Controlled Potential Enzymology of Methyl Transfer Reactions Involved in Acetyl-CoA Synthesis by CO Dehydrogenase and the Corrinoid/Iron-Sulfur Protein from Clostridium thermosaceticum*

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Many anaerobic bacteria fix CO₂ via the Wood pathway of acetyl-CoA synthesis. Carbon monoxide dehydrogenase (CODH), also called acetyl-CoA synthase, accepts the methyl group from the methylated corrinoid/iron-sulfur protein (C/Fe-SP), binds a carbonyl group from CO, CO₂, or the carbonyl of pyruvate, and binds coenzyme A. Then CODH catalyzes the synthesis of acetyl-CoA from these enzyme-bound groups. Here, we have characterized the methyl transfer steps involved in acetyl-CoA synthesis. We have studied the reactions leading to methylation of CODH by methyl iodide and shown an absolute requirement of the C/Fe-SP in this reaction. In addition, we have discovered and partly characterized two previously unknown exchange reactions catalyzed by CODH: between the methylated C/Fe-SP and methylated CODH and between methylated CODH and the methyl moiety of acetyl-CoA. We have performed these two exchange reactions, methylation of the C/Fe-SP, and methylation of CODH at controlled potentials. The rates of all these reactions except the exchange between methylated C/Fe-SP and methylated CODH are accelerated (from 1 to 2 orders of magnitude) when run at low potentials. Our results provide strong evidence for a nucleophilic redox-active metal center on CODH as the initial acceptor of the methyl group from the methylated C/Fe-SP. This metal center also is proposed to be involved in the cleavage of acetyl-CoA in the reverse reaction.

Clostridium thermosaceticum and many other anaerobic bacteria perform CO₂ fixation via the acetyl-CoA pathway (Wood pathway) which involves a corrinoid/iron-sulfur protein (C/Fe-SP) and carbon monoxide dehydrogenase (CODH) as central enzymes (see Refs. 1-4 for recent reviews). This autotrophic pathway can be summarized by Equation 1.

\[ 2\text{CO}_2 + 4\text{H}_2 + \text{CO} \rightarrow \text{CH}_3\text{CO}-\text{SCoA} + 2\text{H}_2\text{O} \]  

(1)

In this pathway, CO₂ is reduced to methyldihydrofolate (CH₃-H₄folate) via formate dehydrogenase and several H₂folate enzymes. The individual steps which are involved in the synthesis of acetyl-CoA from CH₃-H₄folate involve the participation of enzyme-bound intermediates and are catalyzed by three enzymes: a methyltransferase, the C/Fe-SP, and CODH. The five genes encoding these three enzymes from C. thermosaceticum have been cloned and found to be part of an ~11-kilobase gene cluster (5).

The methyl group of CH₃-H₄folate is transferred to the C/Fe-SP by methyltransferase forming enzyme-bound CH₃-cob(III)amide. Methylation of the C/Fe-SP involves reduction of both the cobalt and [4Fe-4S] centers from the 2+ to the 1+ state (6), the 2+/1+ couples having midpoint reduction potentials at -504 and -523 mV, respectively (7). The exact mechanism of the cleavage of the CH₃-N bond of CH₃-H₄folate has not been established; however, it appears that the reaction occurs by a nucleophilic attack of Co⁺ on the methyl group (6, 7). This reaction is analogous to the reaction mechanism of methionine synthase (8) which transfers the methyl group of CH₃-H₄folate to homocysteine, forming methionine, via an enzyme-bound methyl-cob(III)alamin intermediate (8-10).

The final steps in the synthesis of acetyl-CoA occur on CODH. Based on studies of the exchange reaction between CO and the carbonyl of acetyl-CoA, it was proposed that CODH should be called acetyl-CoA synthase since it has the ability to bind the methyl group, the carbonyl group, and CoA at separate sites and then to condense these groups to form acetyl-CoA (11). When CODH binds CO or CO₂ (after reduction), an organometallic intermediate consisting of nickel, iron, and CO (4, 12, 13) is formed. This Ni-Fe-C intermediate has been studied by a number of spectroscopic methods, including EPR (12, 13), Mössbauer, electron nuclear double resonance, and x-ray absorption spectroscopies (17, 18). Based on Mössbauer spectra, the magnetic properties of the iron associated with the Ni-Fe-C center resemble those of [4Fe-4S] clusters in the 2+ core oxidation state. The nickel site consists partly (17) or primarily (18) of Ni-S bonds and evidence for a Ni-Fe distance consistent with a Ni-X-Fe bridge has been presented (18). An analogous Ni-Fe-C intermediate apparently is involved in cleavage of acetyl-CoA by the CODH from methanogenic bacteria (19) and by the aetogenic CODH.

