyl–Ni or acetyl–Fe would be formed by carbonyl insertion or methyl migration, respectively. We favor a carbonyl insertion mechanism that would be more consistent with stereochemical studies of acetyl-CoA synthesis.[12,13] In addition, a methyl migration mechanism would require formation of a high-valent Ni species. After acetylation of Ni or Fe, thiolytic cleavage by CoA would form acetyl-CoA.

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