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Infrared and EPR Spectroscopic Characterization of a Ni(I) Species Formed by Photolysis of a Catalytically Competent Ni(I)-CO Intermediate in the Acetyl-CoA Synthase Reaction†

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Abstract

Acetyl-CoA synthase (ACS) catalyzes the synthesis of acetyl-CoA from CO, coenzyme A (CoA), and a methyl-group from the CH₃-Co³⁺ site in the corrinoid iron-sulfur protein (CFeSP). These are the key steps in the Wood-Ljungdahl pathway of anaerobic CO and CO₂ fixation. The active site of ACS is the A-cluster, which is an unusual nickel-iron-sulfur cluster. There is significant evidence for the catalytic intermediacy of a CO-bound paramagnetic Ni species, with an electronic configuration of [Fe₄S₄]²⁺-(Ni_p¹⁺-CO)-(Ni_d²⁺), where Ni_p and Ni_d represent the Ni centers in the A-cluster that are proximal and distal to the [Fe₄S₄]²⁺ cluster. This well-characterized Ni_p¹⁺-CO intermediate is often called NiFeC species. Photolysis of the Ni_p¹⁺-CO state generates a novel Ni_p¹⁺ species (A_{red}^{*}) with a rhombic electron paramagnetic resonance spectrum (*g*-values of 2.56, 2.10, 2.01) and an extremely low (1 kJ/mol) barrier for recombination with CO. We suggest that the photolytically generated A_{red}^{*} species is (or is similar to) the Ni_p¹⁺ species that binds CO (to form the Ni_p¹⁺-CO species) and the methyl group (to form Ni_p-CH₃) in the ACS catalytic mechanism. The results provide support for a binding site (an “alcove”) for CO near Ni_p, indicated by X-ray crystallographic studies of the Xe-incubated enzyme. We propose that, during catalysis, a resting Ni_p²⁺ state predominates over the active Ni_p¹⁺ species (A_{red}^{*}) that is trapped by the coupling of a one-electron transfer step to the binding of CO, which pulls the equilibrium toward Ni_p¹⁺-CO formation.

Keywords

CO dehydrogenase; acetyl-CoA synthase; photolysis; nickel; EPR spectroscopy; enzyme kinetics; iron-sulfur cluster

Carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS) catalyzes the key steps in the Wood-Ljungdahl pathway of anaerobic CO₂ fixation, which provides carbon and energy for a variety of anaerobic microbes (1–4). This bifunctional enzyme consists of two

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central CODH subunits, each of which is attached to an ACS subunit, forming an $\alpha_2\beta_2$ complex (5,6). In the CODH subunit, CO₂ is reduced to CO, which then travels through a 70 Å tunnel to the A-cluster of ACS (7). Here, CO, a methyl-group from the CH₃-Co³⁺ cofactor in the corrinoid iron-sulfur protein (CFeSP) and coenzyme A (CoA) (1) are converted to acetyl-CoA. Based on the results of X-ray crystallographic (5,8) and biochemical (9,10) studies, the active A-cluster is known to be composed of a [Fe₄S₄] cluster bridged through cysteine to the proximal Ni (Ni_p) of a dinuclear Ni center (Figure 1), an arrangement similar to the Fe-only hydrogenases in which a [Fe₄S₄] cluster and a binuclear Fe site are bridged by a Cys residue (11,12). The substrate binding site is ambiguous; however, computational results (13) combined with biochemical and spectroscopic experiments (10,14–16) and studies of model complexes (17,18) suggest that Ni_p is the binding site. Therefore, the purpose of discussion, we will refer to the CO complex as involving Ni_p-CO, as shown in the mechanism in Figure 2. Ni_p changes ligation and oxidation states during catalysis, whereas the distal Ni (Ni_d), which is ligated by two deprotonated amides and two cysteine thiolates in a Cys-Gly-Cys motif, appears to remain square planar in the +2 oxidation state (17). Various details of the mechanism of acetyl-CoA synthesis are not yet established; for example, whether Ni_p forms paramagnetic or diamagnetic intermediates during the reaction is debated (1,19–21). The paramagnetic mechanism, which is so-called because it includes a paramagnetic Ni(I)-CO species, is outlined in Figure 2. For simplicity, the ACS mechanism is described as an ordered reaction; however, recent isotope trapping experiments demonstrate that CO and the methyl group bind randomly to ACS, forming a ternary CH₃-CO-ACS (or binary acetyl) intermediate that reacts with CoA to form acetyl-CoA (20).

