Methylation of Carbon Monoxide Dehydrogenase from *Clostridium thermoaceticum* and Mechanism of Acetyl Coenzyme A Synthesis

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Abstract: Carbon monoxide dehydrogenase from *Clostridium thermoaceticum* was methylated such that all bound methyl groups could subsequently react with CO and coenzyme A (or OH^-) to yield acetyl-coenzyme A (acetyl-CoA) (or acetate). Methyl groups could not bind enzyme lacking the labile Ni of the A-cluster, but could bind such samples after incubation in aqueous Ni^{2+}, a process known to reinsert the labile Ni and reactivate the enzyme. Bound methyl groups inhibited the ability of 1,10-phenanthroline to remove the labile Ni, and the amount bound approximately correlated with the amount of labile Ni. This is strong evidence that the methyl group used in acetyl-CoA synthesis binds the labile Ni. Evidence is presented that a redox site (called the D site) other than the spin-coupled metals that define the A-cluster must be reduced before methylation can occur. Both methyl and acetyl intermediates appear to be EPR-silent. The acetyl intermediate reacted slowly with OH^- to yield acetate and rapidly with $CoAS^-$ to yield acetyl-CoA. When enzyme in a state with the A-cluster reduced and bound with CO (the $S = {}^1/_2$ A_{red}-CO state) was methylated, the resulting acetyl intermediate was also EPR-silent, indicating that the order of substrate addition had no effect on the EPR silence of the resulting acetyl intermediate. The D site appears to be an $n = 2$ redox agent that functions to reduce the oxidized A-cluster upon methylation and to oxidize the A-cluster as the product acetyl-CoA dissociates. D is EPR-silent in both of its oxidation states and is not any of the known metal clusters in the enzyme. D may be a special pair of cysteines coordinated to the labile Ni that can be oxidized to cystine at unusually low potentials (∼−530 mV vs NHE). Catalytic mechanisms that do not include D or its functional equivalent, or that employ the reduced $S = {}^1/_2$ CO-bound form of the A-cluster as an intermediate, are inconsistent with the present data. A new catalytic mechanism incorporating the results of this study is proposed.

Introduction

Acetogenic bacteria such as *Clostridium thermoaceticum* can grow autotrophically on CO_2 and H_2, utilizing the so-called Wood−Ljungdahl pathway to synthesize acetyl coenzyme A (acetyl-CoA), a starting material for the anabolic processes of these cells.[1] The central enzyme of this pathway, carbon monoxide dehydrogenase (CODH[2]), catalyzes the synthesis of acetyl-CoA by condensing the methyl group of a methylated corrinoid−iron−sulfur protein (CoFeSP) with CO and coenzyme A.[3] The methyl group originates from CO_2, which is first converted to methyltetrahydrofolic acid (CH_3-THF) and then transferred to CoFeSP. The CO also originates from CO_2, as CODH catalyzes the reversible reduction of CO_2 to CO at a separate active site.[4,5]

CODH contains 4 Ni, ∼24 Fe, and ∼30 S^{2-} ions per $\alpha_2\beta_2$ tetramer,[6] organized into three types of clusters named A, B, and C.[7,8] The B-cluster is an unexceptional $[Fe_4S_4]^{2+/1+}$ cluster that exhibits a $g_{av} = 1.94$ EPR signal in its reduced (B_{red}) state.[9,10] The C-cluster is the active site for CO oxidation/CO_2 reduction catalysis.[4,5,11−13] It can be stabilized in an oxidized diamagnetic state (C_{ox}), a one-electron-reduced $S = {}^1/_2$ state (C_{red1}) that exhibits an EPR signal with $g_{av} = 1.82$, and what is apparently a three-electron-reduced $S = {}^1/_2$ state (C_{red2}) that exhibits an EPR signal with $g_{av} = 1.86$.[4,9,14,15] Cyanide is a

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(2) Abbreviations: CODH, carbon monoxide dehydrogenase; acetyl-CoA, acetyl coenzyme A; $CoAS^-$ or CoA, coenzyme A; THF, tetrahydrofolic acid; CoFeSP, corrinoid−iron−sulfur protein; CH_3-Co^{3+}FeSP, the methylated form of CoFeSP; Co^{1+}FeSP, the reduced state of CoFeSP; MTr, methyltransferase; EPR, electron paramagnetic resonance; DTT, dithiotheitol; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; NHE, normal hydrogen electrode; A_{ox}, the diamagnetic oxidized A-cluster state; A_{red}-CO, the A-cluster state exhibiting the NiFeC EPR signal; B_{ox}, the diamagnetic oxidized B-cluster state; B_{red}, the reduced B-cluster state, exhibiting the $g_{av} = 1.94$ EPR signal; C_{ox}, the oxidized, diamagnetic state of the C-cluster; C_{red1}, the C-cluster state exhibiting the $g_{av} = 1.82$ EPR signal; C_{red2}, the C-cluster state exhibiting the $g_{av} = 1.86$ EPR signal; phen, 1,10-phenanthroline; D_{ox} and D_{red}, the oxidized and reduced states of the D site.

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potent, tight-binding reversible inhibitor of CO oxidation that binds to the C-cluster and renders it redox-inactive.[4,11,16]

The A-cluster consists of a Ni complex exchange-coupled to an Fe_4S_4 cluster through an unidentified bridge.[9,10,17] When the enzyme is exposed to CO, an electron originating from the oxidation of CO at the C-cluster reduces the diamagnetic oxidized form of the A-cluster (A_{ox}) to a one-electron-reduced form with CO bound (A_{red}-CO).[4,5] This $S = {}^1/_2$ state exhibits the so-called NiFeC EPR signal ($g_\perp = 2.08$ and $g_\parallel = 2.03$). Since CO is a substrate for acetyl-CoA synthesis, the presence of CO bound at the A-cluster provided the first indication that the A-cluster may be the active site for that reaction and that A_{red}-CO might be a catalytic intermediate. CO is thought to bind to one of the irons of the Fe_4S_4 cube.[18]

CH_3-THF is attacked by CoFeSP in the nucleophilic Co^{1+} state to yield CH_3-Co^{3+}FeSP in a reaction (eq 1) catalyzed by methyltransferase (MTr). The methyl group is then transferred to CODH (eq 2), and combined with CO and $CoAS^-$ to form acetyl-CoA (eq 3).

$$[Co^{1+}FeSP]^{m+} + CH_3\text{-}THF + H^+ \rightleftharpoons [CH_3\text{-}Co^{3+}FeSP]^{(m+1)+} + H\text{-}THF \quad (1)$$

$$[CH_3\text{-}Co^{3+}FeSP]^{(m+1)+} + [CODH]^{n+} \rightleftharpoons [Co^{1+}FeSP]^{m+} + [CH_3\text{-}CODH]^{(n+1)+} \quad (2)$$

$$[CH_3\text{-}CODH]^{(n+1)+} + CO + CoAS^- \rightleftharpoons CH_3\text{-}C(O)\text{-}SCoA + [CODH]^{n+} \quad (3)$$

The methyl group of CH_3-THF transfers to acetate with *retention* of configuration, consistent with mechanisms involving an even number of S_N2-based methyl group transfers.[19] Subsequent stopped-flow studies indicate that the methyl groups in eqs 1 and 2 do transfer in S_N2 fashion.[20,21] These results imply that the site to which methyl binds is also that to which CO and $CoAS^-$ bind and react to form acetyl-CoA.

Ragsdale and Wood used ^{14}C-labeled acetyl-CoA to demonstrate that CODH (by itself) catalyzes the exchange of the carbonyl carbon of acetyl-CoA with free CO[22] (eq 4). This

$$CO + CH_3\text{-}{}^{14}C(O)\text{-}SCoA \rightleftharpoons {}^{14}CO + CH_3\text{-}C(O)\text{-}SCoA \quad (4)$$

seminal discovery implies that CODH (a) binds CH_3-$^{14}C(O)$-SCoA, (b) breaks both the CH_3-^{14}C and ^{14}C-SCoA bonds, (c) binds CH_3^+, ^{14}CO, and $CoAS^-$ moieties at separate coordination sites, and (d) reforms the CH_3-C and C-SCoA bonds of unlabeled acetyl-CoA (after bound ^{14}CO exchanges with free CO).

Since the reduced A-cluster was known to bind CO in the A_{red}-CO state, Ragsdale and Wood suggested that this cluster served as the CO-binding site in the exchange and synthase reactions and proposed a mechanism of acetyl-CoA synthesis analogous to the Monsanto process used to synthesize acetic acid.[22,23] Possibly because of the rarity of Ni in enzymes, they proposed that (a) CO and the methyl group bind the Ni of the A-cluster, (b) a migratory insertion leads to a nickel−acetyl intermediate; and c) that this intermediate is cleaved by $CoAS^-$, forming the product acetyl-CoA.

Pezacka and Wood reported that a stable methylated intermediate of CODH could be isolated by incubating *reduced* CODH with either CH_3-Co^{3+}FeSP or CH_3I.[24] CH_3I apparently methylates trace amounts of contaminating Co^{1+}FeSP in purified CODH preparations (rather than methylating CODH directly), and the resulting CH_3-Co^{3+}FeSP methylates CODH.[25] The authors found that a potent thiol-modifier, *p*-chloromercuribenzoate, completely inhibited methylation, and the transferred methyl group was attached to a cysteine after the methylated enzyme was acid-hydrolyzed. These results suggested that cysteine was the methyl group binding site. Lu *et al.*[25] subsequently argued that the binding site was probably not cysteine but a redox-active nucleophilic metal site that could be methylated only in its reduced form. They suggested that this site was the reduced A-cluster (presumably A_{red}-CO).

Kumar *et al.* recently used resonance Raman spectroscopy to identify the methyl group binding site.[21] They first examined CH_3-Co^{3+}FeSP, and identified a CH_3-Co stretching frequency at 429 cm^{-1}. After CODH was added, this peak disappeared and one at 422 cm^{-1} appeared. The 422 cm^{-1} peak shifted frequency when either $^{13}CH_3$ or C^2H_3 was used in the methylation reaction (to 410 and 392 cm^{-1}, respectively), and it shifted with samples enriched in ^{64}Ni (to 417 cm^{-1}). The peak did not shift with samples enriched in ^{54}Fe or ^{58}Fe. The authors concluded that the methyl used in the synthesis of acetyl-CoA binds to the Ni associated with the A-cluster.