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CODH binds CoA at a site involving arginine and tryptophan residues (20, 21) near the Ni Fe C site (12). Wood and coworkers have recently sequenced the residues surrounding the CoA binding site and found some homology to ATP-binding enzymes. Recently, Ramer et al. (22) characterized an exchange reaction between $[^3P]CO$ and the CoA moiety of acetyl-CoA, earlier studied by Pezacka and Wood (23). This reaction occurs 6.3-fold faster than the exchange between CO and the carbonyl of acetyl-CoA (22). CODH accepts the methyl group of the methylated C/Fe-SP, forming a methylated CODH intermediate. We favor a mechanism of methyl transfer to CODH involving a heterolytic cleavage via nucleophilic attack by a group on CODH on the methyl group of the methylcobalt (7). The chemistry involved in the methylation of CODH is the major focus of the work presented in this paper. Pezacka and Wood (24) were successful in methylating CODH with methyl iodide, methylated C/Fe-SP, and methyl-B$_2$ as methyl donors. An enzyme-bound CH$_3$-CODH intermediate was proposed to be S-methylcysteine based on amino acid analysis of $^{13}$CH$_3$-labeled CODH, which was located on the $\beta$ (71 kDa) subunit, and was converted to acetyl-CoA in the presence of CO and CoA (24). In the work presented in this paper, we have further characterized the reactions leading to methylation of CODH and also the subsequent demethylation reaction to form an acetyl-CODH intermediate, followed by the synthesis of acetate. We propose that a nucleophilic metal center on CODH is the active site which accepts the methyl group from the methylated C/Fe-SP.

**Experimental Procedures**

**Growth of C. thermoaceticum**—C. thermoaceticum was grown in 20-liter carboys at 55 °C under CO$_2$ as described by Ljungdahl andAndreassen (25).

**Purification of Proteins**—The C/Fe-SP (6), CODH (11), and ferredoxin (26) were purified in a Coy anaerobic chamber maintained at 16 °C. As judged by SDS-gel electrophoresis, the purity of the C/Fe-SP was ~98% and of CODH was generally ~96% and is specified below for individual experiments. The specific activity of the CODH used in these experiments was 210 units/mg in the CO oxidation reaction. The amount of disulfide reductase (or any other protein with the subunit molecular weight of this protein) in the purified C/Fe-SP was determined by SDS-gel electrophoresis, i.e. less than 1%, as judged by SDS-gel electrophoresis. The molar concentration of CODH was expressed as the $\alpha$ form (M, $\sim$149,000) throughout the paper. Ramer et al. (22) recently suggested that CODH actually was a three-subunit enzyme, the third subunit most likely being a disulfide reductase, purified and characterized earlier by Pezacka and Wood (23). In the CODH used in all the reactions described here, we were unable to detect, by SDS-gel electrophoresis, any protein with a molecular weight equivalent to that of the disulfide reductase after our final step in purification of CODH. The two-subunit $\alpha$ form of CODH is sufficient for all the reactions described here, which includes synthesis of acetyl-CoA, the CO:acetyl-CoA exchange, methylation of the C/Fe-SP, methylation of CODH, and the two methyl transfer reactions.