Because binding of CO is not a redox reaction, the Ni_p center in A_{red}* (prior to binding substrates, CO or CH₃) should have the same redox state as Ni_p¹⁺-CO, namely Ni_p¹⁺, as shown in Fig. 2. However, it is a conundrum that ACS lacks an electron paramagnetic resonance (EPR) signal before binding CO and becomes EPR-active afterwards, because this would indicate that CO binding converts the enzyme from a diamagnetic to a paramagnetic state. Ironically, the best-characterized intermediate in the ACS mechanism is the most controversial one; this is the paramagnetic CO-bound Ni_p species (Ni_p¹⁺-CO) (called A_{red}-CO or the “NiFeC species”), which has been trapped, spectroscopically characterized and shown to be catalytically competent in acetyl-CoA synthesis (22). Based on density functional theory (DFT) computations that incorporate the results of EPR (15), Mössbauer (23,24), electron nuclear double resonance (ENDOR) (25), infrared (26,27) and X-ray crystallographic experiments (11,12), the electronic configuration of A_{red}-CO has been described as [Fe₄S₄]²⁺-(Ni_p¹⁺-CO)-(Ni_d²⁺) (13). The assignment of this as a catalytically competent intermediate is based on its formation and decay rates being faster than the overall rate of acetyl-CoA formation (22,27,28). Furthermore, the results of combined freeze quench EPR and stopped-flow fourier transform infrared (FTIR) experiments demonstrate that the NiFeC species is the sole metal-carbonyl species formed upon reaction of ACS with CO (27).

Besides Ni_p-CO, both methyl- (14,22) and acetyl-ACS (5,29) species have been trapped and these forms of the enzyme are EPR-silent and likely diamagnetic. The diamagnetic nature of methyl-ACS represents a challenge for the paramagnetic mechanism because rapid kinetic studies indicate that methyl transfer is an S_N2 reaction in which the methyl group is transferred formally as a cation; thus, nucleophilic attack of Ni_p¹⁺ or Ni_p¹⁺-CO on a methyl cation should generate a paramagnetic methyl-Ni³⁺ (or acetyl-Ni³⁺) state. However, it is recognized that such a species would be highly oxidizing, possessing a Ni^{3+/2+} couple perhaps as high as +1.0 V (18,19) and it has been proposed that a rapid coupled one-electron transfer could reduce the methyl-Ni³⁺ species to methyl-Ni²⁺ (1). Thus, the predominant observable species shown in Figure 2 that would accumulate to detectable levels during the

catalytic mechanism would be Ni_p^{2+} , $\text{Ni}^{1+}\text{-CO}$, methyl- Ni^{2+} and acetyl- Ni^{2+} , while the unstable Ni_p^{1+} and methyl- Ni^{3+} states would be present in low amounts and observable only by special methods, like those described in the present manuscript.

Characterization of the photolysis of M-CO complexes, e.g., of the $\text{Fe}^{2+}\text{-CO}$ state of heme in myoglobin or hemoglobin (30,31), has yielded important insights into protein structure, function and dynamics. Detailed analysis of CO rebinding kinetics can give information on different ligand binding sites and mechanisms (32). Here, we show that photolysis of the catalytically competent $\text{Ni}_p^{1+}\text{-CO}$ species at low-temperatures generates a novel paramagnetic Ni_p^{1+} species with an extremely low barrier for recombination with CO. The photolysis and recombination have been characterized by EPR and IR measurements. The photolysis and spectroscopic studies described here provide significant novel insights into the mechanism of CO binding to ACS and into the electronic structure of the A-cluster.

MATERIALS AND METHODS

Protein Purification

The His-tagged α -subunit (ACS) of the *Moorella thermoacetica* CODH/ACS was prepared, purified and Ni-reconstituted under strictly anaerobic conditions below 1 ppm of O_2 as described (20). For some ACS samples, Ni reconstitution was done for longer periods (2–3 days) and at higher temperature (45 °C), which gave a higher NiFeC EPR spin quantity (60–75%).