Given the abundance of supporting evidence that the A-cluster Ni is the methyl group binding site, the above conclusion was and is the most reasonable. However, no evidence actually linking the Ni-associated vibration with the A-cluster was reported. This is of some concern because Shin and Lindahl found that only a small fraction (∼15%) of the 4 Ni ions in the enzyme are from the A-cluster.[26] The chelating agent 1,10-phenanthroline (phen) selectively removes the A-cluster Ni, thereby abolishing the NiFeC signal and CO/acetyl-CoA exchange activity. Ni can be reinserted by incubating phen-treated enzyme in aqueous Ni, causing near quantitative redevelopment of the NiFeC signal and exchange activity. Only ∼0.3 $Ni^{2+}/\alpha\beta$ could be removed from or reinserted into the labile Ni site of the A-cluster. These results, in conjunction with the fact that the NiFeC signal intensity from A_{red}-CO quantifies to only ∼0.3 spin/$\alpha\beta$, suggest that only ∼30% of α subunits contain functional A-clusters and that the remaining α subunits contain structurally similar but nonfunctional clusters.[26] The cause(s) and exact nature of this apparent heterogeneity have not been elucidated, and this certainly complicates the analysis of many experiments performed on the enzyme, including the Raman study by Kumar *et al.*[21]

A related concern is whether the methyl groups of the samples used in the Raman study were functionally bound (i.e., bound in a manner that would yield acetyl-CoA or acetate if CO and CoA were added). None of the methylations reported to date has afforded completely functional methylated enzyme. Pezacka

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and Wood found that ≤60% of the methyl groups bound in their samples were functional, while Lu *et al*. obtained ∼70%.[24,25] The proportion of functionally-bound methyl groups in the resonance Raman samples was not reported, but the substantial percentage of nonfunctional groups reported in earlier studies raises the possibility that the vibration assigned to Ni−CH_3 actually arose from nonfunctionally-bound methyl groups. Consistent with this is the fact that the Raman samples for which methyl group quantification was reported had 0.89 methyl groups bound/$\alpha\beta$.[27] Pezacka and Wood's samples had similar proportions of methyl groups bound (up to 1.3 methyl groups/$\alpha\beta$), but a maximum of only 0.4 methyl groups/$\alpha\beta$ were functional.[24] Lu *et al.*'s samples had as many as 0.85 methyl groups/$\alpha\beta$ bound, but a maximum of only 0.43 methyl groups/$\alpha\beta$ were functional.[25] Given the evidence for ∼0.3 labile Ni/$\alpha\beta$,[26] only ∼0.3 methyl groups/$\alpha\beta$ would be expected to bind functionally if the methyl group bound at the labile Ni.

Qiu *et al.* and Kumar *et al*. have suggested a heterobimetallic mechanism in which the CO bound at the Fe_4S_4 portion of the A-cluster either inserts into the Ni−CH_3 bond, forming a nickel−acetyl intermediate, or the CH_3 migrates to Fe-CO, forming an iron-acetyl intermediate.[18,21] A schematic reaction describing this is shown in eq 5. In the final step of acetyl-

$$[CH_3\text{-CODH}]^{(n+1)+} + CO \rightleftharpoons [CH_3(CO)\text{-CODH}]^{(n+1)+} \rightleftharpoons [CH_3\text{-C(O)-CODH}]^{(n+1)+} \quad (5)$$

CoA synthesis, $CoAS^-$ attacks the acetyl intermediate to afford acetyl-CoA, as shown in eq 6.

$$[CH_3\text{-C(O)-CODH}]^{(n+1)+} + CoAS^- \rightleftharpoons CH_3\text{-C(O)-SCoA} + [CODH]^{n+} \quad (6)$$

Holm and co-workers have demonstrated a similar reaction sequence using synthetically prepared complexes. They found that $[Ni^{2+}(NS_3^R)(Cl)]^{1+}$, where NS_3^R is the tetradentate tripodal ligand $N(CH_2CH_2SCH(CH_3)_2)_3$, reacts with CH_3MgCl to yield $[CH_3\text{-}Ni^{2+}(NS_3^R)]^{1+}$.[28] This methylated compound reacts with CO to yield an acetyl intermediate, which then reacts with thiolates to yield thioesters and Ni^0. A second complex, square planar $[CH_3\text{-}Ni^{2+}(SR)(bpy)]^0$ (bpy = 2,2′-bipyridyl; R = 2,6-$C_6H_3Cl_2$), reacts with 1 equiv of CO to yield an acetyl intermediate and with 3 equiv of CO to yield the thioester and $[Ni(CO)_2(bpy)]^0$.[29] The thioester forms when the bound thiolate reacts with the acetyl group. These CODH mimics are not catalysts, however. They react with methyl *anions* (at the Ni^{2+}) and are reduced by 2 e^- (forming Ni^0) as product forms. In contrast, CODH reacts with methyl *cations*, and does so catalytically because it is able to return to its original electronic state as product forms. Ram and Riordan have recently demonstrated the first example of a methyl group transfer from a Co complex to a Ni complex yielding a stable Ni^{2+} methyl species.[30]

In summary, tremendous progress has been made in identifying the methyl group binding site used in the synthesis of acetyl-CoA and in elucidating the mechanism of catalysis. Available evidence suggests that the Ni and an Fe of the reduced A-cluster bind the methyl group and CO substrates, respectively, and that catalysis occurs by a heterobimetallic migratory insertion mechanism via methylation of the A_{red}-CO intermediate state. However, CODH is unusually complicated and the concerns detailed above prompted us to reinvestigate the substrate binding sites and the catalytic mechanism. In this paper, we provide substantial evidence that (a) the labile Ni of the A-cluster is, in fact, the methyl group binding site, (b) the methyl group binds the A_{ox} state (not A_{red}-CO), and (c) another redox-active species called D must be reduced before A_{ox} can be methylated. Species D may be a pair of special cysteines that oxidize to cystine and thus maintain the labile Ni of the A-cluster in the Ni^{2+} state during each step of catalysis.

Experimental Procedures

CODH Purification. *Clostridium thermoaceticum* cells were grown in two 20 L carboys, as described,[31] and harvested anaerobically using a CO_2/H_2 atmosphere chamber (Coy Labs, Inc.). CODH was purified *immediately* from the pelleted cell paste, using a slight modification of a published procedure (10 mM DTT was included in all buffers).[32] All purification steps were performed anaerobically in a Vacuum Atmospheres HE-453 glovebox containing <0.5 ppm O_2, as monitored continuously (Teledyne model 310 analyzer). A second batch of CODH was purified similarly except that the bacteria were harvested in the glovebox. Batches 1 and 2 of CODH were 90−95% pure according to SDS-PAGE. They displayed CO oxidation activities[32] of 270 and 320 μmol min^{-1} mg^{-1} and CO/acetyl-CoA exchange activities (using 11.9 mM acetyl-CoA)[33] of 0.41 and 0.45 μmol min^{-1} mg^{-1}, respectively.

CoFeSP Purification. Purifications were performed by a modification of published methods.[34,35] The red-brown fractions eluting prior to CODH on the first DEAE column were combined, concentrated, diluted with an equal volume of buffer A (50 mM Tris-HCl, pH 8.0, 2 mM dithionite, 10 mM DTT), and loaded onto a second DEAE Sephacel column (5 cm × 18 cm) equilibrated in 1 L of buffer A. The column was washed with 600 mL of buffer A containing 0.1 M NaCl and eluted with a linear gradient composed of 500 mL of buffer A with 0.1 M NaCl and 500 mL of buffer A with 0.4 M NaCl. Red-brown fractions were combined, made 10% (w/v) in ammonium sulfate, and loaded onto a phenyl Sepharose (Pharmacia) column (5 cm × 16 cm) equilibrated in buffer A with 10% ammonium sulfate. The column was washed with 500 mL of buffer A containing 10% ammonium sulfate, and the proteins were eluted with a linear gradient of buffer A containing 10−2.5% ammonium sulfate (750 mL of each). The combined red-brown fractions were >90% pure CoFeSP according to SDS-PAGE. Batches 1 and 2 contained 0.97 and 0.90 cobalamins/$\alpha\beta$, respectively[36] (assuming a molecular mass of 89 000 Da for CoFeSP[37]). Both batches had acetyl-CoA synthase activities of 150 nmol min^{-1} mg^{-1} at 55 °C.[38]

MTr Purification. Initial purification steps (up to the DEAE Bio-

(27) The authors methylated some of their samples using methyl iodide at concentrations sufficient to completely inhibit both CO/acetyl-CoA exchange and CO oxidation activities.[21,22] The mechanism by which methyl iodide inhibits CO oxidation is not known, but the presence of Ni in the C-cluster raises the possibility that the methyl−nickel bond observed by Kumar *et al.*[21] arose from the Ni of the C-cluster rather than from that of the A-cluster.

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Gel A column) followed a literature procedure[39] except that (i) cell extracts were sonicated (Heat Systems model W-380) in the glovebox for 5 min instead of being disrupted with a French pressure cell, (ii) a folate Sepharose column (2.5 × 4 cm) was used, (iii) a Centricon-30 (Amicon, Inc.) was used to concentrate the MTr fraction to 1 mL prior to loading the Bio-Gel A 0.5 cm column (1.6 × 100 cm) run at 24 mL per hour. Active fractions were combined and loaded onto a DEAE Sephacel column (0.9 cm × 5 cm) equilibrated in Buffer B (50 mM potassium phosphate pH 6.3, 1 mM DTT) and 50 mM NaCl. The column was washed with 50 mL portions of Buffer B containing 150, 225, 300, and 400 mM NaCl. Active fractions (225 and 300 mM NaCl) were combined, concentrated, made 15% in ammonium sulfate, and loaded onto a phenyl Sepharose column (0.9 cm × 6 cm) equilibrated in Buffer B with 10% ammonium sulfate. A stepwise gradient of Buffer B containing 10, 8, 6, 4, 2, and 0% ammonium sulfate (50 ml each) was applied. The most active fraction (4% ammonium sulfate) was colorless and >95% pure MTr by SDS-PAGE. MTr had an acetyl-CoA synthase activity of 8300 nmol min^{-1} mg^{-1}.[38]

After purification, protein samples were immediately fractionated into vials and frozen in liquid nitrogen. Protein concentrations were determined by the biuret method using bovine serum albumin as a standard.[40] Concentrations of CODH are expressed on an $\alpha\beta$ (M_r = 154 700) basis.[41] EPR spectra were recorded, quantified, and simulated as described.[9,42]

Methylation of CoFeSP. CoFeSP and CODH were methylated according to a modification of published methods.[25] Dithionite-reduced CoFeSP was thawed, concentrated, and applied to a Sephadex G-25 (Pharmacia) column (1 × 20 cm) equilibrated in 50 mM potassium phosphate, pH 6.8. The eluted protein was collected into a 0.5 cm pathlength quartz cuvette (Starna) modified with a double-septum seal. Most of the dithionite was removed by this process.[43] Residual dithionite was removed by slowly titrating eluted samples with aliquots of 1 mM thionin until a feature at 470 nm (Perkin-Elmer Model λ3B spectrometer) due to Co^{2+} and oxidized iron−sulfur cluster appeared. Dithionite-free CoFeSP samples were concentrated, resubjected to G-25 chromatography, collected back into the cuvette, and reduced with 1 mM titanium(III) citrate.[44] After a peak at 390 nm due to Co^{1+} developed fully, 0.2−0.4 μM MTr and 2−4 equiv/$\alpha\beta$ of $^{14}CH_3$-THF (Amersham) were added. The extent of reaction was followed by the decline at 390 nm which required ∼2 h. Samples were then brought back into the glovebox, concentrated, and subjected to G-25 chromatography (to remove excess radioactivity). The amount of protein methylated was quantified using a scintillation counter (Beckman LS 6000SE). Two batches of $^{14}CH_3$-THF were used with specific activities of 5870 dpm/nmol and 2200 dpm/nmol.

