A Methylnickel Intermediate in a Bimetallic Mechanism of Acetyl–Coenzyme A Synthesis by Anaerobic Bacteria

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Resonance Raman (RR) spectroscopy was used to identify a methylnickel adduct (ν_{Ni-C} = 422 wave numbers) of carbon monoxide dehydrogenase (CODH) from *Clostridium thermoaceticum*. Formed at a nickel/iron-sulfur cluster on CODH called center A, the methylnickel species is the precursor of the methyl group of acetyl–coenzyme A in an anaerobic pathway of carbon monoxide or carbon dioxide fixation. Rapid kinetic and RR studies demonstrated that methylation of nickel occurs by heterolysis of the methyl-cobalt bond (ν_{Co-C} = 429 wave numbers) of a methylated corrinoid/iron-sulfur protein. In combination with the earlier finding of an iron-carbonyl adduct at center A, detection of the methylnickel intermediate establishes a bimetallic mechanism for acetyl–coenzyme A synthesis.

Carbon monoxide dehydrogenase is a metalloenzyme that catalyzes the final steps in the reductive acetyl–coenzyme A (acetyl-CoA) pathway. This pathway allows anaerobic bacteria to grow on CO_2 or CO as a sole carbon source and methanogenic archae to convert acetic acid to methane. The final steps involve activating and combining a methyl group and CO to form an acetyl group which is then incorporated into acetyl-CoA. Here we establish that the C-C bond is formed by an organometallic mechanism.

CODH contains 2 Ni, 11 to 14 Fe, ~1 Zn, and ~14 inorganic sulfides per heterodimeric unit (*1*). The metals are organized into three clusters, centers A, B, and C (*2*). Centers A and C (*3*, *4*) are Ni-FeS clusters. Center A catalyzes the final steps in acetyl-CoA synthesis (*2*, *5*, *6*). A minimal structure for center A has been defined by spectroscopic studies to be Ni-X-[4Fe-4S], where X is an unknown ligand bridge (*7*). An adduct between CO and center A was shown to be kinetically competent as the precursor of the carbonyl group of acetyl-CoA (*2*, *5*). Surprisingly, this adduct was found to be a complex between CO and Fe, not Ni (*3*, *8*, *9*). What, then, is the role of nickel, which is required for acetyl-CoA synthesis (*6*, *10*)? A bimetallic mechanism for acetyl-CoA synthesis was proposed that included iron-carbonyl and methylnickel intermediates (*9*).

The methyl group of acetyl-CoA is derived from CO_2 through steps that involve formate dehydrogenase, a series of tetrahydrofolate (H_4folate) enzymes, and a corrinoid/iron-sulfur protein (corrinoid-FeS protein), forming a methylcobamide species on the corrinoid-FeS protein (*11*). This methylcobalt species donates the methyl group to CODH. Our first goal was to characterize the methylcobalt bond. We methylated the corrinoid-FeS protein (*12*) with CH_3I and identified the methyl-Co stretching band at 429 cm^{-1} in the resonance Raman (RR) spectrum (Fig. 1). This band moved to 420 cm^{-1} when the corrinoid-FeS protein was methylated with $^{13}CH_3I$ (*13*). The band position indicates that the Co-C bond is weaker and longer than that of free methylcobalamin in solution and other six-coordinate organocobalt B_{12} model compounds that exhibit Co-C stretching modes at ~500 cm^{-1} (*14*).

Although it has been presumed that methylation of CODH occurs at center A (*15*), direct evidence has been lacking. When CODH (*16*) was reacted with the $^{12}CH_3$-corrinoid-FeS protein (*17*), the 429-cm^{-1} band from methyl-Co diminished and a new band at 422 cm^{-1} appeared (Fig. 2). This band moved to 410 cm^{-1} or to 392 cm^{-1} when $^{13}CH_3$-corrinoid-FeS protein or CD_3-corrinoid-FeS protein was the methyl donor (*18*) . Therefore, this band was assigned as the stretching frequency for the methylmetal bond at center A of CODH. The 422-cm^{-1} band was also observed when CODH was reacted with $^{12}CH_3I$ and catalytic amounts of the corrinoid-FeS protein (*19*).