**CO/Acetyl-CoA Exchange Activity Catalyzed by CODH**—This reaction was performed as described (11).

**Assay for Acetyl-CoA Synthesis from Methyl Iodide, CO, and CoA by the C/Fe-SP and CODH**—The amount of methyl iodide added is not equivalent to the amount in solution. Due to the high vapor pressure of methyl iodide (400 mm at 25 °C) only ~50% of methyl iodide added was found in the reaction solution of the sealed vial under the experimental conditions. Thus, whenever the concentrations of methyl iodide in solution are stated, the values are one-half of the amount added. The reaction was performed anaerobically at room temperature ($\sim$25 °C) in a capped vial, and in a total volume of 100 μl containing CODH (0.14 mg), C/Fe-SP (0.05 mg), ferredoxin (15 μg), methyl iodide (0.75 μmol), CoA (0.5 μmol), diethiothreitol (DTT) (100 nmol), and Tris maleate buffer (5 μmol), pH 7.3. When radioactive methyl iodide was used, the specific activity of the $^{13}$CH$_3$-I was 3800 dpm/nmol. After flushing with CO for 5 min, the reaction vial was wrapped with aluminum foil and methyl iodide (7.5 μl) added to start the reaction. At various time points, 5–10 μl of the reaction mixture was removed and acetyl-CoA was quantitated by one of the following two methods.

**A Coupled Enzyme Assay for Acetyl-CoA**—Acetyl-CoA was quantitated essentially as described by Williamson and Corkeby (27) and Schulman and Wood (28). The assay mixture (1 ml) contained malate (4 μmol), NAD (1.2 μmol), NADH (0.1 μmol), citrate synthetase (0.6 units), malate dehydrogenase (2 units), and Tris-Cl buffer (25 μmol), pH 7.5. After a stable absorbance reading at 340 nm was reached, 10 μl of reaction mixture containing 10–30 nmol of acetyl-CoA was added. The increase in absorption at 340 nm (i.e. the formation of NADH) was proportional to the amount of acetyl-CoA present in the 10-μl reaction mixture.

**HPLC Analysis of Acetyl-CoA**—Acetyl-CoA was chromatographed essentially as described by Corkeby et al. (29) on a Waters μBondapak C$_18$ (30 μm x 30 cm) HPLC column with a Waters system including a Waters model 501 pump and a 2454 nm detector. The reaction mixture (5–50 μl) was directly injected into the system and eluted isocratically with 15% methanol and 0.1 M phosphate buffer, pH 5.5, at a flow rate of 1 ml/min. Retention times for protein, CoA, and acetyl-CoA were found to be 3–4, 6–8, and 16–18 minutes, respectively. Quantitative analysis of acetyl-CoA was achieved by (a) determination of the specific activity of the CODH fraction of the reaction mixture of the HPLC column when radiolabeled substrate was used and (b) comparison of the peak height of acetyl-CoA eluted from the reaction mixture with that of the standard acetyl-CoA.

**A Coupled Enzyme Assay for Acetate**—Acetate was quantitated by addition of acetyl-CoA synthetase (0.12 unit), CoA (0.2 μmol), ATP (0.2 μmol), and MgCl$_2$ (10 μmol) into the enzyme solution for the spectrophotometric assay of acetyl-CoA as described above. Acetate was totally converted to acetyl-CoA by acetyl-CoA synthetase and further to citrate with concomitant reduction of NAD catalyzed by citrate synthase and malate dehydrogenase. The amount of acetate in the reaction mixture added was determined by following the reduction of NAD and subtracting the reading due to the acetyl-CoA present in the reaction mixture. Because of a high Km value (0.51 mM) of acetyl-CoA synthetase for acetate and low amounts of acetate added to the sample mixture (~5 nmol/10 μl), the rate of the reaction was very slow (~20 times slower than the acetyl-CoA assay).

**Method for Quantifying the C/Fe-SP Activity**—C/Fe-SP activity was determined essentially as described above for determining the rate of acetyl-CoA synthesis except that less (10–40 μg) C/Fe-SP was used and reaction time was 60 min.

