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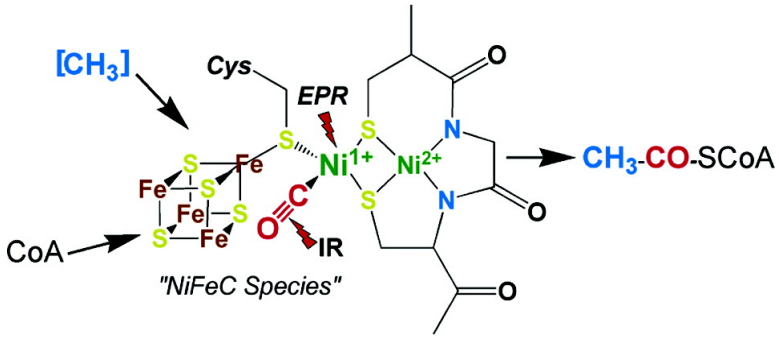

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EPR and Infrared Spectroscopic Evidence That a Kinetically Competent Paramagnetic Intermediate is Formed When Acetyl-Coenzyme A Synthase Reacts with CO

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CO dehydrogenase/acetyl-CoA synthase (CODH/ACS[1]) is a bifunctional enzyme that enables archaea and bacteria to grow autotrophically on CO and H_2/CO_2 using the Wood−Ljundahl pathway.[2,3] The best characterized CODH/ACS from *Moorella thermoacetica*[2,4] is an $\alpha_2\beta_2$ heterotetrameric protein in which the β subunit (CODH) catalyzes the reduction of CO_2 to CO, while the α subunit (ACS) catalyzes the synthesis of acetyl-CoA (1) from Coenzyme A, CO, and a methyl cation donated by a methylated corrinoid iron−sulfur protein ($CH_3-Co^{3+}-$CFeSP).[5]

$$CoAS^{1-} + CO + CH_3-Co^{3+}-\text{CFeSP} \leftrightarrow H_3C-CO-SCoA + Co^{1+}-\text{CFeSP}^{1-} \quad (1)$$

The X-ray structures of all ACS proteins reveal an active site A-cluster that contains an $[Fe_4S_4]$ cubane bridged by a cysteine sulfur atom to a binuclear center, which is composed of two Ni atoms.[6,7] The proximal Ni (Ni_p), is attached to the cluster by a cysteinyl sulfur and to the distal Ni (Ni_d) by two additional cysteine residues. Ni_d, which assumes a square-planar geometry, is coordinated by the two bridging Cys and two backbone amide nitrogens.

In the oxidized state of the A-cluster, the $[Fe_4S_4]$ cubane, the Ni_p, and the Ni_d atoms are all diamagnetic with charges of +2.[2,4,9] However, to catalyze acetyl-CoA synthesis, reductive activation of the A-cluster is required to form one of several proposed states: $[Fe_4S_4]^{2+}-Ni_p^0-Ni_d^{2+}$,[10] $[Fe_4S_4]^{1+}-Ni_p^{1+}-Ni_d^{2+}$ [11], and $[Fe_4S_4]^{2+}-Ni_p^{1+}-Ni_d^{2+}$.[5,12] Only the latter state is EPR active ($S = {}^1/_2$), and this paramagnetic species (termed the *NiFeC species*) has only been observed when the enzyme is incubated with CO.[13,14] It has remained an unresolved question, addressed here, whether the active carbonylated form of the A-cluster is the paramagnetic or one of the diamagnetic metal−carbonyl (M−CO) states. The aim of these studies is to measure the rate of formation of the M−CO species by stopped-flow infrared spectroscopy (SF−IR)[15] and to determine whether this rate coincides with the rate of formation of the paramagnetic *NiFeC* species by rapid-freeze quench EPR (RFQ−EPR). Both rates are compared with that of steady-state catalysis to determine catalytic competence.

A monofunctional ACS has been isolated from *Carboxydothermus hydrogenoformans* (ACS_{CH}).[6] We cloned and overexpressed ACS_{CH} in *E. coli* (see Supporting Information) to yield a purified ACS_{CH} containing 3−4 Fe atoms per monomer but no Ni. Upon reconstitution with $NiCl_2$ and treatment with CO and DTH, ACS_{CH} exhibits the *NiFeC* EPR signal with typical *g* values of 2.067 and 2.026 (0.38−0.67 spins/ACS_{CH} monomeric unit). The sample used in the IR experiment had 0.67 spins/ACS_{CH} (indicating that at least 67% of $ACS_{CH}-CO$ is paramagnetic) and exhibited the following activities (at 30 °C): CO/acetyl-CoA exchange, 0.25 U/mg (0.34 s^{-1}); acetyl-CoA synthesis, 0.029 U/mg (0.04 s^{-1}); and dephosphoCoA/acetyl-CoA exchange,[16] 23 U/mg (32 s^{-1}).

To explore CO binding to ACS, SF−IR experiments were performed at 25 °C by rapidly mixing 100 μM ACS_{CH} with CO-saturated buffer, with both solutions containing 2 mM DTH and 2 mM DTT (Figure 1). When ACS_{CH} is reacted with ^{12}CO, the only significant metal−CO band that forms at a rate consistent with catalysis is at 1994.7 cm^{-1}. A similar band observed with the *M. thermoacetica* CODH/ACS was assigned to a terminally coordinated Ni−CO.[17,18] When ACS_{CH} is reacted with ^{13}CO, the band shifts 45 cm^{-1} to 1949.7 cm^{-1}, which is consistent with the 44.3 cm^{-1} predicted using a simple harmonic oscillator for a bound CO. The band increases with time of CO incubation, following a single exponential with a rate constant of 0.83 ± 0.06 s^{-1} (Figure 2). A weaker band forms at a rate much slower than that of catalysis at 2044 cm^{-1} (3% of the 1994.7 cm^{-1} band after 800 s). When DTH is absent, the same M−CO bands are seen, but these form much more slowly, presumably due to the weaker reducing power of DTT compared to that of DTH.