FTIR Spectroscopy

The FTIR cell consisted of a 50 μm thick, air-tight, transparent sample compartment and an outside metal frame. The samples used for FTIR were prepared by incubating 100 μL of dithionite-reduced ACS (609 μM) with CO for 5 minutes, filling the FTIR cell in the anaerobic chamber using a blunt-ended syringe, removing the cell from the chamber, and freezing immediately in liquid nitrogen. Then, the FTIR cell was transferred into a liquid helium cooling cryostat to start the experiment. Identical samples were prepared in H_2O and D_2O . Similar results were obtained with the H_2O - and D_2O -prepared samples; however, having D_2O as the solvent provided better access to the 1800–1600 cm^{-1} region of the spectrum, which includes the so-called ‘amide-I region’ where conformational changes in the polypeptide are observed.

EPR Spectroscopy

For the EPR-based detection of the photolysis reaction, the $\text{Ni}^{1+}\text{-CO}$ intermediate was formed by incubating 50–150 μM of ACS with 2 mM dithionite and then purging with 100% CO for 15 min in an EPR tube. The spin concentration of the resultant $\text{A}_{\text{red}}\text{-CO}$, measured by double integration relative to a Cu-perchlorate standard, was between 30–75% in different samples. Dithionite-reduced and methylated ACS samples were similarly prepared, but not treated with CO. Methylated ACS was prepared by incubating 65 μM of ACS with 2.5 equivalents of methylcobinamide and 4 mM Ti(III) citrate at 45 °C for 2.5 hours, and then removing excess methylcobinamide by ultrafiltration. Acetylated ACS was prepared by purging the methylated ACS sample with 100% CO for 15 minutes in the EPR tube. X-band (9.4 GHz) continuous-wave EPR spectra were recorded under non-saturating, slow-passage conditions using a Bruker ECS106 spectrometer equipped with a TE₁₀₂ cavity. Cryogenic temperatures were achieved and controlled using an Oxford Instruments ESR900 liquid helium cryostat in conjunction with an Oxford Instruments ITC503 temperature and gas flow controller. Spectrometer settings for the data presented in Figure 6 include: temperature = 4.7 K, excitation frequency = 9.480 GHz, microwave power = 20 mW, modulation amplitude = 0.5 mT, modulation frequency = 100 kHz, sweep rate = 4.1 mT/s.

The microwave power had to be reduced to 320 nW to give the NiFeC signal shown in the Inset under non-saturating conditions.

Photolysis and recombination experiments

For the FTIR experiments, the sample photolysis was conducted in an Oxford cryostat with CaF₂ external windows, ZnSe intermediate windows, and ZnS windows for the IR cell. Spectra were recorded at 4 cm⁻¹ resolution with a Bruker V-70 FT-IR spectrometer using an MCT detector. For photolysis, a 100 W XENOPHOT® lamp (OSRAM HLX) source was located at the alternate source position of the Bruker spectrometer, and the sample was irradiated by turning the spectrometer mirrors to illuminate the sample with the alternative source. The estimated power on the sample was approximately 100 mW. The FTIR cell was irradiated at 4.7 K for different amounts of time as indicated in the Figure 3 legend. The recombination was observed after turning off the lamp, adjusting the temperature to one of the higher points indicated in Figure 4 and recording the kinetics at which the FTIR difference peak disappeared after the new temperature stabilized (within 90 sec).

For the EPR experiments, photolysis was performed by focusing the IR-filtered (10 cm of an aqueous 10 mM Cu(II)SO₄ solution) light from a 300 W tungsten filament lamp into the light port of the EPR cavity. The sample was rotated four times in 90 ° intervals each followed by 15 min of continuous illumination to maximize photolysis. No further intensity changes in the NiFeC signal were observed after this one hour of illumination. The addition of 50 % glycerol (v/v) to samples of A_{red}-CO led to a significant increase in the efficacy of the photolysis. Without glycerol, we observed only a 20 % decrease in the NiFeC signal upon illumination. We attribute this change in yield to an increase in the optical quality of the frozen glass upon addition of glycerol. This has the effect of decreasing light scattering and allowing the incident light to penetrate more deeply into the 3 mm diameter EPR sample. For all reported data, a spectrum of the buffer collected under identical spectrometer conditions was subtracted. Data manipulations and spectral simulations were performed in MatLab using EasySpin 3.1 (33,34). Parameters used for the EPR spectral simulation presented in Figure 6 included: $S = 1/2$, $g = [2.558, 2.10, 2.01]$, $g\text{-strain} = [0.06, 0.015, 0.015]$, line-width = 7 mT.