**Methylation of the C/Fe-SP with Methyl Iodide**—The formation of methylated C/Fe-SP was accomplished in an electrochemical titrator used for redox titration of proteins as described before (7, 30). The reaction solution contained C/Fe-SP (~10 μg) in 10 mM sodium trimethoxide, 0.2% dipyrindil bromide (30 μg), N,N$'$_trimethylecine, 2,2' dipyrindil bromide (TRIQUAT) (30 μg), and 0.05 M Tris maleate, 0.2 M KCl, pH 7.3, in a final volume of ~600 μl. After poised the potential of the solution to ~500 mV versus NHE the reaction was initiated by adding 10 μl of 0.2 M radioactive methyl iodide (11°C) 1700 dpm/nmol or [H] 6460 dpm/nmol. Waa gasvag syringe and incubated at ~900 mV for 7 min. The titrator was then transferred into an anaerobic chamber and the protein was completely separated from free methyl iodide and the dyes within a few (3–4) min by a spun column technique (31) with two Sephadex G-50 columns (bed volume = 3 ml) equilibrated with 0.1 M Tris maleate, pH 7.0. Radioactivity and protein content in the fraction were determined, from which the ratio of bound methyl group to C/Fe-SP was calculated. The ratio of the methylation was also indirectly derived from the comparison of the signal intensity of the EPR spectrum of the methylated C/Fe-SP with that of the protein in the Co$^{3+}$ state. Co$^{2+}$ is EPR-active, and methyl-Co$^{3+}$ is EPR-silent. Because the EPR signals for Co$^{3+}$ and Co$^{2+}$ are not well resolved and in a total volume of ~420 ± 200 mV is equivalent to the amount of methylation of the C/Fe-SP. Results from the two methods were consistent.

**Methylation of CODH with Methyl Iodide at Ambient or Low Potential**—The reaction mixture for the methylation at ambient potentials (~0 mV) contained CODH (4 nmol), [11°C]methyl iodide (0.1–0.4 μmol) (1700 or 3800 dpm/nmol), and 0.05 M Tris maleate, pH 7.3, in a final volume of 100 μl. The temperature was 22 °C. After the reaction, free methyl iodide was separated from protein by the
spun column technique as described above. Reaction conditions for the methylation of CODH at low potentials were the same as described for the methylation of the C/Fe-SP. For analytical experiments at a potential $\sim$300 mV, $\sim$5 nmol of CODH and benzyl viologen (30 mg) instead of the two more negative dyes (TRIQUAT and P,N,N'-trimethylene-2,2'-dipyridinium bromide) were used. For the large scale preparation of methylated CODH, from 5 to 15 mg of CODH was used and a potential of $\sim$550 mV applied. The methylated CODH was completely active when stored at 16°C in an anaerobic chamber and used within 10 days.

**Methyl Transfer from CH$_3$/C/Fe-SP to CODH at Ambient or Low Potentials**—The basic reaction mixture contained approximately equal amounts (5-25 nmol) of [33$^3$]CH$_3$/C/Fe-SP (1700 dpm/nmol) and CODH in 0.05 M Tris maleate, 0.2 M KCl buffer, pH 7.3, in a final volume of $\sim$500 l. DTT was removed from the enzyme solution prior to the experiment by the spun column technique. Methyl transfer reactions performed at ambient potentials were performed in an anaerobic chamber at 16°C, while those at low potentials were carried out at room temperature ($\sim$22°C) in the electrochemical titrator as described above. At the end of the reaction, the reaction mixture was applied to a Phenyl-Sepharose column (bed volume = 1.5 ml) equilibrated with 0.35 M (NH$_4$)$_2$SO$_4$ in 0.1 M phosphate buffer, pH 7.0. The C/Fe-SP was eluted with 5 ml of 0.3 M followed by 3 ml of 0.25 M (NH$_4$)$_2$SO$_4$ in the same buffer. CODH was then eluted with 5 ml of 0.1 M phosphate buffer, pH 7.0. Radioactivity and protein in the CODH and C/Fe-SP fractions were analyzed both before and after concentration of the fractions. After chromatography, the C/Fe-SP and CODH fractions were more than 95% and $\sim$90% pure, respectively, as judged by polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) and by immunoblotting with purified antibodies (5).

**Analytical and Spectroscopic Techniques**—Protein was determined by the Rose-Bengal dye-binding assay (32) with lysozyme as standard. SDS-PAGE was performed as described (33). UV-visible spectra were obtained and manipulated using the PECUV software with a Perkin-Elmer Lambda 4C spectrophotometer. EPR spectra collected at liquid nitrogen temperature were obtained with a Varian model EI 115 spectrometer as described earlier (7).