Methyl Group Transfer from $^{14}CH_3$-Co^{3+}FeSP to CODH. An Altex model 110 A pump, Scientific Systems model LP-21 pulse dampener, Rheodyne model 7125 injector, TosoHaas G3000SW size-exclusion column (7.5 mm × 30 cm) (with guard column) and Varian UV-200 detector were combined in series in an argon atmosphere glovebox (Vac/Atm HE 493). An isocratic buffer system of 100 mM potassium phosphate, pH 6.3, 0.2 M NaCl, and 0.1% sodium azide was used with a flow rate of 0.8 mL/min. Buffers were filtered through a 0.45 μm filter (Supelco) and then degassed on a Schlenk line. Once inside the glovebox, buffers were further degassed by bubbling with box atmosphere (<1 ppm of O_2) for ∼2 h using an aquarium air pump.

CODH was thawed, concentrated, and rendered dithionite-free with a G-25 column (1 × 20 cm) equilibrated in 50 mM potassium phosphate, pH 6.8. All methylations of CODH were performed at room temperature in a glovebox. In a typical methyl group transfer reaction, $^{14}CH_3$-Co^{3+}FeSP and CODH were mixed at equimolar concentrations with 2−5 equiv/$\alpha\beta$ of dithionite (standardized against potassium ferrocyanide) in a minireaction vial (total volume of 60 μL) for 2 min. Samples were injected into the HPLC, and fractions were collected into scintillation vials at 30 s intervals 2−20 min after injection. Vials were removed from the glovebox, filled with 4.5 mL of scintillation cocktail (Beckman), and scintillation-counted. Elution times of CODH and CoFeSP were monitored at 400 nm.

The amounts of methyl group bound to CODH and the CoFeSP were calculated as follows: (a) the HPLC fractions associated with CODH and CoFeSP were identified; (b) a background (average dpm per fraction outside the protein peak region) amount of radioactivity was subtracted from each fraction (∼25 dpm per fraction); (c) the dpm values of the background-subtracted peak fractions were summed; (d) the summed dpm values associated with each protein peak were converted to nanomoles of "methyl" using the ^{14}C specific activity; (e) the amount of each protein injected was calculated from the protein concentration and the injection loop size (calibrated with radioactivity to be 26.3 μL); (f) the total dpm values collected in all fractions was compared to that calculated from the amount of $^{14}CH_3$-Co^{3+}FeSP injected. Results were discarded if >10% of the radioactivity could not be accounted for.

Functional Activity of the $^{14}CH_3$-CODH. CODH was methylated as described above, mixed with a 30-fold molar excess of CoA, injected into a reaction vial containing 1 atm of CO, incubated for a prescribed time, and then injected into the HPLC. Fractions were collected and analyzed as described above. To identify the products formed, reaction mixtures were subjected to reversed-phase C_{18} (Rainin, 4.6 mm × 25 cm) HPLC chromatography (Beckman Model 128/166). Samples were eluted into scintillation vials at 1 min intervals using a linear gradient from 30 mM potassium phosphate pH 7.5, 5% MeOH (HPLC grade) to 100% MeOH at a flow rate of 1 mL/min.

Requirement of Labile Ni for Methylation. $^{14}CH_3$-Co^{3+}FeSP (39 μM) was reacted for 5 min with 4 equiv/$\alpha\beta$ of dithionite and (a) native CODH (39 μM), (b) phen-treated CODH (38 μM) prepared by incubating native CODH in 5 equiv/$\alpha\beta$ phen for 1 h, and (c) Ni-reconstituted CODH (36 μM) prepared by incubating phen-treated CODH with 10 equiv/$\alpha\beta$ aqueous Ni for 2 h. In a second experiment, CODH (35 μM) was reacted with CH_3-CoFeSP (35 μM) for 2 min, analyzed by HPLC, then treated with 11 equiv/$\alpha\beta$ of phen for 1 h, and reanalyzed.

Methylation of Cyanide-Inhibited CODH. One aliquot of CODH (35 μM) was incubated with 1 equiv/$\alpha\beta$ of $^{14}CH_3$-CoFeSP for 2 min. A second aliquot was inhibited with 10 equiv/$\alpha\beta$ of CN^- and then methylated as above. A third aliquot was inhibited as above, but incubated for 15 min during the methylation procedure.

Results

Methylation of CODH. CODH was methylated such that essentially all of the bound methyl groups were functional (i.e., they reacted with CO and either $CoAS^-$ or OH^- to yield acetyl-CoA or acetate). The first step of the procedure involved mixing reduced Co^{1+}FeSP with an excess of $^{14}CH_3$-THF, separating unreacted $^{14}CH_3$-THF, and then analyzing by HPLC. The resulting chromatogram, shown in Figure 1A, consisted of one absorbance peak due to CoFeSP and one co-migrating radioactivity peak. Co-migration of the two peaks indicates that CoFeSP was methylated according to eq 1, affording $^{14}CH_3$-Co^{3+}FeSP. The proportion that methylated was calculated from the ratio of quantified radioactivity to the amount of CoFeSP in peak fractions. Lack of radioactive counts in the small-molecule region of the chromatogram (17−18 min) demonstrates that unreacted $^{14}CH_3$-THF had been removed. This sample bound 0.92 methyl groups per CoFeSP $\alpha\beta$. In the six reactions of this type performed, an average of 0.77 methyl groups/$\alpha\beta$ bound.

In the second step of the methylation procedure, CODH was reduced with dithionite and mixed with an equimolar amount

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(42) Shin, W.; Stafford, P. R.; Lindahl, P. A. *Biochemistry* **1992**, *31*, 6003−6011.

(43) A residual amount of dithionite inevitably remained after size-exclusion chromatography. This prevented the Co^{1+} feature in the absorption spectrum of CoFeSP from developing when reductants were added and inhibited methylation of CoFeSP. The possibility that dithionite binds to CoFeSP should be evaluated further.

(44) Seefeldt, L. C.; Ensign, S. A. *Anal. Biochem.* **1994**, *221*, 379−386.

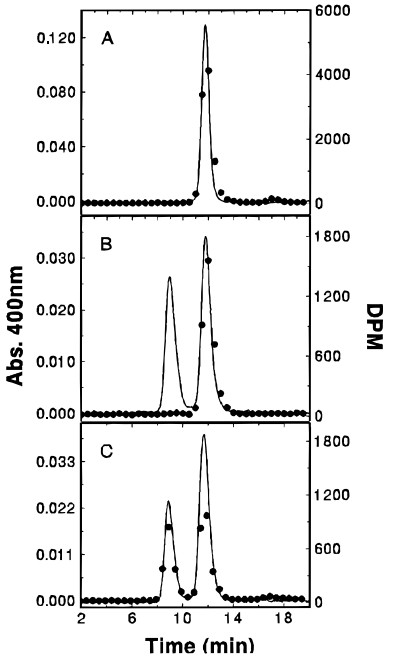

Figure 1. Sizing HPLC chromatograms demonstrating methyl group transfer from $^{14}CH_3$-Co^{3+}FeSP to CODH. Fractions were monitored at 400 nm (solid line) and by scintillation counting (solid circles). (A) $^{14}CH_3$-Co^{3+}FeSP (88 μM; 0.92 methyl groups/CoFeSP $\alpha\beta$ bound). (B) Reductant-free CODH (27 μM) reacted with $^{14}CH_3$-Co^{3+}FeSP (29 μM) for 2 min. (C) Sample similar to B except reduced with 15 equiv/$\alpha\beta$ dithionite (0.40 methyl groups/CODH $\alpha\beta$ bound). HPLC conditions described in Experimental Procedures.

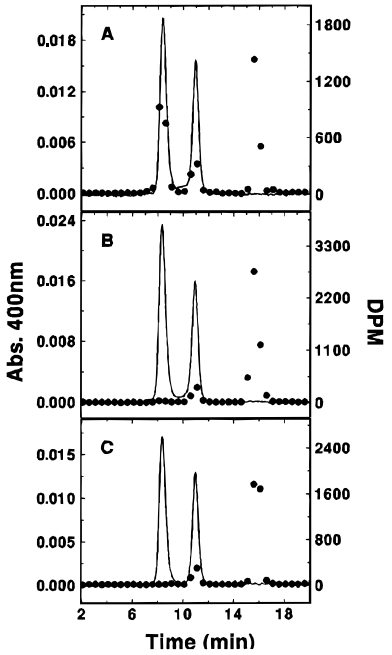

Figure 2. Sizing HPLC chromatograms demonstrating functionally active methylation. CH_3-CODH (29 μM) prepared as in Figure 1 and reacted with (A) CO (1 atm) for 2 min; (B) CO (1 atm) for 30 min; (C) CO (1 atm) and 0.98 mM CoA for 2 min. Other details are as in Figure 1.

of the $^{14}CH_3$-Co^{3+}FeSP solution generated in the first step of the procedure. After incubation, the mixture was analyzed by HPLC. The resulting chromatogram, shown in Figure 1C, consists of two absorbance peaks. The peak at ~9 min corresponds to CODH while that at ~12 min corresponds to CoFeSP. The two co-migrating radioactivity peaks indicate that some $^{14}CH_3$ transferred from $^{14}CH_3$-Co^{3+}FeSP to CODH, apparently in accordance with eq 2, and that some $^{14}CH_3$-Co^{3+}-FeSP did not react. The proportion of CODH that was methylated (0.4 methyl groups per CODH $\alpha\beta$ in this example) depended on the exchange activity of CODH and not the amount of excess CoFeSP used in the reaction. In other samples using a larger excess of CoFeSP (data not shown), the same amount of methyl group was transferred. In the 27 reactions of this type performed, an average of 0.49 ± 0.14 methyl groups bound per CODH $\alpha\beta$.