The recombination kinetics data obtained via FTIR spectroscopy were supported by analogous EPR experiments that monitored the recovery of the NiFeC signal—indicating successful recombination of CO and the transiently formed Ni¹⁺ species—as a function of time once the tungsten lamp was extinguished. The sample was allowed to thermally equilibrate at 20.6 K for at least 15 minutes prior to initiating the experiment. This temperature was chosen for convenience and to allow for facile comparison of the kinetics measured via FTIR. The entire NiFeC signal was acquired in approximately 40 sec. Successive scans were collected individually and the intensity of the corresponding NiFeC signal was determined via double integration. Upon onset of the illumination period of eight minutes, the observed NiFeC signal diminishes significantly (10–12%, see Figure 8). Once the light is extinguished, we observed an immediate jump in the signal intensity followed by a much slower increase. It is likely that the first phase is due to sudden temperature changes in the sample caused by the cessation of illumination. Temperature stability returns rapidly, within one data point (i.e. 40 sec). This second phase is well-fit by a signal exponential function with a rate constant $\approx 0.0006\text{--}0.0012\text{ sec}^{-1}$, in agreement with the 20.8 K recombination rate constant of 0.001 sec^{-1} obtained from fitting the recovery of the $\nu(\text{C}\equiv\text{O})$ band in the FTIR spectrum once the light is turned off (vide supra).

RESULTS

FTIR studies of the photolysis of $A_{\text{red}}\text{-CO}$ and recombination of CO with A_{red}^*

Low-temperature (4.5 K) photolysis of $A_{\text{red}}\text{-CO}$ generates a negative band at 1998 cm^{-1} in the difference FTIR spectrum (Figure 3). This is the first observation of any effect of photolysis on the FTIR (or EPR) spectra of $A_{\text{red}}\text{-CO}$. This feature shifts to lower frequency (1953 cm^{-1}) when ACS is treated with ^{13}CO . This band and a similar isotope shift were first observed in FTIR studies of CO binding to CODH/ACS and assigned as a terminal carbonyl bound to the Ni center in ACS2 (26). We attribute this loss of the $\nu(\text{C}\equiv\text{O})$ stretching mode intensity to photo-induced breaking of the Ni–CO bond to generate CO and an A_{red}^* species. Indeed, upon photolysis, a small positive band appears at 2129 cm^{-1} (2081 cm^{-1} when prepared with ^{13}CO) that is associated with unbound CO. Upon raising the temperature, the negative CO bands disappear as the original $\text{Ni}^{1+}\text{-CO}$ species reappears (Figure 4); thus, photolysis must be conducted at low temperatures for A_{red}^* to accumulate. Studies of the temperature dependence of the recombination rate reveal an exceptionally low activation energy (E_a) of 1 kJ/mol (Figure 5). For comparison, CO recombination to the Fe^{2+} -heme in myoglobin has an E_a of 8.4 kJ/mol (35).

EPR studies of the photolysis of $A_{\text{red}}\text{-CO}$ and recombination of CO with A_{red}^*

The photochemistry of $A_{\text{red}}\text{-CO}$ was followed by EPR spectroscopy. The pre-photolysis spectrum of $A_{\text{red}}\text{-CO}$ (black trace, inset Figure 6A) is identical to that previously observed for the NiFeC signal (15). After 1 h of continuous white-light illumination at 4.7 K, the NiFeC signal intensity diminishes by 66 % (red trace, inset Figure 6) as a new set of resonances at ca. 260, 310 and 325 mT appear (Figure 6A). After subtracting residual NiFeC contributions, the resultant A_{red}^* signal is well simulated as an $S = 1/2$ spin system with g -values of 2.56, 2.10, 2.01 (Figure 6B). These signals are absent from the spectra of pre- or post-photolyzed ACS samples (A_{red} , $A_{\text{red}}\text{-CH}_3$ or $A_{\text{red}}\text{-COCH}_3$) lacking CO (Figure 7).

As in the FTIR experiment, photolysis of $A_{\text{red}}\text{-CO}$ is only observed at low temperatures and, after photolysis, the NiFeC signal returns at a rate consistent with that measured by FTIR (Figure 8). Thus, the formation of the paramagnetic photoproduct (A_{red}^*) observed by EPR spectroscopy appears to be correlated with loss of the CO ligand in $A_{\text{red}}\text{-CO}$, observed by IR.