**RESULTS**

1. **Acetyl-CoA Synthesis from Methyl Iodide, CO, and CoA by CODH and the C/Fe-SP**—This reaction is summarized by Equation 2.

\[
\text{CH}_3 + \text{CO} + \text{CoA} \rightarrow \text{CH}_3-\text{CO}-\text{SCoA} + \text{HI} \tag{2}
\]

The synthesis of acetyl-CoA using methyl iodide as methyl donor is performed at 22°C due to the volatility of methyl iodide. Even at this temperature, we required a correction for the concentration of methyl iodide in solution. Acetyl-CoA was synthesized by CODH and the C/Fe-SP in the presence of methyl iodide, CO, and CoA, as was reported earlier (20, 24). The synthesis was linear with time for 30 min to 1 h. Quantitation of the amount of acetyl-CoA formed in the reaction mixture by the coupled enzyme assay and by the HPLC method gave approximately equivalent results. There was no detectable acetyl-CoA formed in the absence of CODH or the C/Fe-SP and methyl iodide, CO, and CoA were all essential. DTT or other reducing agents were not required in the synthesis. Acetate was formed as an additional product accounting for $\sim$20% of the total products and approximately the same amount of acetate was formed both in the absence or presence of CoA. Acetyl-CoA synthesis from methyl iodide was stimulated by ferredoxin with a maximum of $\sim$40% stimulation occurring with 0.8 l. of ferredoxin (1 nmol of ferredoxin/15 nmol of CODH). Ferredoxin also stimulated the synthesis from CO, CoA, and CH$_3$-H$_2$folate (11).

The $K_m$ value for CoA was determined from a Lineweaver-Burk plot to be 4.3 mM, which is consistent with the value for CoA (4.7 mM) reported before (34) with CH$_3$-H$_2$folate as the methyl donor, suggesting that the nonphysiological substrate, methyl iodide, does not alter the kinetic properties of the enzyme system. Inhibition of acetyl-CoA synthesis was observed when concentrations of CoA higher than 10 mM were used; 50% inhibition occurred at $\sim$15 mM. A $K_s$ value of 14.7 mM was determined for methyl iodide. Concentrations of methyl iodide higher than 10 mM were inhibitory to acetyl-CoA synthesis with 50% inhibition occurring at 17.5 mM; therefore, 5-7.5 mM methyl iodide was used in the acetyl-CoA synthesis reactions. The pH dependence of the synthesis followed a bell-shaped curve with an optimum pH between pH 7.2 and 7.5 and 50% of the activity seen at pH values of 5.2 and 9.3. At higher pH values, Pesacka and Wood (24) found that nonspecific methylation of CODH occurred and less than stoichiometric amounts of the bound methyl groups could be converted to acetyl-CoA in the presence of CO and CoA.

A specific activity of 15 nmol of acetyl-CoA formed min$^{-1}$ mg$^{-1}$ CODH was obtained. By varying the amount of the C/Fe-SP from 20 to 100 l. under the same experimental conditions, we obtained a maximal velocity of 44 nmol of acetyl-CoA formed min$^{-1}$ (mg CODH)$^{-1}$ from a double reciprocal plot, which would be equivalent to $\sim$350 nmol min$^{-1}$ (mg CODH)$^{-1}$ at 55°C assuming a rate doubling for each 10°C increase in temperature. In comparison, rates of $\sim$150 nmol min$^{-1}$ (mg CODH)$^{-1}$ at 55°C have been measured for acetyl-CoA synthesis from CO, CoA, and CH$_3$-H$_2$folate (23).

2. **Determination of Activity of the C/Fe-SP and the Essential Role of the C/Fe-SP in Acetyl-CoA Synthesis from Methyl Iodide, CO, and CoA**—The assay of acetyl-CoA synthesis described above could be used to quantitatively measure the activity of the C/Fe-SP by adding enough CODH that the C/Fe-SP was rate limiting. When the amount of C/Fe-SP was in the range of 10 40 l. the rate was proportional to the concentration of C/Fe-SP and the same specific activity (in terms of C/Fe-SP) was obtained (Fig. 1). At higher levels of C/Fe-SP, the reaction was limiting in CODH and the specific activity decreased.