In a similar experiment in which no reductant was added during the second step of methylation, no methyl group transferred from $^{14}CH_3$-Co^{3+}FeSP to CODH (Figure 1B). This confirms the result and conclusion of Pezacka and Wood[24] and of Lu *et al.*[25] that some site on CODH must be reduced before the enzyme can be methylated.

Functional Activity of Methylated CODH. A sample of $^{14}CH_3$-CODH was prepared as above (i.e., resulting in a solution that contained a mixture of $^{14}CH_3$-Co^{3+}FeSP and Co^{1+}FeSP). A portion was exposed to CO for 2 min and then analyzed by HPLC. The resulting chromatogram, shown in Figure 2A, exhibited the same two absorption peaks obtained previously (arising from CODH and CoFeSP) and three radioactivity peaks. Two of the radioactivity peaks co-migrated with the proteins. The third eluted significantly later and did not absorb at 400 nm, suggesting that the ^{14}C radioactive label was now associated with one or more low-molecular-weight species.

Another portion of the $^{14}CH_3$-CODH sample was exposed to CO for 30 min and then injected into the HPLC. The resulting chromatogram (Figure 2B) exhibited the same two protein peaks as above, as well as two radioactivity peaks. The minor radioactivity peak co-migrated with CoFeSP (corresponding to 0.04 methyl groups/CoFeSP $\alpha\beta$), while the major peak eluted later and co-migrated with low-molecular-weight species. No radioactivity co-migrated with the CODH peak. These experiments indicate that the CO-induced transfer of the methyl group, from the two proteins to form low-molecular-weight species, was incomplete after 2 min and essentially complete after 30 min.

Another experiment was performed under the same conditions, except that the $^{14}CH_3$-CODH sample was exposed to CO and CoA and analyzed after 2 min. As shown in Figure 2C, the resulting chromatogram was essentially the same as that of Figure 2B. Thus, CoA *accelerated* the CO-induced transfer of methyl group from the proteins to form one or more small molecules. In *none* of the other five experiments of this type performed (adding CO and CoA) did *any* radioactivity (apart from background) co-migrate with the CODH peak (i.e., the methyl groups bound to every CODH sample examined were completely functional).

The low-molecular-weight radioactive species in the last experiment were identified using C_{18} reversed-phase HPLC. The resulting chromatogram of the $^{14}CH_3$-CODH sample exposed to CO and CoA (Figure 3A) exhibited two significant 254 nm peaks and three radioactivity peaks. A minor radioactivity peak and authentic acetate co-migrated at ~1 min. Thus, when CH_3-CODH was exposed to CO and $CoAS^-$, a small proportion of the bound methyl groups must have reacted with CO and OH^- to form acetate. The first major 254 nm peak eluted ~10 min after injection and co-migrated with authentic CoA. The second 254 nm peak eluted at ~12 min and co-migrated with the major radioactivity peak and authentic acetyl-CoA. This indicates that the majority of methyl groups bound to CODH reacted with CO and $CoAS^-$ to yield acetyl-CoA.[45] The third radioactivity peak eluted at ~20 min and corresponded to unreacted $^{14}CH_3$-Co^{3+}FeSP.

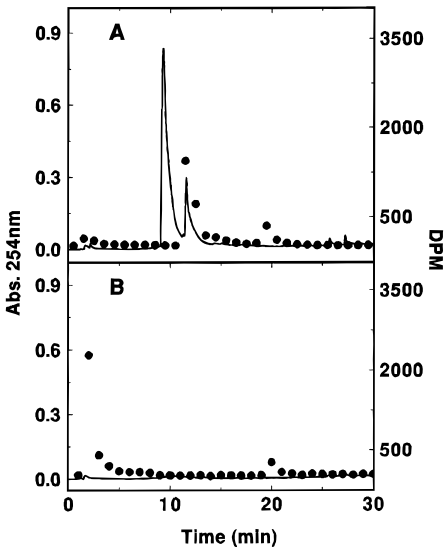

Figure 3. Reversed-phase HPLC chromatograms identifying the products of reacting CH_3-CODH with CO and CoA. Elutant was monitored at 254 nm (solid line) and by scintillation counting (solid circles). Authentic acetate, CoA, acetyl-CoA, and unreacted $^{14}CH_3$-CoFeSP had retention times of ~2, 9, 12, and 20 min, respectively. (A) Sample used for Figure 2C, spiked with 0.78 mM nonradioactive acetyl-CoA. (B) Sample of Figure 2B incubated with CO for an additional 60 min.

A similar C_{18} HPLC analysis was performed to identify the low-molecular-weight species obtained by exposing methylated CODH to CO (in the absence of CoA) for 90 min. In this case, the major radioactivity peak co-migrated with authentic acetate (Figure 3B). The minor radioactivity peak at ~20 min in Figure 3B was from unreacted $^{14}CH_3$-Co^{3+}FeSP.

The CO- and CO/$CoAS^-$-induced loss of radioactivity from the $^{14}CH_3$-Co^{3+}FeSP in the reaction mixture is probably an indirect consequence of $^{14}CH_3$-CODH reacting with CO and $CoAS^-/OH^-$ to form acetyl-CoA/acetate. Once the methyl group is displaced from CODH, an additional methyl group from the remaining $^{14}CH_3$-Co^{3+}FeSP probably binds, eventually depleting the solution of $^{14}CH_3$-Co^{3+}FeSP. A small proportion of $^{14}CH_3$-Co^{3+}FeSP did not react in this manner (note the minor radioactive peaks co-migrating with CoFeSP in Figures 2B,C and 3A,B), and may have been nonfunctional.

Requirement of the Labile Ni of the A-Cluster for Methylation. We wanted to determine whether functional methyl groups bind to the labile Ni of the A-cluster. If so, phen-treated enzyme (which is devoid of labile Ni) should not methylate when treated as above, while a similar sample subsequently incubated in aqueous Ni^{2+} (Ni-reconstituted enzyme) should methylate. To test this, a stock solution of reductant-free CODH was prepared, and a control aliquot was assayed for (a) methylation, (b) NiFeC spin intensity, and (c) CO/acetyl-CoA exchange activity. The control exhibited properties of active native enzyme. Another aliquot treated with phen was almost completely devoid of the labile nickel, as evidenced by the lack of NiFeC spin intensity and CO/acetyl-CoA exchange activity. Essentially none of phen-treated enzyme was methylated, supporting the hypothesis that the methyl group binds to the labile nickel. The Ni-reconstituted enzyme, prepared from the phen-treated aliquot, exhibited a NiFeC signal, CO/acetyl-CoA exchange activity, and methylation ability similar to the control. These results are organized in Table 1. Thus, when the labile Ni is present, CODH can be methylated; when it is absent, CODH cannot be methylated. This is exactly the reactivity pattern expected if functional methyl groups bound at the labile Ni of the A-cluster.

Table 1. Effect of Labile Nickel on Methylation of CODH

sample	methyl group transfer (methyl groups/$\alpha\beta$)	NiFeC (spins/$\alpha\beta$)	exchange activity (units/mg)
native	0.54	0.49	0.39
Phen-treated	0.04	0.03	0.03
Ni-reconstituted	0.51	0.25	0.28

Bound Methyl Group Inhibits Phen-Induced Removal of Labile Ni. We conjectured that if phen and the functional methyl group bind the labile Ni using the same coordination site, then phen may be unable to remove the labile Ni when the methyl group is bound. To test this, a control aliquot of CODH was assayed for methylation (0.50 methyl groups/$\alpha\beta$) and NiFeC spin intensity (0.29 spins/$\alpha\beta$). A second aliquot was methylated and then treated with phen. With a control sample of CODH (i.e., treated with unmethylated CoFeSP), these conditions were sufficient to completely remove the labile Ni (0 spin/$\alpha\beta$ NiFeC). However, with methylated CODH these conditions yielded a sample that still had 0.37 methyl groups/$\alpha\beta$ bound and a NiFeC signal intensity of 0.12 spins/$\alpha\beta$ (after the sample was exposed to CO and CoA). The presence of the NiFeC signal and bound methyl group indicates that a substantial fraction of the labile nickel was *not* removed by phen when the enzyme was methylated. This indicates that the bound methyl group inhibited phen from removing the labile Ni.[46]

EPR Spectra of Methylated CODH before and after Exposure to Either CO or CO/CoA. The EPR spectrum of methylated CODH is shown in Figure 4A. It exhibited the $g_{av} = 1.82$ signal from the C_{red1} state of the C-cluster and the $g_{av} = 1.94$ signal from the B_{red} state of the B-cluster. No other EPR signals were observed. Thus, in the absence of CO, the methylated A-cluster appears to be EPR-silent.

Another CH_3-CODH sample was exposed to CO for 2 min and frozen in an EPR tube. As shown in Figure 4B, the sample exhibited the NiFeC signal (0.17 spin/$\alpha\beta$), the $g_{av} = 1.86$ signal (from C_{red2}), and the $g_{av} = 1.94$ signal (B_{red}). A third CH_3-CODH sample was exposed to CO for 30 min and then analyzed by EPR. The resulting spectrum (Figure 4C) was the same as that in Figure 4B, except that the NiFeC signal was two times as intense (0.33 spins/$\alpha\beta$). A fourth CH_3-CODH sample was exposed to CO and CoA for 2 min and then analyzed by EPR (Figure 4D). This spectrum was virtually identical to that of Figure 4C (0.29 spins/$\alpha\beta$ for the NiFeC signal).

These samples almost certainly exhibited the NiFeC signal because CH_3-CODH was "demethylated" by reaction with either CO and $CoAS^-$ to yield acetyl-CoA (see Figures 2C and 3A) or with CO and OH^- to yield acetate (see Figures 2A,B and 3B) and because the demethylated enzyme subsequently reacted

(45) One reviewer suggested that this method of determining whether the methyl groups bound to CODH were functional was flawed in that it actually assessed the functionality of the methyl groups bound to CoFeS, not those bound to CODH. He correctly pointed out that acetyl-CoA would be obtained quantitatively even if only 1% of our CODH was active. This criticism would be valid had we not determined that the methyl groups on CoFeSP quantitatively transferred to CODH before we added CO and CoA to generate acetyl-CoA (and acetate). The methyl groups did transfer quantitatively to CODH (as determined by HPLC and scintillation counting) prior to adding CO and CoA. Past this step, the presence of excess CH_3-Co^{3+}FeSP in the solution had no bearing as to whether the methyl groups already bound to CODH were functional. Those methyl groups on CODH reacted with CO and CoA to yield acetyl-CoA (or acetate), demonstrating that they must have been functionally bound *to CODH*.