Other minor features at $g = 2.40, 2.20$ are observed in the difference EPR spectrum. These minor signals may arise from a slightly altered A_{red}^* state, which is consistent with the appearance, in some samples of CO-treated ACS, of an altered form of the NiFeC signal with g -values at 2.05, 2.028 (15). It is also possible that these low-intensity resonances represent a small amount of Ni_d reduction; however, this is unlikely because spectroscopic and electrochemical studies of the $\text{Ni}^{2+/1+}$ couple in model complexes with an N_2S_2 thiolato- and carboxamido coordination environment reveal a very low midpoint potential ($\sim -1.26\text{ V}$ versus NHE) that would not be accessible in aqueous solution with biological reductants (18,36). Thus, we suggest that the existence of these low intensity features in the EPR spectra for the Ni_p center indicate variations in electronic and geometric environments, as has been observed after low temperature photolysis of Mb-CO (37).

²In this citation, the M-CO band was assigned to CODH because it was not known until later (Kumar, M., Lu, W.-P., Liu, L., and Ragsdale, S. W. (1993) J. Am. Chem. Soc. 115, 11646-11647) that CO oxidation and acetyl-CoA synthesis occur at separate sites.

DISCUSSION

This manuscript describes photolysis studies of a catalytically competent metal-carbonyl species on ACS, the key enzyme in the Wood-Ljungdahl pathway of anaerobic CO₂ and CO fixation. Low-temperature (4.5 K) photolysis of the A_{red}-CO species generates a negative band at 1998 cm⁻¹ in the difference FTIR spectrum (Figure 3). This feature shifts by 45 cm⁻¹ to lower frequency (1953 cm⁻¹) when ACS is incubated with ¹³CO. This IR band has been previously observed in studies of CO binding to the *M. thermoacetica* CODH/ACS (26) and to the *Carboxydotherrmus hydrogenoformans* ACS (27) and was assigned to the stretching mode of a terminal carbonyl bound to the Ni center in ACS. The 1953 cm⁻¹ IR band of A_{red}-¹³CO undergoes isotope substitution when the ¹³CO-incubated enzyme is reacted with natural abundance acetyl-CoA, indicating that the CO group of A_{red}-CO undergoes isotope exchange with the carbonyl group of acetyl-CoA (26). Furthermore, this IR band develops at catalytically relevant rates upon incubation of ACS with CO (27). These isotopic exchange experiments indicate that A_{red}-CO is catalytically relevant to the mechanism of acetyl-CoA synthesis (26,27).

The IR band associated with A_{red}-CO forms at the same rate as an EPR signal with *g*-values at 2.074, 2.028 (27). This has been called the NiFeC signal because it undergoes hyperfine-induced broadening when the metal centers in ACS are isotopically substituted with ⁶¹Ni or ⁵⁷Fe and when ACS is incubated with ¹³CO versus ¹²CO (15). Here we describe EPR spectroscopic studies of the photolysis of A_{red}-CO. When we had earlier irradiated samples of CO-incubated CODH/ACS at 77 K in EPR tubes that were transferred into the EPR cavity for analysis, we did not observe any effects of photolysis on the NiFeC EPR signal (unpublished results). We now recognize that, as seen in the IR experiment, in order to observe photolysis, the experiment must be performed at low temperatures; for example, after 1 h of continuous white-light illumination at 4.7 K, the EPR signal intensity of A_{red}-CO diminishes by 66 %.

These photolysis results have important implications on the structure of the CO binding site in ACS. We attribute the loss of the $\nu(\text{C}\equiv\text{O})$ stretching mode intensity to photo-induced breaking of the Ni¹⁺-CO bond to generate CO and a Ni¹⁺ species (designated as A_{red}*), a concept that is supported by the *g*-values in the EPR experiments being similar to those of other Ni¹⁺ complexes (see below) and by the appearance of a small positive IR band (at 2129 cm⁻¹ when ACS is reacted with ¹²CO and 2081 cm⁻¹ with ¹³CO) that is associated with unbound CO. The reason that such low temperatures are required to observe photolysis is that recombination of CO with A_{red}* to generate the original Ni¹⁺-CO species has an activation energy (*E*_a) of only 1 kJ/mol. The minimal *E*_a for recombination of CO with A_{red}* suggests that the nascent CO molecule cannot diffuse far from the Ni_p site. For comparison, CO recombination to the Fe²⁺-heme in myoglobin has an *E*_a of 8.4 kJ/mol (35). FTIR (38,39) and X-ray crystallographic (40) studies of these heme-based systems indicate that, after photolysis, CO exits the active site and recombination kinetics depend on CO migration inside the protein as well as on protein dynamics accompanying the movement of CO. As shown in Figure 1B, a hydrophobic pocket located by crystallographic studies of Xe-incubated CODH/ACS is only 3.9 Å from Ni_p and has been proposed to represent the entry point of CO to the A-cluster (7). Assuming a Ni_p-CO bond distance of 1.8 Å (41), CO would travel ~2 Å to reach this hydrophobic binding pocket after the bond is broken. We propose that photolysis of A_{red}-CO could relocate CO into this “alcove” from where it can rebound to Ni_p¹⁺ with a minimal *E*_a. The low *E*_a for CO recombination also implies that the photolysis and rebinding of CO at liquid He temperatures is not accompanied by any major reorganization of the active site conformation.