Although no detectable acetyl-CoA was formed without addition of either the C/Fe-SP or CODH under the experimental conditions described above, synthesis of acetyl-CoA was observed with the purified fraction of CODH alone at concentrations higher than 0.35 mg/100 l. of reaction mixture. We investigated whether this was due to remaining C/Fe-SP present in the purified CODH sample or if the methyl binding site of CODH actually was being directly methylated.

![Fig. 1. Dependence of acetyl-CoA synthesis on the C/Fe-SP.](http://www.jbc.org/) The reaction was performed as described under "Experimental Procedures" except that the amount of the C/Fe-SP was varied. Acetyl-CoA was determined by the coupled enzyme assay. Acetyl-CoA synthesis is expressed by total acetyl-CoA formed in the reaction mixture (O) and specific activity (A, nmol of acetyl-CoA formed min$^{-1}$ (mg C/Fe-SP)$^{-1}$).
by methyl iodide as had been suggested earlier by Pezacka and Wood (24). First, we varied the amount of C/Fe-SP and determined the amount of acetyl-CoA formed (Fig. 2). Extrapolation of the plots of the rates of acetyl-CoA synthesis against the amount of C/Fe-SP added gave intercepts of 12.5 and 8.2 µg on the abscissa for the reaction containing 0.36 and 0.216 mg of CODH, respectively. From these values the C/Fe-SP present in the CODH sample were calculated to be ~3.5% (mg/mg). Second, by SDS-PAGE followed by staining with Coomassie Blue or by immunoblotting with purified antibody against the C/Fe-SP, similar amounts of the C/Fe-SP were found in several batches of the purified CODH. Third, from the acetyl-CoA synthesis activity (37 nmol/h) of 0.36 mg of CODH alone and its C/Fe-SP content (12 µg), a specific activity of 49 nmol of acetyl-CoA formed min⁻¹ (mg C/Fe-SP)⁻¹ was calculated, which is very close to the values shown in Fig. 1. Fourth, we obtained a highly purified CODH sample (but still containing 0.8% of the C/Fe-SP) after exhaustive washing of CODH bound to phenyl-Sepharose to attempt the total separation of C/Fe-SP from CODH. This sample showed no “C/Fe-SP-independent” activity except at very high levels of CODH (1 mg/100 µl).

3. Methylation of the C/Fe-SP with Methyl Iodide—This reaction is summarized in Equation 3. When poised electrochemically at ~−500 mV as described under “Experimental Procedures,” the C/Fe-SP was rapidly methylated with methyl iodide as methyl donor. Usually a ratio of 0.9 to 1.0 methyl bound to C/Fe-SP was obtained and the UV-visible spectrum of the methylated C/Fe-SP was identical to that of the protein methylated by CHJ₂H₃folate (6). The methylated C/Fe-SP was fully active in acetyl-CoA synthesis when assayed by the method described above. When the C/Fe-SP was methylated using higher concentration of methyl iodide and/or longer (over 30 min) incubation times, significant inactivation was observed.

Although the midpoint potential for Co²⁺/Co⁺ couple of the C/Fe-SP is ~−904 mV (i), substantial methylation occurred at ~−350 mV (Fig. 3). We studied a series of methylation reaction at various potentials and at short reaction times (6 min) and found that the extent of methylation was redox potential-dependent (Fig. 3). From the results of Fig. 3, a rate of 3.6 nmol of C/Fe-SP methylated min⁻¹ (mg C/Fe-SP)⁻¹ was calculated for the methylation reaction at ~−547 mV. The methylation reaction is not at equilibrium since increasing the reaction time increased the methylation, e.g. ~80% methylation of C/Fe-SP was obtained when the reaction time was extended from 6 to 12 min for the reaction at ~−400 mV. No significant methylation of the protein was found at redox potentials higher than ~300 mV during this time period.

4. Methylation of CODH with Methyl Iodide at Various Redox Potentials and Stimulation of the Methylation by the C/Fe-SP at Low Potentials—The measured potential of a solution of CODH dissolved in anaerobic Tris maleate buffer, pH 7.3, free of reducing agents was ~−0 mV. In a CODH sample containing a 3.5% contaminant of C/Fe-SP (~17 nmol of CODH)/(nmol C/Fe-SP)), methylation by methyl iodide was seemingly independent of C/Fe-SP when the reaction was incubated at ambient potential, the standard conditions used by Pezacka and Wood (24) (Table I). Even after a 1-h incubation, the methylation was still incomplete.