(46) The NiFeC signal intensity of the methylated sample treated with phen, CO, and CoA may have been lower than that of the control sample (which lacked phen) because the phen probably removed some of the labile Ni during the period after the sample was "demethylated" by CO and CoA and before it was frozen.

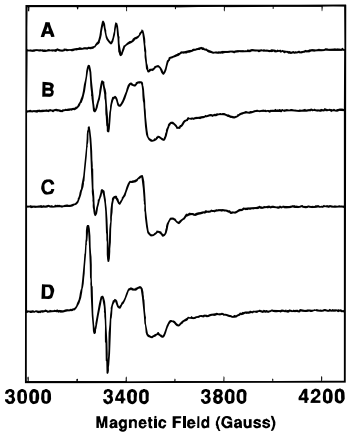

Figure 4. EPR spectra of methylated CODH, before and after treatment with CO or CO/CoA. (A) Sample of Figure 1C. (B) Sample of Figure 2A. (C) Sample of Figure 2B. (D) Sample of Figure 2C. EPR conditions: temperature 10 K; microwave power 0.05 mW; frequency 9.43 GHz; modulation amplitude 11.8 G; 3 scans averaged. The NiFeC signal has $g = 2.08$ and $g = 2.03$. The $g_{av} = 1.94$ signal has g-values of 2.04, 1.94, and 1.90. The $g_{av} = 1.82$ signal has g-values of 2.01, 1.81, and 1.65. The $g_{av} = 1.86$ signal has g-values of 1.97, 1.87, and 1.75.

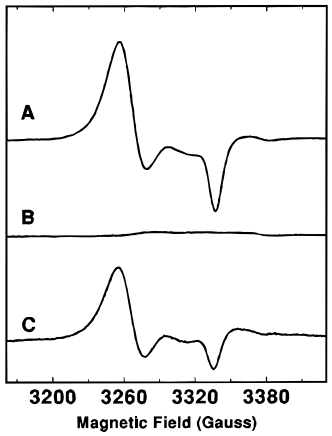

Figure 5. EPR spectra of CO-reduced CODH before and after methylation. (A) CODH (93 μM) under 1 atm CO (0.09 spin NiFeC/$\alpha\beta$). (B) Sample A reacted with $^{14}CH_3$-Co^{3+}FeSP (93 μM) for 2 min (0.11 methyl groups bound per CODH $\alpha\beta$). (C) Sample B reacted with 10 equiv/$\alpha\beta$ of CoA for 5 min (0.05 spin NiFeC/$\alpha\beta$). EPR conditions: temperature 130 K; microwave power 80 mW; frequency 9.44 GHz; modulation amplitude 11.8 G.

with CO to yield the A_{red}-CO state. Thus, the NiFeC signal intensity of methylated samples (reacted with CO) reflects the proportion of A-clusters without methyl groups bound.

Since the substrate CO is known to react very rapidly with the enzyme,[4,5] these experiments seem to indicate that methylated CODH exposed to CO reacts slowly with OH^- and rapidly with $CoAS^-$. The low NiFeC spin intensity for the sample incubated with only CO for 2 min (and the lack of any new EPR signals) suggests that CO and CH_3-CODH reacted rapidly to give an EPR-silent product. This product then reacted rapidly with $CoAS^-$ to yield acetyl-CoA and slowly with OH^- to yield acetate. We argue in the Discussion that this EPR-silent product is the acetyl intermediate.

Methylation of CO-Reduced CODH. We were somewhat surprised that CH_3-CODH and the product of reacting CH_3-CODH with CO (i.e., the acetyl intermediate) were both EPR-silent. It has been widely assumed that the $S = {}^1/_2$ A_{red}-CO state is methylated during catalysis and that the methylated (and acetylated) intermediates should have half-integer spin states. We considered the possibility that reversing the order of substrate addition might yield an EPR-active species (essentially "forcing" the A_{red}-CO state to be methylated). To test this, CODH was reduced with CO (yielding the NiFeC signal shown in Figure 5A), reacted with $^{14}CH_3$-Co^{3+}FeSP, and frozen for EPR analysis. The sample bound radioactive methyl group in *some* form, but the resulting EPR spectrum lacked the NiFeC signal (Figure 5B). After adding $CoAS^-$, the enzyme lost the radioactive methyl group and once again exhibited the NiFeC signal (Figure 5C). The methyl group had presumably reacted with CO and $CoAS^-$ to form $^{14}CH_3$-C(O)-SCoA. This experiment indicates that methylating the enzyme in the A_{red}-CO state yields an EPR-silent methyl-bound state. This EPR-silent state and the one obtained by exposing the CH_3-CODH to CO are probably the same acetyl intermediate.

Redox Requirements for Methylation. A titration was performed to correlate the redox state of CODH to the ability of the enzyme to be methylated. A sample of reductant-free CODH was divided into nine aliquots reduced by increasing amounts of dithionite. Small portions of each were reacted with $^{14}CH_3$-Co^{3+}FeSP and assayed for methylation by HPLC. The remaining amounts were frozen for EPR analysis (at the same time as the methylated samples were injected into the HPLC). The quantified signal intensities of the resulting EPR spectra along with the amounts methylated are plotted in Figure 6 vs the amount of dithionite added. The extent of methylation increased monotonically with dithionite, reaching a plateau of

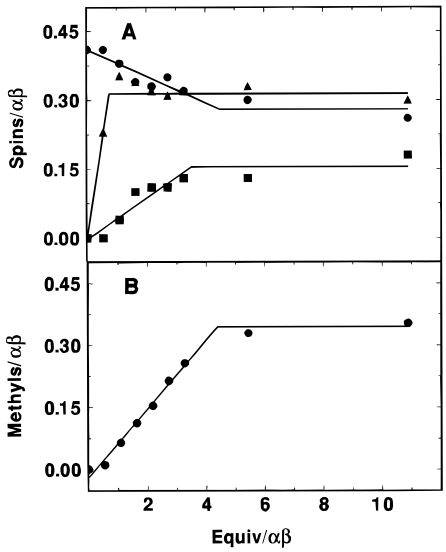

Figure 6. Plots of CODH EPR signal intensities prior to methylation (A) and the resulting methyl groups bound (B) vs amount of dithionite added to CODH. Reductant-free CODH (29 μM) was reduced with 0, 0.5, 1.1, 1.6, 2.2, 2.7, 3.3, 5.4, and 10.9 equiv/$\alpha\beta$ dithionite. After 5 min of incubation, aliquots of each sample were analyzed by HPLC and the remainders frozen for EPR analysis. (A) Intensities of the $g_{av} = 1.86$ (squares), 1.82 (circles), and 1.94 (triangles) EPR signals plotted vs equiv/$\alpha\beta$ of dithionite added. (B) Amounts of methyl groups bound. The line in A nearest the squares was constructed by joining the least-squares best-fit horizontal line obtained using the last 2 points to the least-squares-best-fit first-order polynomial line obtained using the remaining points. The two lines intersected at 0.16 spin/$\alpha\beta$ and 3.5 equiv/$\alpha\beta$ of dithionite. The line in A nearest the circles was constructed identically and afforded an intersection point at 0.28 spin/$\alpha\beta$ and 4.5 equiv/$\alpha\beta$ of dithionite. The line in B was constructed identically and afforded an intersection point at 0.35 methyl groups/$\alpha\beta$ and 4.4 equiv/$\alpha\beta$ of dithionite. The line in A nearest the triangles was constructed similarly, except that the polynomial line was obtained using the first two points, and the horizontal line was obtained using the remaining points. The intersection point was at 0.31 spin/$\alpha\beta$ and 0.74 equiv/$\alpha\beta$ of dithionite.

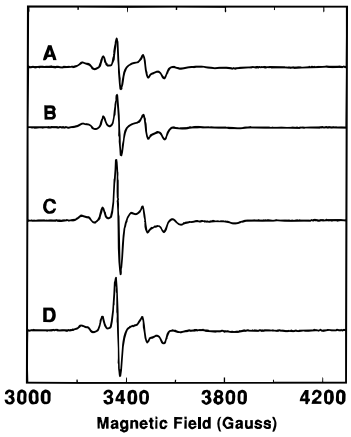

Figure 7. EPR spectra of CODH before and after methylation. (A) CODH (53 μM) reduced with 1.5 equiv/$\alpha\beta$ of dithionite (0.8 equiv/$\alpha\beta$ of methyl viologen). (B) Sample A reacted with 1 equiv/$\alpha\beta$ of $^{14}CH_3$-CoFeSP for 2 min (0.17 methyl groups/$\alpha\beta$ transferred to CODH). (C) CODH (51 μM) reacted as in A except reduced with 2.9 equiv/$\alpha\beta$ of dithionite. (D) Sample C reacted with 1 equiv/$\alpha\beta$ of $^{14}CH_3$-Co^{3+}-FeSP for 2 min (0.43 methyl groups/$\alpha\beta$ transferred to CODH). EPR conditions are as in Figure 4.

$\sim$0.33 methyl groups/$\alpha\beta$ at 5 equiv/$\alpha\beta$ of dithionite. The reductant-free sample exhibited only the $g_{av} = 1.82$ signal and could not be methylated. The sample reduced by 1.1 equiv/$\alpha\beta$ of dithionite exhibited the fully-developed $g_{av} = 1.94$ signal but was methylated only 18% of maximal. Methylation correlated most closely with the development of the $g_{av} = 1.86$ signal.[47] No other EPR signals were observed. Since the $g_{av} = 1.86$ signal develops in accordance with $E_m' = -530$ mV vs NHE,[9] this potential is probably within $\sim$50 mV of the reduction potential of the species that must be reduced before CODH can be methylated.