The photolysis results also have important implications with respect to the electronic and electrochemical properties of the A-cluster of ACS. Coupled to photoinduced loss of the $\text{Ni}^{1+}\text{-CO}$ species is the formation of a novel $S=1/2$ species with g values of 2.558, 2.1 and 2.01, which are appropriate for assignment of the photolyzed state as a Ni(I) species (42–44). The unusually large g -anisotropy found for A_{red}^* indicates that the unpaired electron is almost wholly localized on Ni_p with very little spin density on the adjacent metal centers. Complexes that exhibit similar EPR spectra include a three-coordinate bisphosphine diethylether Ni complex ($g = 2.45, 2.11, 2.11$) (42) and a tetrahedral tris-thioether Ni complex with bound CO ($g = 2.64, 2.02, 1.95$) (45). The Ni-L form of [NiFe] hydrogenase ($g = 2.3, 2.12, 2.05$), which is formed by photolyzing the H_2 -reduced enzyme at 40 K (46), has much less g -anisotropy because of the near square-pyramidal geometry of the Ni site and because exchange interactions with a neighboring Fe delocalize the electron density (47).

We do not observe any indication of the reduced $[\text{Fe}_4\text{-S}_4]$ cluster (g -values at 2.06, 1.92, 1.80) (48) or of a Ti(III)citrate-reduced form of ACS that exhibits an axial EPR signal ($g = 2.10, 2.03$) (21). There also is no evidence for the reduction of the Ni_d site. As described above, reduction of Ni_d would be highly unlikely based on the very low midpoint potential of the $\text{Ni}^{2+/1+}$ couple in model complexes with a similar coordination environment (18,36). Because an electron from the photolytically generated Ni_p^{1+} species could potentially transfer to either of the metal centers to which it is bridged, the predominance of the Ni_p^{1+} state suggests that, even though the redox potential for the $\text{Ni}^{2+/1+}$ couple of Ni_p must be extremely low, those for the $[\text{Fe}_4\text{S}_4]^{2+/1+}$ cluster and for $\text{Ni}_d^{2+/1+}$ must be even lower (or their reductions are kinetically disfavored).

Importantly, the photolysis experiments allow the observation of a novel EPR-active form of the A-cluster of ACS that is generated when CO is released; thus, this is the state that binds CO. As shown in Figure 2, we suggest that A_{red}^* is a thermodynamically unfavorable Ni_p^{1+} state. The EPR signal of A_{red}^* is not observed when ACS treated with reductants like Ti(III)-citrate or dithionite because, in the absence of CO, the $\text{Ni}_p^{2+/1+}$ equilibrium strongly favors Ni(II) (Equation 1). In the presence of CO, the equilibrium shifts to favor the formation of Ni(I)-CO because CO tends to bind strongly to the low valent states of metal centers (the K_m for CO in the CO/acetyl-CoA exchange is 10 μM (49)) and because there is a gas binding pocket near Ni_p (7). Thus, it appears that, even in the presence of low potential reductants with midpoint potentials below -500 mV, there is too little accumulation of the Ni_p^{1+} state to be observed; however, by kinetically coupling the reduction (Eq. 1) and CO binding (Eq 2) processes, a low valent $\text{Ni}^{1+}\text{-CO}$ complex becomes favorable. In a similar fashion, it was observed by cyclic voltammetry that the midpoint redox potentials of nickel(I) macrocyclic complexes shifted to a more positive value under 1 atm CO (50).