![Figure 2. Effect of the C/Fe-SP on the activity of acetyl-CoA synthesis at high concentration of CODH. The assay was done as described except that 0.36 mg (C) or 0.216 mg (L) of CODH and 0.1 M phosphate buffer, pH 7 were used and ferredoxin omitted from the reaction mixture. The time of reaction was 60 min. The coupled enzyme assay was used for acetyl-CoA analysis.](http://www.jbc.org/)

![Figure 3. Effect of redox potential on the methylation of the C/Fe-SP or CODH with CHJ. The methylation of the C/Fe-SP (L) was performed as described under “Experimental Procedures” except that 40 nmol of the C/Fe-SP, 0.3 µmol of methyl iodide and two additional mediators (40 µg each), methyl viologen and benzyl viologen, were used in the reaction mixture. Methyl iodide (in 3 µl) was added to initiate the reaction after a desired stable potential had been reached. Reaction time was 6 min except the reaction at ~−547 mV, which was 3 min. For the methylation of CODH (Δ), the reaction mixture contained highly purified CODH, 4.6 nmol; C/Fe-SP, 1 nmol and methyl iodide, 0.25 amol in a final volume of 500 µl. Reaction time was 6 min. For other details see the legend to Table II.](http://www.jbc.org/)
methylation at low potentials was complete within 10 min (Table I), yielding a ratio of ~1 (0.9–1.2) methyl group bound per mol CODH, but still appeared to be independent of C/Fe-SP. With a highly purified CODH sample containing ~0.8% C/Fe-SP (76 nmol of CODH)/(nmol C/Fe-SP) (Table II), at all potentials studied, even as low as ~550 mV, the rate of methylation of CODH was stimulated by addition of the C/Fe-SP and quantitative yields of methylated CODH were obtained. Thus, the methylation of CODH, as was shown for acetyl-CoA synthesis (above), is strictly dependent on the C/Fe-SP to act as a methyl carrier. Methylated CODH generated by the reactions at low potentials was fully active in acetyl-CoA synthesis.

Since a cysteinyl thiol had been proposed to be the methyl acceptor site on CODH, we determined the effect of p-hydroxymercuribenzoate (pHMB) on methylation of CODH (Table I). When this reaction was studied earlier (24), 100% inhibition of methylation of CODH was observed at 0.05 mM concentration of pHMB. With our low potential system, we observed little inhibition even at final concentrations of 0.66 mM (molar ratio of pHMB/CODH of 50/1). We determined that the pHMB inhibition was not reversed during the experiment since 18–20 nmol of Hg²⁺/S/nmol CODH were bound to CODH both before and after poising the potential and performing the methylation. DTT was found to slowly demethylate CODH with 50 and 85% of the bound methyl groups lost on incubation with 0.5 mM DTT for 6 and 15 h, respectively. When the methylated CODH which had been treated previously with pHMB was incubated with CO and CoA, the methyl group was converted to acetyl-CoA. These results of the pHMB-treated enzyme indicate that thiol groups which are accessible to pHMB are not on either the

| Table II | Stimulation of C/Fe-SP by pHMB of the Methylation of CODH with CH₃⁻ at Poised Low Potential |
|---|---|
| The methylation was performed as described above under “Experimental Procedures” with 0.35 μmol of CH₃⁻ (1700 dpm/nmol). The redox potential was ~550 mV (±20) and incubation time was 8 min. Highly purified CODH (containing 0.8% (mg/mg) C/Fe-SP) were used in Expn. 1 and 2. |  |
| Expt. | CODH | C/Fe-SP | CH₃⁻ bound to CODH |
|---|---|---|---|
| 1 | 4 | 0.03 | 0.36 |
| 2 | 4 | 0.13 | 0.88 |
| 3 | 4 | 1.03 | 0.77⁹ |

* The value was corrected for methylated C/Fe-SP (nmol) by assuming that C/Fe-SP added was 100% methylated.