This last experiment monitored the CODH EPR signals present just prior to methylation. We also wanted to determine the EPR signals present just after methylation. A sample of reductant-free CODH was mixed with a trace of methyl viologen, reduced with 1.5 equiv/$\alpha\beta$ of dithionite, and then divided into two aliquots. One aliquot was frozen for EPR analysis. The resulting spectrum, shown in Figure 7A, consists of the $g_{av} = 1.94$ and 1.82 signals, as well as an isotropic signal at $g = 2.00$ due to reduced methyl viologen. The methyl viologen served as an internal indicator of the redox status of the clusters. The other aliquot was immediately methylated. Most of the material was frozen for EPR analysis, and the rest was analyzed by HPLC. The sample had 0.17 methyl groups/$\alpha\beta$ bound and exhibited the EPR spectrum of Figure 7B. The spectrum consisted of the same signals as those of Figure 7A. Thus, methylation caused virtually no change in the EPR spectrum of CODH.

The experiment was repeated using 2.9 equiv/$\alpha\beta$ of dithionite. The resulting EPR spectra are shown in Figure 7C,D. In this case, 0.43 methyl groups/$\alpha\beta$ bound, and the EPR spectra of both samples consisted of the $g_{av} = 1.94$, 1.82, and 1.86 signals. Again, there was no significant difference between the two spectra, indicating that methylation does not affect the redox state of EPR-active B- and C-clusters.

Methylation of Cyanide-Inhibited CODH. We considered the possibility that the C-cluster had to be functional for methylation to occur and tested this by inhibiting the CO oxidation activity of the enzyme using cyanide and then determining whether CN^--inhibited CODH could be methylated. A sample of reductant-free CODH was divided into three aliquots. The control had 320 units/mg of CO oxidation activity and bound 0.48 methyl groups/$\alpha\beta$. The second aliquot was inhibited with KCN, reducing its CO oxidation activity to 2% of the control. The CN^- -inhibited sample was reacted as above with $^{14}CH_3$-Co^{3+}FeSP for 2 min, and it bound 0.15 methyl groups/$\alpha\beta$. The CO oxidation activity of the sample was unchanged after methylation. Thus, CN^--inhibited CODH can be methylated. The third aliquot was also inhibited with CN^-, this time yielding enzyme with no detectable CO oxidation activity. This CN^- -inhibited sample was methylated as above, except for a longer time (15 min). Analysis by HPLC revealed that this sample bound substantially more methyl groups (0.35 methyl groups/$\alpha\beta$). These experiments indicate that a non-functioning C-cluster (bound with CN^-) slows either the rate of methylation at the A-cluster or the rate at which the enzyme is reductively-activated toward methylation. However, CN^- inhibition does not appear to alter the final extent of methylation.

Discussion

Identity of the Methyl Group Binding Site. All relevant evidence now supports the view that *the labile Ni of the A-cluster binds the methyl group used in the synthesis of acetyl-CoA*. First, the resonance Raman results of Kumar *et al.* provide compelling evidence that methyl groups (functional and/or nonfunctional) bind Ni (either the labile Ni or another Ni) in the enzyme.[21] Second, the inability of phen-treated enzyme to be methylated and the ability of Ni-reconstituted enzyme to be methylated demonstrate that the presence of the labile Ni is critical in methylation. Third, the ability of the bound methyl group to inhibit phen from abstracting the labile Ni again suggests that the labile Ni is the binding site. Fourth, the amount of functional methyl group bound (ranging from 0.23 to 0.73 methyl groups/$\alpha\beta$, with an average of 0.49 methyl groups/$\alpha\beta$) supports this conclusion, since only about 30−50% of $\alpha\beta$ dimeric units appear to contain functional A-clusters with labile Ni sites.[48] Finally, the lability of the A-cluster Ni is itself evidence for this role. If synthesis of acetyl-CoA proceeds by a migratory insertion step, then the binding site for the methyl group and CO would most likely have two cis open coordination sites.[49] This property may well be responsible for the ability of the bidentate phen ligand to remove the Ni.

Redox Requirements of Methylation. Previous studies had suggested that the methyl group binding site had to be reduced (at potentials < -350 mV) before it could be methylated, and arguments were made that this site might be the A-cluster.[24,25,50,51] On the basis of these reports, we initially assumed that, prior to methylation, A_{ox} had to be reduced to the $S = {}^1/_2$ A_{red}-CO state (yielding the NiFeC signal). Since the reaction of a diamagnetic methyl cation with the A_{red}-CO state must afford an half-integer spin state that should be EPR-active, we

(47) The C-cluster did not convert fully from C_{red1} to C_{red2} during this experiment. This behavior has been observed previously, but is not well understood.[9,14] This incomplete conversion may have lowered the amount of methyl group bound, but it does not affect the conclusion that methylation correlated most closely with the development of the $g_{av} = 1.86$ signal.

(48) Shin *et al.*[26] measured 0.2−0.3 labile Ni/$\alpha\beta$, for samples with average NiFeC spin intensities and exchange activities of 0.14 spins/$\alpha\beta$ and 0.16 units/mg, respectively. Due to improved bacterial harvesting and enzyme purification methods (harvesting anaerobically and purifying rapidly and immediately after harvesting), our NiFeC spin intensities (0.3−0.5 spins/$\alpha\beta$) and exchange activities ($\sim$0.4 units/mg) are significantly higher. Since we obtained an average of $\sim$0.5 methyl groups bound per $\alpha\beta$, the CODH used here may have contained more labile nickel than the lower-activity samples studied previously.

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were initially surprised that the methylated A-cluster was EPR-silent. We subsequently realized that A_{red}-CO could not possibly be the state that is methylated, because A_{ox} cannot be reduced by dithionite or electrochemically-reduced redox mediators,[9] yet these reductants are effective in the methylation process. *This implies that A_{ox} is the state of the A-cluster that is methylated and that there is another site in the enzyme (to be called D), which can be reduced by dithionite or redox mediators and must be reduced before methylation can occur.*

According to our analysis, the potential dependence of methylation corresponds to the reduction of D_{ox}, not A_{ox} (i.e., $E^{0\prime} < -350$ mV for D_{ox}/D_{red}). The reduced D site, then, may provide the electrons needed for binding CH_3^+ to A_{ox} (since the resulting state is viewed as having a coordinated methyl anion), and A_{ox} need not change its redox state. Since CH_3-A_{ox} is EPR-silent, D is probably a two-electron redox agent. These relationships are given in eqs 7−9.

$$[A_{ox}D_{ox}]^{n+} + [^{-}CH_3\text{-}Co^{3+}FeSP]^{m+} \rightleftharpoons \text{no reaction} \quad (7)$$

$$[A_{ox}D_{ox}]^{n+} + 2\ e \rightleftharpoons [A_{ox}D_{red}]^{(n-2)+} \quad (8)$$

$$[A_{ox}D_{red}]^{(n-2)+} + [CH_3\text{-}Co^{3+}FeSP]^{m+} \rightleftharpoons [CH_3\text{-}A_{ox}D_{ox}]^{(n-1)+} + [Co^{1+}FeSP]^{(m-1)+} \quad (9)$$

The oxidation state of the Ni in A_{ox} is almost certainly Ni^{2+}. The evidence for this is that A_{ox} is diamagnetic and its Fe_4S_4 portion has Mössbauer parameters typical of diamagnetic $[Fe_4S_4]^{2+}$ clusters.[10] This indicates that the labile Ni is diamagnetic (or $S = 1$) as well. Of the three oxidation states of Ni demonstrated to be stabilized by biologically-relevant ligands (+1, +2, +3), only Ni^{2+} can be diamagnetic or $S = 1$ (Ni^{1+} and Ni^{3+} have half-integer spin states). Moreover, Ni^{2+} reinserts into the labile site of reductant-free phen-treated enzyme to afford an EPR-silent state that is presumably A_{ox}. Linking the oxidation of a neighboring D site with the methylation of A_{ox} could also explain why a non-nucleophilic Ni^{2+} can attack a methyl cation from CH_3-Co^{3+}FeSP, leaving Co^{1+}, a well-known nucleophile. Reduction of the methylated A-cluster by D_{red} may drive what would otherwise be an unfavorable methylation step.

Evidence for an Acetyl Intermediate. According to our results, both CH_3-CODH and the product obtained by exposing this intermediate to CO are EPR-silent. That product may be any of the three possibilities given in eq 10, including the methyl intermediate, a methyl−carbonyl intermediate, and the acetyl intermediate.

$$[CH_3\text{-}A_{ox}D_{ox}]^{(n-1)+} + CO \rightleftharpoons [CH_3(CO)\text{-}A_{ox}D_{ox}]^{(n-1)+} \rightleftharpoons [CH_3\text{-}C(O)\text{-}A_{ox}D_{ox}]^{(n-1)+} \quad (10)$$

The first possibility seems unlikely, as our results and those of Lu *et al.* demonstrate that acetate forms when methylated CODH is exposed to CO in the absence of CoA (eq 11).[25]

$$[CH_3\text{-}C(O)\text{-}A_{ox}D_{ox}]^{(n-1)+} + OH^- \rightleftharpoons CH_3C(O)OH + [A_{ox}D_{red}]^{(n-2)+} \quad (11)$$

This suggests that the acetyl intermediate is formed when methylated samples are exposed to CO, although it does not establish what proportion is present relative to the uncombined carbonyl−methyl intermediate. It is possible that most of the molecules are in the carbonyl−methyl intermediate form and that, by removing the tiny proportion in the acetyl form from solution (by attack with hydroxide), the remaining molecules shift into the acetyl form. It is also possible that most of the molecules are in the acetyl form. In support of this last possibility, some well-characterized Ni−CH_3 complexes with biologically-relevant ligands react rapidly with CO to yield stable acetyl−nickel species,[28,29] and we are unaware of any such complexes that yield stable carbonyl−methyl intermediates at room temperature. The study by Ramer *et al.*[52] also suggests that the acetyl intermediate is stable, as does a study of acetyl-CoA decarbonylase synthase from *Methanosarcina barkeri*.[53] This enzyme is quite similar to CODH in that it contains a cluster with spectroscopic and functional properties virtually identical to the A-cluster. For example, the CO-reduced enzyme exhibits an EPR signal mimicking the NiFeC signal of CODH. However, the enzyme is involved in fermenting acetate to CH_4 and CO_2 (acetoclastic methanogenesis). Grahame *et al.* recently reported that when N^5-methyltetrahydrosarcinapterin (an analog of CH_3-THF) is added to the CO-reduced enzyme, the NiFeC-like signal disappears.[53] They attributed this to the formation of an acetyl intermediate and concluded that this intermediate was EPR-silent. Our experiment of Figure 5 is analogous and supports their conclusion.