Figure 2 shows two one-electron transfer steps that occur during the catalytic cycle - one is used to reductively activate Ni^{2+} to Ni^{1+} in the one-electron coupled CO binding step and the other electron is used in the internal one-electron transfer associated with the methylation of ACS by the methylated CFeSP. These electrons ultimately come from CoA-dependent thiolysis of the acetyl-ACS intermediate to generate acetyl-CoA; thus, the overall reaction cycle does not involve net electron transfer. There is ample evidence for internal

redox chemistry during acetyl-CoA synthesis; in fact, an unknown factor that was isolated based on its ability to stimulate the CO/acetyl-CoA exchange reaction turned out to be ferredoxin, but it could be replaced by other mediators (51). Although CoA is the ultimate electron donor, the immediate donor(s) of the reducing equivalents involved in conversion of Ni^{2+} to Ni^{1+} -CO and of methyl- Ni^{3+} to methyl- Ni^{2+} during the transmethylation reaction is unknown. Because the $[\text{Fe}_4\text{S}_4]$ cluster that is attached to Ni_p is a low potential center, we speculate that it could act as a conduit in the formation of A_{red} -CO. Although electron transfer to and from the $[\text{Fe}_4\text{S}_4]^{2+/1+}$ cluster has been shown to be 200-fold slower than the rate of methyl group transfer (52), coupling the electron transfer step to carbonylation in a so-called “EC reaction” would significantly enhance the rate because the thermodynamic driving force would be greater. We speculate that Ni_d may be involved in the redox-coupled methyl transfer reaction. Like other cobalamin-dependent transmethyations (53), this is a nucleophilic $\text{S}_{\text{N}}2$ -type reaction (54,55) that would initially generate a methyl- Ni^{3+} intermediate; however, unlike methyl- Co^{3+} , methyl- Ni^{3+} is expected to be highly oxidizing and to undergo reduction to the methyl- Ni^{2+} state in the presence of even moderate reducing agents. The coordination environment of Ni_d resembles that of Ni-superoxide dismutase (Ni-SOD) (56,57), which accesses both the Ni^{2+} and Ni^{3+} states during the catalytic cycle (56,58). Furthermore, model studies have shown that Ni^{3+} is stabilized by the anionic amidate ligands found in Ni_d (59,60). Regardless, the paramagnetic pathway and the internal electron transfer loop in Figure 2 describe a speculative, but testable, working hypothesis that would include roles for all three components of the A-Cluster.

In summary, we have demonstrated the photodissociation of CO from the Ni_p site in A_{red} -CO, which is a catalytically competent intermediate in acetyl-CoA synthesis (22,27,28). This is the first time a photolysis event has been observed on a catalytically competent metal-carbonyl intermediate on an enzyme, even though CO photodissociation was observed in Ni-based inorganic compounds (61) and in Mo- and Ni-dependent nitrogenase and hydrogenase, respectively, for which CO acts as an inhibitor rather than a substrate (62,63). The other photoproduct (A_{red}^*) is a Ni^{1+} species that is only long-lived at low temperatures ($t_{1/2} = 24.8$ min at 18 K) and has EPR properties similar to those of Ni^{1+} compounds with trigonal planar or tetrahedral geometries. The facile recombination of CO with A_{red}^* can be attributed to the capture of photolyzed CO in a gas-binding pocket near Ni_p that has been observed in the crystal structure (7). The $\text{Ni}_p^{2+/1+}$ couple must have a sufficiently low redox potential that the Ni_p^{1+} photoproduct trapped in this study at low temperatures does not accumulate during catalysis. We propose that, during the ACS catalytic mechanism, binding of CO traps this unstable Ni_p^{1+} species in a coupled one-electron transfer step, pulling the redox equilibrium toward formation of Ni^{1+} -CO. Further spectroscopic and computational studies on A_{red}^* are underway and are expected to yield essential insight into changes in the electronic and geometric structures of the A cluster that occur upon substrate binding.

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Abbreviations

CODH	CO dehydrogenase
ACS	acetyl-CoA synthase
FTIR	Fourier transform infrared
Ni_p	proximal nickel in the binuclear site of the A-cluster of ACS
Ni_d	distal nickel in the binuclear site of the A-cluster of ACS
DFT	density functional theory
EPR	electron paramagnetic resonance
A_{red}	the state of the A-cluster when ACS is treated with dithionite
A_{red}[*]	the photolyzed state of A _{red} -CO

Figure 1.