To identify the methyl acceptor, we isolated CODH from cells grown in medium containing ^{54}Fe (98%), ^{58}Fe (85%), or ^{64}Ni (95%). (Natural abundance masses are 55.9 for Fe and 58.7 for Ni.) When ^{54}Fe or ^{58}Fe was substituted into CODH, the metal-CH_3 stretching band remained at 422 cm^{-1}, the position observed with ^{56}Fe-CODH (Fig. 2). Therefore, the methyl group does not bind to an iron site in center A. However, when ^{64}Ni-CODH was methylated with the methylated corrinoid-FeS protein, the band moved to 417 cm^{-1}, establishing the mode as a Ni-methyl stretch (*20*).

These results demonstrate that Ni accepts the methyl group from the methylated corrinoid-FeS protein. The transfer reaction could involve homolytic or heterolytic cleavage of the methyl-Co^{3+} bond. These two mechanisms were distinguished by determining whether the transmethylation reaction generated Co^{2+} or Co^{+} as the prod-

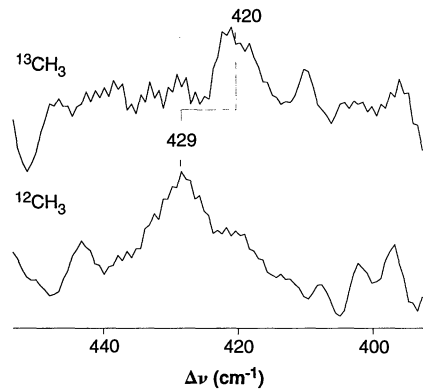

Fig. 1. RR spectra at 77 K of methylated corrinoid-FeS protein, methylated with $^{12}CH_3I$ (bottom) and $^{13}CH_3I$ (top). The scattered light from a 476.5-nm Ar^+ ion laser line (~70 mW) was focused into a triple monochromator equipped with a diode array multichannel detector. The collection time was ~3 hours per spectrum. Spectra were calibrated with carbon tetrachloride and dimethyl formamide.

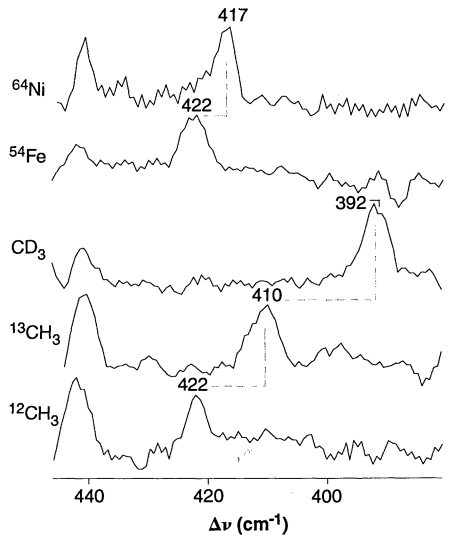

Fig. 2. RR spectra (476.5 nm–excited, 77 K) of the indicated isotopomers of reduced CODH treated with methylated corrinoid/Fe-SP (corrinoid-FeS protein spectrum subtracted digitally). The top two spectra show the effect on the 422-cm^{-1} band (metal-CH_3 stretching) of labeling CODH with ^{64}Ni and ^{54}Fe, whereas the bottom three spectra show the effect of increasing the mass of the methyl group. Conditions as in Fig. 1.

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uct. The product of a heterolytic cleavage would be Co^+, characterized by a sharp absorption peak at 390 nm [$\Delta\epsilon$ (extinction coefficient change) = 5 mM^{-1} cm^{-1}]. The product of homolytic fission would be Co^{2+}. Co^{2+} in the corrinoid/FeS protein adopts a four-coordinate geometry in which the benzimidazole base is displaced (termed base-off) resulting in a broad absorption band centered at 470 nm (*21*). A Co^+ product was established by stopped-flow kinetics (*22*). When CODH was rapidly mixed with CH_3-Co^{3+}-corrinoid-FeS protein, Co^+ was formed (Fig. 3). Clean isobestic points were observed at the expected wavelengths, indicating that methyl-Co^{3+} was converted to Co^+ without an intermediate. That Co^+ is the product of the transmethylation reaction is supported by single wavelength–monitored kinetics (*23*). The decay rate for the 450-nm peak of base-off methyl-Co^{3+} was equivalent to the formation rate for the 390-nm peak that characterizes Co^+ (1.2 s^{-1} at 25°C) (Fig. 3, inset). Extrapolating this rate constant to 55°C, the optimal growth temperature for *Clostridium thermoaceticum*, gave a rate constant of ~10 s^{-1} (*24*). Thus, formation of Co^+ is catalytically relevant because it occurred approximately fivefold faster than the k_{cat} for acetyl-CoA synthesis (*25*). Generation of Co^+ would be advantageous for the cell because it can then react with CH_3-H_4 folate to regenerate the methylated corrinoid-FeS protein. For a homolytic radical mechanism, a one-electron reduction of Co^{2+} would be required with each cycle of acetyl-CoA synthesis.