Properties of the D Site. In the final step of catalysis, CoA binds the enzyme and attacks the acetyl intermediate, affording the product acetyl-CoA (eq 12).

$$[CH_3\text{-}C(O)\text{-}A_{ox}D_{ox}]^{(n-1)+} + CoAS^- \rightleftharpoons CH_3\text{-}C(O)\text{-}SCoA + [A_{ox}D_{red}]^{(n-2)+} \quad (12)$$

D_{ox} is re-reduced as $CoAS^-$ attacks the acetyl group, while maintaining the A-cluster in its A_{ox} state. Thus, one unexpected property of the D site is that D_{ox} must *not* be reducible when the A-cluster is methylated or acetylated. If D was reduced in these intermediate states during catalysis, the reaction in eq 12 could not proceed and catalysis would halt. This redox requirement for D is unusual because D_{ox} *can* be reduced when A_{ox} is not methylated or acetylated. We have not been able to circumvent this requirement because the catalytic cycle operates in the presence of a reductant that is able to reduce D_{ox} in samples prior to methylation.

The second property of D is that it must be reducible by low-potential redox mediators, dithionite and CO, since CODH reduced by any of these agents can be methylated. DTT-reduced CODH can not be methylated,[25] implying that DTT cannot reduce D.

The third property of D is that it is almost certainly an $n = 2$ redox agent. We conclude this for the following reasons. A_{ox} and the methyl cation that reacts with this state are both diamagnetic. The resulting methyl intermediate is EPR-silent and most likely diamagnetic or paramagnetic with an integer spin. If D was an $n = 1$ redox agent, and D_{red} donated one electron to the methyl intermediate, the resulting intermediate and D_{ox} would both have half-integer spin states and would likely exhibit EPR signals. However, no appropriate signals were observed. Related to this is the fourth property of D, namely, that it is EPR-silent in both oxidized and reduced states. We conclude this because no EPR signals other than those from A-, B-, and C-clusters developed during methylation, and none of these clusters appear to be the D site (see below).

Fifth, D appears to undergoing redox changes during each catalytic cycle, reducing the methyl group via the A-cluster as A is methylated and oxidizing the CoA via the A-cluster as

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acetyl-CoA is formed. D_{ox} is apparently reduced at potentials substantially lower than that required to reduce C_{ox} to C_{red1} (-220 mV[9]), somewhat lower than that required to reduce B_{ox} ($E^{0\prime}$ (B) ≈ -440 mV[9]) and similar to that required to convert C_{red1} to C_{red2} ($E_m' \approx -530$ mV[9]).

Identity of the D Site. Might one of the known clusters in the enzyme be the D site? The A-cluster cannot be D, since it cannot be reduced by redox mediators or dithionite.[9,10] The B-cluster is probably not D because it is an $n = 1$ redox agent and its reduction potential is too positive. We have considered the possibility that the C-cluster is the D site and that C_{red2} corresponds to D_{red} and C_{red1} corresponds to D_{ox}. In support of this, C_{red2} is probably two electrons more reduced than C_{red1} (as required for the D_{ox}/D_{red} couple), and the extent of methylation is proportional to the development of C_{red2} (i.e., E_m' ($C_{red1-to-}C_{red2}$) $= -530$ mV must be near to $E^{0\prime}$ (D), the redox potential for the D_{ox}/D_{red} couple). However, the C-cluster is probably not D because cyanide-inhibited CODH methylated to almost the same extent as native CODH (albeit at a slower rate). With CN^- bound, the C-cluster is nonfunctional and locked in the C_{red1} state,[4] and it would be unable to function in the role required of D. Moreover, C_{red1} can be reduced to C_{red2} when the A-cluster is methylated (in contrast to the required irreducibility of D_{ox} under this circumstance). Since the CO/acetyl-CoA exchange and acetyl-CoA synthase reactions are performed in a CO atmosphere, and CO transforms C_{red1} to C_{red2}, C_{red2} is probably present at each step of catalysis. In the C_{red2} state, the C-cluster would be unable to accept an electron pair from A as product is released, another requirement of D.

D is probably another redox-active species in the enzyme, possibly a special pair of low-potential cysteines that can be readily oxidized to cystine. Such a pair would undergo n = 2 redox and be EPR-silent in both oxidation states, as required for D. $E^{0\prime}$ (D), estimated to be -530 ± 50 mV, is substantially lower than that expected for a cystine/cysteine couple.[26] It is possible that one S of cystine coordinates to the labile nickel of the A-cluster. Binding a metal ion should lower the midpoint potential of the couple,[54] and it could conceivably inactivate the disulfide toward reduction when the Ni is methylated or acetylated, a requirement discussed above.

Analogous chemistry is exhibited by chloro(bis{2-[(2-pyridylmethyl)amino]ethyl}disulfide)nickel(II) monocation and a number of macrocyclic bis(disulfide)tetramine Ni^{2+} complexes.[55] These six-coordinate Ni disulfide complexes have Ni−(R)SSR bond lengths of ∼2.5 A, demonstrating substantial Ni−S bonding interactions. They are prepared by oxidizing the corresponding thiolate complexes. When the oxidant used is I_2, (think of I_2 as $I^- \rightarrow I^+$) the coordinating thiolates are oxidized by two electrons (reducing I^+ to I^-), and the resulting I^- and a disulfide-sulfur coordinate to Ni^{2+}. This chemistry is analogous to that proposed here: a two-electron oxidation of D_{red} (two cysteines free or coordinated to the labile Ni^{2+} of the A-cluster) by "CH_3^+", resulting in CH_3^- and a disulfide coordinated to Ni^{2+}.

Since the A-cluster is located in the α subunit,[6,17] and this subunit appears to function only in the synthesis of acetyl-CoA (not CO/CO_2 redox),[26] the D site is probably located in α as well. The Ni of the A-cluster appears to be coordinated by 2 S donors and 2 N/O donors, while the Fe_4S_4 cluster is coordinated by cysteine ligands.[7] Comparing the amino acid sequence between the α subunits of CODHs from *C. thermoaceticum, Methanobacterium thermophilia,* and *Methanococcus jannischii* reveals a 100 amino acid region with unusually high homology, including six cysteines.[41,56,57] Assuming that three or four of these cysteines coordinate the Fe_4S_4 cluster leaves two or three cysteines to coordinate the labile Ni and conceivably constitute the D site.

The Electronic and Catalytic Nature of A_{red}-CO and Deficiencies of the Generally Accepted Mechanism of Acetyl-CoA Synthesis. There is either disagreement or confusion regarding the oxidation state of the Fe_4S_4 portion of the A-cluster in the A_{red}-CO state. In our view, the strongest evidence regarding this is the Mössbauer parameters (δ and ΔE_Q) associated with these irons. These parameters, in *both* the A_{ox} and A_{red}-CO states, are characteristic of $[Fe_4S_4]^{2+}$ clusters.[10] These parameters are easily understood for the diamagnetic A_{ox} state (a low-spin $S_{Ni} = 0$ Ni^{2+} complex linked to an $S_c = 0$ $[Fe_4S_4]^{2+}$ cluster) but not for the $S = {}^1/_2$ A_{red}-CO state. Their invariance when the A-cluster was reduced suggested to Münck and co-workers that the unpaired electron used to reduce A_{ox} does not localize on the cube. They proposed that the A_{red}-CO state arises from a Ni^{1+} ion spin-coupled through a bridge to an $[Fe_4S_4]^{2+}$ cluster, using a mechanism analogous to that by which siroheme $Fe^{3+/2+}$ in *Escherichia coli* sulfite reductase couples to a bridging $[Fe_4S_4]^{2+}$ cluster.[10,58] In contrast, Qiu *et al.* concluded from their resonance Raman study of the enzyme that the Fe_4S_4 cluster is reduced to the +1 state when the enzyme is reduced with CO.[18]

There is also disagreement or confusion regarding the number of electrons used to reduce the A-cluster. As discussed in the Mössbauer study of Lindahl *et al.*[10] and later by Shin and Lindahl,[59] the spin state difference between A_{ox} ($S = 0$) and A_{red}-CO ($S = {}^1/_2$) indicates that A_{red}-CO is one-electron more reduced than A_{ox}. Tucci and Holm suggested that, during catalysis, A_{ox} (Ni^{2+}-X-$[Fe_4S_4]^{2+}$) is reduced by *two* electrons to a state designated Ni^{1+}-X-$[Fe_4S_4]^{1+}$ and that this state bound both CO (at the $[Fe_4S_4]^{1+}$ moiety) and methyl group (at the Ni^{1+}).[29] But a 2 e^- reduced state of the A-cluster has not been observed, and it cannot correspond to the A_{red}-CO state since it would have an even system spin while A_{red}-CO is $S = {}^1/_2$. Thus, if CO binds to an Fe of the A-cluster,[18] it would bind to the oxidized $[Fe_4S_4]^{2+}$ form. However, as far as we are aware, this mode of CO binding has no precedence in the chemistry of well-characterized synthetic $[Fe_4S_4]^{2+}$ clusters.

Another unsettled issue is whether the A_{red}-CO state is an intermediate in catalysis. Our results strongly suggest that it is not. The inability of A_{ox} to be reduced by dithionite or redox mediators and the ability of these reductants to facilitate methylation of the enzyme indicate that A_{ox} can be methylated and that the A_{red}-CO state is not required. This conclusion is reinforced by the EPR-silence of the methylated and acetylated intermediates. Finally, the EPR-silence of the A-cluster obtained by first reducing A_{ox} to the A_{red}-CO state and then methylating it seems to indicate that A_{red}-CO cannot be methylated in a stable fashion. Although further studies are needed to fully explain

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these experiments, we suggest that under a CO atmosphere, the A-cluster is in the equilibrium given in eq 13.

$$A_{ox} + e + CO \rightleftharpoons A_{red}\text{-}CO \qquad (13)$$

The electron in eq 13 originates from the C-cluster. In 1 atm of CO, the reaction appears to lie well to the right.[59] When CODH under 1 atm of CO is methylated, we suspect that the methyl group reacts with the small amount of A_{ox} present, shifting the equilibrium such that the enzyme is eventually fully methylated. Alternatively, the methyl group may react with the A_{red}-CO state, but this reaction appears to cause the loss of an electron (since the intermediate is EPR-silent), yielding the same A_{ox}-C(O)-CH_3 state obtained when A_{ox} is first methylated in the absence of CO. In either case, this result suggests that A_{red}-CO is not a catalytic intermediate. A similar experiment was recently performed by Grahame *et al.*[53] using decarbonylase synthase. They found that methylating A_{red}-CO caused the NiFeC signal to disappear and concluded that A_{red}-CO is the product of a *nonfunctional* side reaction, arising because CO tends to bind to reduced metal centers. They suggested that a reduced A-cluster serves to stabilize positively charged intermediates (we assume they are referring to CH_3^+ and $CH_3C(O)^+$) during catalysis. If the mechanism used by decarbonylase synthase is the same as that used by CODH, these results would provide further evidence that the acetyl intermediate CH_3-C(O)-A_{ox} is either diamagnetic or an integer spin system.