When the bifunctional CODH/ACS is reacted with CO, at least five bands are observed with stretching frequencies in the range of 2200−1800 cm^{-1}. One of these bands (at 1996 cm^{-1}) was proposed to correspond to the A-cluster of ACS, and the others were assigned to CODH.[17,18] Observation of a similar band at 1994.7 cm^{-1} with the monofunctional ACS_{CH} unambiguously demonstrates that the 1996 cm^{-1} band indeed arises from the carbonylated A-cluster and implies that the other bands are correctly assigned to M−CO complexes associated with the C−cluster in CODH/ACS.

To determine whether the M−CO band corresponds to a diamagnetic or paramagnetic state, RFQ−EPR was performed in which 80 μM ACS_{CH} was reacted with CO at 22 °C under otherwise identical conditions as the SF−IR experiments. The *NiFeC* signal developed with a rate constant of 1.4 ± 0.2 s^{-1} (Figure 2). The *NiFeC* species has *g* values identical to those of native *C. hydrogenoformans* ACS [6] and similar to those of the CODH/ACS from *M. thermoacetica* and methanogens.[13,19]

That the 1994.7 cm^{-1} IR band and the *NiFeC* EPR signal form at similar rates and are the predominant species present in solution strongly indicates that they represent the vibrational and magnetic spectral fingerprints of the same M−CO species. Furthermore, the IR band and the *NiFeC* signal develop faster than the rates of acetyl-CoA synthesis and the CO/acetyl-CoA exchange reactions, which satisfies the criterion of catalytic competence of the M−CO species in these reactions. When reacted with the methylated CFeSP, the paramagnetic *NiFeC* species decays 6-fold faster than the steady-state catalytic rate,[5] further satisfying the criterion of catalytic

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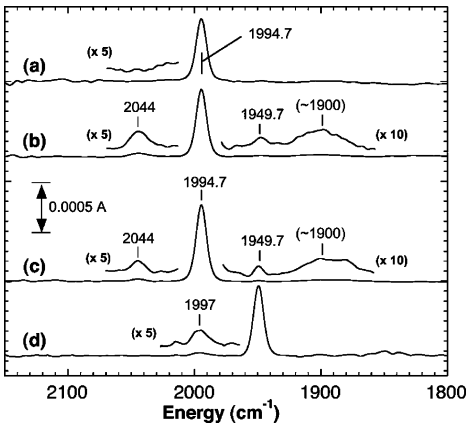

Figure 1. SF−IR spectra of 50 μM ACS_{CH} (final) reacted with CO: (a) 3.0 and (b) 877 s after reaction with ^{12}CO in the presence of 2 mM DTH. Reaction after 1100 s in the absence of DTH (c) ^{12}CO, (d) ^{13}CO. The small band at 1949.7 cm^{-1} in (b) and (c) corresponds to natural abundance ^{13}CO (1.07%). The collection time for (a) was 1 s. The others were collected over 20 s.

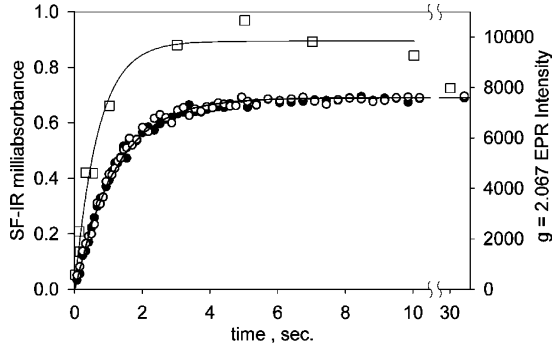

Figure 2. Reaction of ACS_{CH} with CO at 25 °C followed by FQ−EPR and SF−IR. Formation of the 1994.7 cm^{-1} band in two separate experiments is shown as closed and open circles. The data fit to a single exponential with $k_{obs} = 0.83\ s^{-1}$ (solid line). The $g = 2.067$ EPR signal (open squares) followed a single exponential with $k_{obs} = 1.4\ s^{-1}$. EPR spectra were recorded as described in the Supporting Information.

competence. That the M−CO complex involves Ni^{1+}−CO is supported by FT−IR,[17] EPR,[13] ENDOR,[20] and computational studies.[21]

We do not observe reduction of the $[Fe_4S_4]^{2+}$ by UV/visible or EPR spectroscopy when DTH alone is added to Ni-reconstituted ACS_{CH} (data not shown). This agrees with previous EPR, EXAFS, and Mössbauer experiments, indicating the *NiFeC* species contains a diamagnetic $[Fe_4S_4]^{2+}$ cluster.[22] Thus, a $[Fe_4S_4]^{2+}$−Ni^{1+}−CO is supported for the IR band at 1994.7 cm^{-1}. Since the only observed carbonylated form of ACS_{CH} is a *paramagnetic* complex, diamagnetic Ni^{2+}−CO and Ni^{0}−CO states are unlikely intermediates in acetyl-CoA synthesis.

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Supporting Information Available: Description of the cloning and overexpression of ACS_{CH} into pQE70, description of the assays and methodology for SF−IR and RFQ−EPR, as well as original EPR and IR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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(1) Abbreviations: CODH/ACS, CO dehydrogenase/acetyl-CoA synthase; ACS_{CH}, acetyl-CoA synthase from *Carboxydothermus hydrogenoformans*; SF−IR, stopped-flow Fourier transformed infrared spectroscopy; RFQ−EPR, rapid-freeze-quench EPR; DTT, dithiothreitol; DTH, dithionite.

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