Previous studies on the corrinoid-FeS protein indicate that it is designed to facilitate heterolytic Co-C bond cleavage. The cobamide is base-off in all three oxidation states (*21, 26*). Absence of a nitrogenous donor ligand in the methyl-Co(III) state has been reported to predispose the Co-C bond toward heterolytic cleavage and protect against radical chemistry (*27*). Thus, it is possible that the corrinoid-FeS protein modulates the cobalt coordination chemistry to activate the methyl group toward a nucleophilic attack. Heterolytic cleavage of the methyl-Co bond to generate Co^+ as the product requires the participation of a nucleophilic group on CODH.

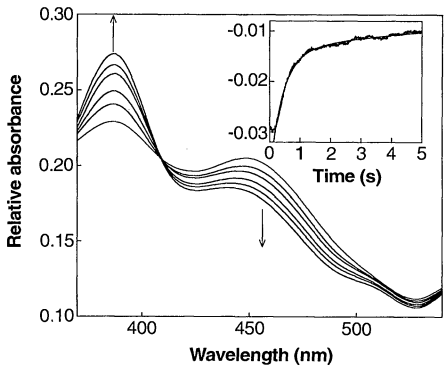

Fig. 3. Reaction of reduced CODH with methylated corrinoid/Fe-SP. CODH was incubated with CO for 15 min and mixed rapidly with the methylated corrinoid/FeS protein. The reaction was repeated as the monochromator was moved in 10-nm increments between 370 and 520 nm. Time slices were obtained by digitally connecting the data points 10 nm apart, using the "point-by-point" software provided by Applied Photophysics. (**Inset**) Single wavelength–monitored kinetics of the reaction of 10 μM CODH with 10 μM methylated corrinoid/FeS protein followed at 390 nm.

The bio-organometallic catalytic cycle catalyzed by CODH can be summarized [see figure 3 of (*9*) or figure 1 of (*28*)] as follows: (i) An Fe-CO species is formed at center A (*29*). The CO originates from the atmosphere or from the reduction of CO_2 to CO by center C of CODH. (ii) The low-valent Ni site in center A performs a nucleophilic attack on the methylated corrinoid-FeS protein, generating Co^+ and methylnickel. (iii) The next step in acetyl-CoA synthesis involves either a carbonyl insertion to form acetyl-Ni or a methyl migration to generate an acetyl-Fe intermediate. (iv) The final step involves thiolysis of the acetyl-metal intermediate to form acetyl-CoA. A suggested mechanism for this step involves a metal-SCoA intermediate (*28*).

We have demonstrated a bimetallic enzymatic mechanism of C-C bond formation. There are several examples of bimetallic catalysts in the organometallic chemical literature. A heterobimetallic complex containing CH_3-Zr and Mo-CO undergoes further carbonylation to form an acetyl complex (*30*). Reaction of methyl-$Mn(CO)_5$ with an Fe-carbonyl complex yields a heterobimetallic bridging acetyl complex (*31*). In addition, there are examples of heterolytic (*32*) and homolytic cleavage of an alkylmetal complex by another metal (*33*). Examples of CO insertion and methyl migration reactions have been reviewed (*34*). Another example is the "Monsanto process" for acetic acid synthesis from methanol and CO in which a rhodium or iridium catalyst undergoes methylation, carbonylation, and methyl migration to form acetyl-rhodium (*35*).

Nickel is an essential trace element for bacteria, plants, animals, and humans (*36*). It is an essential component of four enzymes (CODH, urease, methyl-CoM reductase, and hydrogenase). Our results provide convincing direct evidence for a new biological role of nickel. The methylnickel intermediate represents the first example of a Ni-C bond in nature. Adenosylcobalamin and methylcobalamin are the only other known examples of alkylmetal bonds in enzymes (*37*). Demonstration of a methylnickel intermediate in acetyl-CoA synthesis sets a biological precedent relevant to the mechanism of methane synthesis from methyl-CoM. It has been suggested that this reaction could occur through a methylnickel intermediate (*38*).