In contrast, a number of earlier studies have concluded that A_{red}-CO is a catalytic intermediate. Lu *et al.* concluded that the A-cluster has to be reduced to the A_{red}-CO state for the enzyme to be activated for catalysis.[25] Gorst and Ragsdale found that A_{red}-CO develops faster upon exposing the enzyme to CO than free CO exchanges with acetyl-CoA, and they concluded that A_{red}-CO is catalytically competent to serve as an intermediate for this exchange reaction and for the synthesis of acetyl-CoA.[51] Kumar *et al.* established that the apparent first-order rate constant for development of A_{red}-CO from A_{ox} is similar to the k_{cat} for CO/acetyl-CoA exchange and acetyl-CoA synthesis.[5] Qiu *et al.* later stated that these experiments "established that the CO adduct of center A is a catalytically relevant intermediate in the enzymatic pathway" used to synthesize acetyl-CoA.[18] However, development of A_{red}-CO at rates fast enough for it to be an intermediate in the synthesis of acetyl-CoA is a necessary but insufficient condition for it to be one.

Gorst and Ragsdale found that electrochemically-reduced CODH in the presence of acetyl-CoA yields the NiFeC signal and explained their results in a manner that supports the catalytic intermediacy of A_{red}-CO.[51] They also reported that the NiFeC signal was hyperfine-broadened when electrochemically-reduced enzyme was incubated in CH_3-^{13}C(O)-SCoA and viewed this result as further evidence that A_{red}-CO was an intermediate. However, both results can be explained more simply without assuming that A_{red}-CO is an intermediate.[60]

Lu *et al.* have argued that the three substrates used to synthesize acetyl-CoA can bind in any order, since each can bind in the absence of the other two (i.e., A_{red}-CO can be prepared without methylating the enzyme or adding CoA, methylated enzyme can be prepared in the absence of CO or CoA, and CoA can bind unmethylated enzyme in the absence of CO).[25] However, since A_{red}-CO no longer appears to be an intermediate in catalysis, the issue of whether the enzyme proceeds by an ordered or random mechanism needs to be reevaluated. Since adding CO to native enzyme leads to a nonfunctional A_{red}-CO state, we favor an ordered mechanism

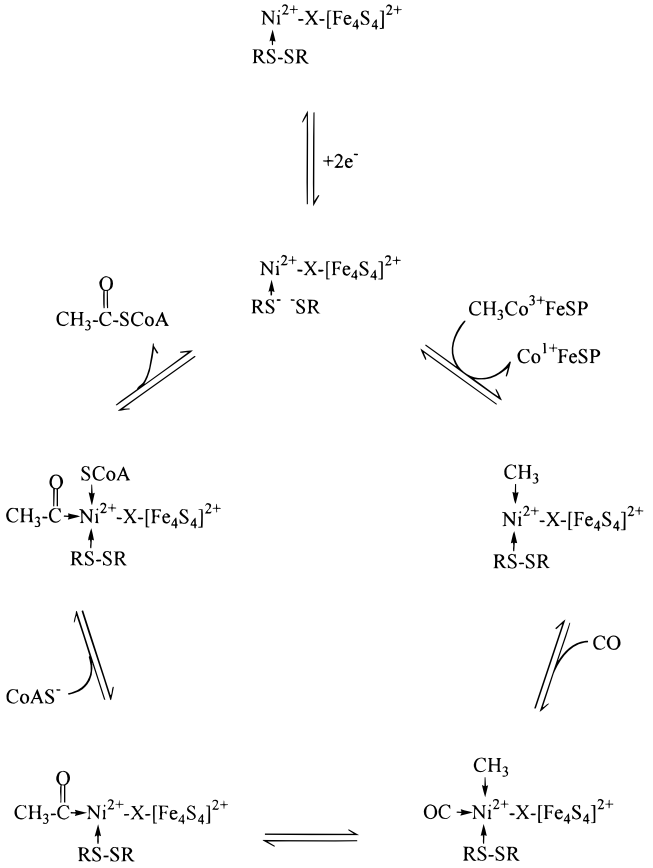

Figure 8. Proposed mechanism of Acetyl-CoA synthesis. D_{ox} is assumed to be a cystine coordinated to the labile Ni of the A-cluster. RSS(R)Ni^{2+}-X-$[Fe_4S_4]^{2+}$ corresponds to $[A_{ox}D_{ox}]^{n+}$. Two-electron reduction of D_{ox} activates the assembly for catalysis. Beginning at the top of the catalytic cycle and moving clockwise are the states $[A_{ox}D_{red}]^{(n-2)+}$, $[CH_3\text{-}A_{ox}D_{ox}]^{(n-1)+}$, $[CH_3\text{-}(CO)\text{-}A_{ox}D_{ox}]^{(n-1)+}$, $[CH_3C(O)\text{-}A_{ox}D_{ox}]^{(n-1)+}$, and $[CH_3C(O)\text{-}(CoA)\text{-}A_{ox}D_{ox}]^{(n-2)+}$.

in which the methyl group binds first, followed by CO, and then CoA. However, further studies are required to examine this issue.

In summary, the generally accepted heterobimetallic mechanism is inconsistent with the present data because it involves the A_{red}-CO state and lacks the D site or its functional equivalent. Moreover, this mechanism includes intermediates with little or no precedence in the chemistry of well-characterized synthetic metal complexes. We are unaware of any Ni^{1+} complexes with biologically relevant ligands that support methylation and form

(60) This result was explained by assuming that redox mediators reduced A_{ox} to A_{red} which reacted with acetyl-CoA to yield methyl(CoA-)-A_{red}-CO and that this latter species happened to exhibit the same NiFeC EPR signal as A_{red}-CO. Thus, it was concluded that A_{red} can be carbonylated and methylated simultaneously, supporting the view that A_{red}-CO (and methyl-(CoA-)-A_{red}-CO) are catalytic intermediates. However, it seems unlikely that methyl(CoA-)-A_{red}-CO would exhibit the same NiFeC EPR signal as A_{red}-CO, since the bound methyl group would probably perturb the ligand field of the A-cluster such that the *g*-values of the resulting signal would be altered. This result can also be explained by assuming that redox mediators reduced the D site and that $[A_{ox}D_{red}]$ reacted with acetyl-CoA to yield an EPR-silent methyl(carbonyl-)(CoA-)-$[A_{ox}D_{ox}]$ state from which CO dissociated. The dissociated CO then reduced and bound A-clusters of other CODH molecules, yielding the observed NiFeC signal. In support of this explanation, the spin intensity of the resulting NiFeC signal was only about 10% of normal (0.04 spins/$\alpha\beta$), indicating that most CODH molecules in solution were in fact EPR-silent. Their other experiment could be viewed as supporting the catalytic intermediacy of A_{red}-CO by assuming that A_{red} bound CH_3-^{13}CO-SCoA, forming a methyl(CoA-)-A_{red}-^{13}CO state that yielded the hyperfine-broadened NiFeC signal. However, we suspect that $[A_{ox}D_{red}]$ reacted with acetyl-CoA to yield the CH_3(^{13}CO-)(SCoA-)-$[A_{ox}D_{ox}]$ state from which ^{13}CO dissociated and bound to other CODH molecules, yielding the A_{red}-^{13}CO state and the hyperfine-broadened signal.

Ni^{3+}-CH_3. By contrast, there are a number of well-characterized Ni^{2+}-CH_3 complexes, some of which react with CO to form nickel(II)−acetyls.[28−30,61,62] We are unaware of any $[Fe_4S_4]^{2+}$ clusters that are known to react with CO. By contrast, there are a number of well-characterized Ni^{1+}-CO complexes and one Ni^{2+}-CO complex.[63] The vast majority of migratory insertions reactions involving metal-bound CO and methyl groups occur with these species coordinated to adjacent positions on a single metal ion.[49]

Mechanism of Acetyl-CoA Synthesis Involving a Surrogate Redox Agent. The results and analysis presented here are summarized by the mechanism of acetyl-CoA synthesis illustrated in Figure 8. According to our mechanism, reductant-free enzyme ($[A_{ox}D_{ox}]^{n+}$) becomes catalytically active when D_{ox} is reduced by 2 electrons. The labile Ni^{2+} ion of the A-cluster in the $[A_{ox}D_{red}]^{(n-2)+}$ state is methylated (by CH_3-Co^{3+}FeSP) and simultaneously reduced (by D_{red}), affording a CH_3-Ni^{2+} intermediate. CO binds $[CH_3$-$A_{ox}D_{ox}]^{(n-1)+}$, and inserts into the nickel−methyl bond, affording the acetyl intermediate $[CH_3C(O)$-$A_{ox}D_{ox}]^{(n-1)+}$. $CoAS^-$ coordinates the nickel[64] and attacks the bound acetyl to yield the product acetyl-CoA and the starting state $[A_{ox}D_{red}]^{(n-2)+}$ in a reductive elimination step.

Our mechanism utilizes the same fundamental steps first proposed by Ragsdale and Wood[22] (migratory insertion of CO and methyl group to form an acetyl intermediate, followed by nucleophilic attack by $CoAS^-$). However, it also (a) specifies that a redox site *other than the A-cluster* itself (i.e., D) must be reduced before methylation can occur, (b) assigns oxidation states at each step of catalysis (Ni^{2+} at each step), and (c) does not include the A_{red}-CO state as a catalytic intermediate. *In summary, our mechanism involves a surrogate redox agent that allows the substrate-binding site to remain redox-inactive (Ni^{2+}) at each step of catalysis.* Given the results presented here, this mechanism appears to be the only one that is compatible with all of the published relevant studies of CODH.[65] We hope that our results and analysis will encourage further experiments aimed at elucidating this elegant and most fascinating bioorganometallic catalytic mechanism.

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(64) Removing sulfur from CoA (desulfo-CoA) increases K_I for the acetyl-CoA/CO exchange reaction by nearly 10^3 suggesting that the thiolate portion of $CoAS^-$ coordinates the labile Ni.[33]

(65) Our mechanism may seem incompatible with the Raman study of Kumar *et al.* in which CO was proposed to bind to the Fe_4S_4 portion of the A-cluster[21] rather than to the labile Ni center as proposed here. However, since this binding mode was assigned from spectra of CODH in the A_{red}-CO state, proposing that CO binds to the labile Ni in other states is compatible with, though not supported by, that result.