Structure of the A-Cluster of ACS and the gas-binding site. (A) The structure is based on that of PDB ID1OAO (6), (B) The structure is based on molecule P of PDB ID 2Z8Y (7), which was generated from a crystal structure obtained at high pressures of Xe. In this structure, Cu was present in the proximal site (Cu_p) and Ni was present in the distal site Ni_d. Some of the hydrophobic residues are shown that were proposed (7) to form a gas-binding site (the “alcove”) near the binuclear center (Ni_p and Ni_d).

Figure 2.

Paramagnetic mechanism of ACS. In this so-called “paramagnetic mechanism”, which is written simplistically as an ordered reaction (although it is a random order of CO or methyl addition), CO traps the thermodynamically unfavorable Ni^{1+} state (A_{red}^*). However, this state can be trapped at low temperatures by photolysis of the CO ligand. The $\text{A}_{\text{red}}^*\text{-CO}$ state is favored because of CO backbonding to a low valent Ni and because ACS has a CO binding site (“alcove”) near Ni_p . Transmethylation generates an unstable methyl- Ni^{3+} state that accepts an electron to generate the stable methyl- Ni^{2+} , which undergoes carbonyl insertion to generate an acetyl- Ni^{2+} . As the acetyl group undergoes thiolysis by the nucleophilic attack of CoA, two electrons bifurcate: one goes into the internal electron transfer pathway and the other is used to regenerate Ni^{1+} . In the diamagnetic mechanism, so-called because none of the proposed intermediates are paramagnetic, the substrate(s) bind to a diamagnetic “ $\text{Ni}(0)$ ” state to generate a diamagnetic methyl- $\text{Ni}(\text{II})$ or $\text{Ni}(\text{II})\text{-CO}$ species. The intramolecular electron transfer step would not be required in the “diamagnetic mechanism”. See the text for details.

Figure 3.

Time dependence of IR absorbance changes upon photolysis of $A_{\text{red}}\text{-CO}$ at 4.5 K. Inset: Development of features in the 2050–2150 cm^{-1} region, using ^{12}CO and ^{13}CO . See the Materials and Methods section for details of the FTIR experiment. Time points for ^{12}CO data are: 0 (black), 3 (red), 6 (blue), 12 (green), 20 (magenta) and 40 (cyan) min. Time points for ^{13}CO data are: 0 (black), 0.67 (red), 1.67 (blue) and 4 (green) min. The ^{13}CO signal is enlarged 2.64-fold.

Figure 4.

Time courses of thermal recombination at various temperatures. The temperatures at which the recombinations were observed and the fitted exponential rate constants are indicated next to the curves.

Figure 5. Temperature dependence of the recombination rate of CO with A_{red}^* to reform $A_{\text{red}}\text{-CO}$ Arrhenius plot based on the data shown in Fig. 4.

Figure 6.

EPR spectra of A_{red}^* and $A_{\text{red-CO}}$

(A) 20 mW X-band EPR spectra of $A_{\text{red-CO}}$ in 50 % glycerol (v/v) (black) and $A_{\text{red-CO}}$ after one hour of photolysis at 4.7 K (red line). (Inset) NiFeC signal measured using 320 nW of microwave power (B) Difference of the two spectra in panel A (blue line) and corresponding simulation (green line). See text for spectrometer settings and full simulation parameters.

Figure 7.

X-band EPR spectra of reduced, methylated and acetylated ACS preparations. EPR spectra of A_{red} (top two spectra), $A_{\text{red}}-\text{CH}_3$ (middle), and $A_{\text{red}}-\text{COCH}_3$ (bottom) in 50 % glycerol (v/v) before (dark) and after (“illum”) one hour of white light illumination. In all cases, the spectrum of the buffer was subtracted. Spectrometer settings are identical to those used to acquire the data in Figure 6.

Figure 8.

Time course for recovery of NiFeC signal following illumination.

EPR spectral intensity of the NiFeC signal in $A_{\text{red}}\text{-CO}$ with 50 % glycerol (v/v) as a function of time and white-light illumination (designated by the hash marks). Spectrometer settings: temperature 20.6 K, microwave frequency 9.4744 GHz, microwave power 6.41 μW , modulation frequency 100 kHz, modulation amplitude 0.5 mT, sweep rate 0.73 mT/sec.