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12. The corrinoid-FeS protein was purified to homogeneity as described (*39*). Methylation of the corrinoid-FeS protein [46 mg in 1 ml of 50 mM tris-HCl (pH 7.6)] was achieved by first reducing it with Ti(III) citrate (5 mM, final) for 1 hour, adding 1 μl (16 μmol) of neat CH_3I, and incubating at 16°C for 30 min. The methylated corrinoid-FeS protein was then centrifuged through a Sephadex G-25 column to remove unreacted CH_3I and Ti. The methylated corrinoid-FeS protein was concentrated with an Amicon macrosolute concentrator. By evaluating this reaction with tracer amounts of $^{14}CH_3$-I, we found that the corrinoid-FeS protein was 96% methylated. Earlier studies had demonstrated that CH_3I can be used in the synthesis of acetyl-CoA and that the methylated corrinoid-FeS protein generated by reaction with CH_3I is catalytically active (*39, 40*).
13. If the $^{13}CH_3$ and $^{12}CH_3$ groups are treated as point masses of 16 and 15 atomic mass units (amu), respectively, the calculated frequency ratio of the Co-$^{13}CH_3$ and Co-$^{12}CH_3$ stretching bands would be 0.975, which matches well with the observed value of 0.979.
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15. A methyl-cysteine intermediate on CODH was proposed [E. Pezacka and H. G. Wood, *J. Biol. Chem.* **263**, 16000 (1988)] as the product of this transmethylation. However, evidence against methyl-cysteine and in favor of a methyl-metal intermediate was provided by electrochemical, kinetic, and cysteine modification experiments (*40*) and by stereochemical considerations [S. A. Raybuck, N. R. Bastian, W. H. Orne-Johnson, C. T. Walsh, *Biochemistry* **27**, 7698 (1988)].
16. CODH was purified as described (*1*) to a specific activity measured at 55°C of 521 U/mg in the CO oxidation reaction and 0.960 U/mg in the exchange reaction between CO and [1-^{14}C]acetyl-CoA. One

unit is defined as 1 μmol of CO exchanged per min.
17. CODH [120 to 140 mg ml^{-1} in 50 mM tris-HCl (pH 7.6)] was reduced with CO (1 atm for 10 to 15 min) or with dithionite (4 mM final) and then methylated by adding the methylated corrinoid-FeS protein (1.5 to 2 equivalents) with vigorous shaking. The reaction mixture was placed in the RR sample holder and frozen cryogenically within 2 to 4 min after mixing the two proteins.
18. Treating the $^{13}CH_3$ and $^{12}CH_3$ groups as point masses of 16 and 15 amu, respectively, the calculated frequency ratio of the Ni-$^{13}CH_3$ and Ni-$^{12}CH_3$ stretching bands would be 0.975, which matches well with the observed value of 0.969. The calculated frequency ratio of the Ni-CD_3 and Ni-CH_3 stretching bands is 0.931, in agreement with the observed value of 0.927.
19. We also used CH_3I as the methyl group donor to CODH. This reaction requires the intermediacy of the corrinoid-FeS protein, because the methylated corrinoid-FeS protein is the only known direct methyl donor for CODH (*40*). CODH [40 nmol, 6 mg, in 100 μl of 50 mM tris-HCl (pH 7.6)] was mixed with 0.40 nmol of corrinoid-FeS protein under a CO atmosphere for 15 min. The atmosphere was exchanged with nitrogen, 4 μmol of CH_3I (20 μl of 0.2 M) was added, and the solution was incubated for 30 min. Excess CH_3I was removed by centrifuging the mixture through a Sephadex G-50 column, and the protein was concentrated with Amicon centricon tubes. By monitoring with tracer amounts of $^{14}CH_3I$, it was shown that 0.89 mol of methyl groups were incorporated per mol of CODH. When $^{13}CH_3I$ was the methyl donor, the methyl-metal band was observed at 410 cm^{-1}.
20. The calculated frequency ratio of the ^{64}Ni-$^{12}CH_3$ and ^{58}Ni-$^{12}CH_3$ stretching bands would be 0.990, which matches well with the observed value of 0.988. If an Fe-CH_3 bond had been observed, the position of the ^{58}Fe-CH_3 band would be expected to shift by 4 cm^{-1} relative to the ^{54}Fe-CH_3 sample. Natural abundance Ni consists of ^{58}Ni and ^{60}Ni in ~2:1 ratio. The sharpness of the 417-cm^{-1} band is interpreted to reflect the presence of a single Ni isotope. This provides further evidence that Ni is involved in this resonance.
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22. Stopped-flow experiments were performed on an Applied Photophysics spectrofluorimeter and data were fit with software purchased from Applied Photophysics. Solutions of enzymes and substrates were made in the anaerobic chamber, transferred into tonometers that are isolated from the atmosphere by stopcocks, and connected directly to the drive syringes of the stopped-flow instrument. These syringes were maintained anaerobically in a temperature-controlled bath of anaerobic water.
23. The possibility was ruled out that Co^{2+} is the product of methyl transfer but is rapidly reduced to Co^{+} by CODH in a subsequent step. In a separate experiment with the Co^{2+}-corrinoid-FeS protein (5 μM) and CO-reduced CODH (2.5 μM), the k_{obs} for Co^{2+} reduction was 0.026 ± 0.003 s^{-1} at 30°C and 0.037 ± 0.003 s^{-1} at 40°C. These rates are significantly lower than the rate of formation of Co^{+} in the methyl transfer reaction. We and others earlier ruled out a mechanism involving one-electron reduction of methyl-Co^{3+} followed by homolytic cleavage, which would also generate Co^{+} [B. D. Martin and R. G. Finke, *J. Am. Chem. Soc.* **112**, 2419 (1990); S. A. Harder, W.-P. Lu, B. F. Feinberg, S. W. Ragsdale, *Biochemistry* **28**, 9080 (1989)].
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T Cell Awareness of Paternal Alloantigens During Pregnancy

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During pregnancy a semiallogeneic fetus survives despite the presence of maternal T cells specific for paternally inherited histocompatibility antigens. A mouse transgenic for a T cell receptor recognizing the major histocompatibility (MHC) antigen H-2K^b was used to follow the fate of T cells reactive to paternal alloantigens. In contrast to syngeneic and third-party allogeneic pregnancies, mice bearing a K^b-positive conceptus had reduced numbers of K^b-reactive T cells and accepted K^b-positive tumor grafts. T cell phenotype and responsiveness were restored after delivery. Thus, during pregnancy maternal T cells acquire a transient state of tolerance specific for paternal alloantigens.

In outbred species, inheritance of paternal histocompatibility antigens by the embryo results in genetic mismatches to the mother. The semiallogeneic fetus is in direct physical contact with uterine and blood-borne cells of the mother, and fetal rejection by the maternal immune system is prevented by mechanisms as yet undefined (*1*). In mice, midgestational placenta expresses paternal MHC antigens of the K and D loci (*1*, *2*); when grafted into maternal-strain recipients, it is rejected and induces sensitization to paternal alloantigens (*3*). However, neither ignorance nor tolerance of maternal T cells to paternal alloantigens has been conclusively shown. Impairment of T cell responses has been observed, but its selectivity to paternal alloantigens remains controversial (*1*, *4*, *5*). Midpregnant CBA mice, which are inbred, have unaltered expression of T cell receptor (TCR), CD4, and CD8 (*6*). However, phenotypic changes may go undetected because T cells specific for paternal alloantigens have low frequency in a normal T cell repertoire. Here, we used a TCR transgenic mouse model (Des-TCR) harboring a T cell repertoire skewed toward the paternal alloantigen H-2K^b (*7*) to take advantage of the high frequency of allospecific cytotoxic T cells as well as the ease of monitoring the transgenic TCR with clonotype-specific antibodies.

Virgin H-2^k Des-TCR transgenic females were mated with H-2^b C57BL/6 males, and K^b-specific T cells were phenotypically characterized during pregnancy. Nonspecific gestational effects (*8*) were controlled for by syngeneic and third-party allogeneic matings with H-2^k CBA or H-2^s ASW males (*9*), respectively. Midpregnant Des-TCR mice bearing a K^b-positive conceptus had reduced numbers of T cells with high expression of the clonotype (Fig. 1B, left) and six to nine times more clonotype-positive cells devoid of CD4 and CD8 (Fig. 1B, right) when compared to the results obtained for H-2^k syngeneic (Fig. 1C) and H-2^s third-party allogeneic (Fig. 1D) pregnancies. Therefore, maternal T cells specifically recognize paternal alloantigens.

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