The pathway of methylation of CODH or of acetyl-CoA formation from methylated CODH.

The dependence of the rate of methylation of CODH on redox potential was studied. Within 6 min, ~50% of CODH was methylated when the poised potential was ~390 mV (Fig. 3). This methylation reaction, like the methylation of the C/Fe-SP, was irreversible since complete methylation of CODH occurred at potentials lower than ~300 mV after prolonged incubation. These combined results indicate that the methyl acceptor site(s) on CODH has to be reductively activated before the methylation can occur.

We also studied the effect of methylation of CODH on the activity of the exchange reaction between CO and acetyl-CoA and found that CODH which has been methylated with methyl iodide at ~500 mV for 10 min (with 100% methylation) showed the same exchange activity (150 nmol of CO exchanged min⁻¹ mg⁻¹) as that of the non-methylated CODH. However, methylation of CODH with methyl iodide at ambient potential for 1 h as in Ref. 24 (with ~85% methylation) resulted in ~40% decrease in the exchange activity. Pezacka and Wood (24) concluded that there was only a single methyl binding site by showing that methylation of CODH inhibited this exchange reaction. It is expected that methylation of CODH would not inhibit the CO/acetyl-CoA exchange since methylated CODH is quantitatively converted to acetate in the presence of CO. This hydrolysis would be expected to regenerate active CODH.

5. Transfer of the Methyl Group from the Methylated C/Fe-SP to CODH—This reaction is summarized by Equation 4.

\[
\text{CH}_3\text{-[Co}^3\text{+]} \rightarrow \text{C/Fe-SP} + \text{CODH} \rightarrow \text{CH}_3\text{-CODH} + [\text{Co}^3\text{+}] + \text{C/Fe-SP}
\]

No substantial (less than 5%) transfer of the methyl group from [¹⁴C]CH₃-C/Fe-SP to CODH was observed when the two proteins were incubated at ambient potential in the absence or presence of ferredoxin and/or DTT (Table III), even after overnight incubation. However, significant methyl transfer did occur within 15 min at potentials lower than ~300 mV (Table III), clearly indicating that this reaction involves redox chemistry. There was more methyl transfer at ~300 mV than at even lower potentials. The methyl transfer from methylated C/Fe-SP to CODH never exceeded 50%, even after longer incubation times (Table III).

That a reduction is required to methylate CODH by the methylated C/Fe-SP is not inconsistent with the results of others (24), where a larger amount of CODH was methylated by CH₃-C/Fe-SP in the presence of reduced ferredoxin than with native ferredoxin at ambient potential. There is a minor

| Table III | Effect of redox potential on the methyl transfer from methylated C/Fe-SP to CODH |
|---|---|
| The reaction was performed as described under “Experimental Procedures.” 25 nmol each of CODH and C/Fe-SP was used in Experiments 1, 2, 3, and 8, and about 6 nmol each of CODH and C/Fe-SP was used in Experiments 4–7. |  |
| Potential | DTT | Ferredoxin | Reaction time | CH₃⁻ bound to | Total methyl bound to |
|---|---|---|---|---|---|
| mV | mM | mM | min | C/Fe-SP | CODH | C/Fe-SP | CODH | % |
| 1. | -0 | 0.3 | 100 | 120 | 0.85 | 0.05 | 94 | 5.5 |
| 2. | -0 | 1 | 30 | 120 | 0.91 | 0.05 | 96 | 5 |
| 3. | -0 | - | - | - | 0.56 | 0.34 | 65 | 35 |
| 4. | -300 (±20) | - | - | - | 0.54 | 0.4 | 55 | 45 |
| 5. | -315 (±10) | - | - | - | 0.7 | 0.2 | 76 | 24 |
| 6. | -416 (±30) | - | - | - | 0.8 | 0.14 | 83 | 16 |
| 7. | -490 (±20) | - | - | - | 0.73 | 0.22 | 75 | 24 |
| 8. | -555 (±30) | - | - | - |  

* = not added.
discrepancy in that we were unable to methylate CODH with native ferredoxin at a measured potential of ~0 mV. We do not know the reason for the discrepancy, but there were two major experimental differences between the two studies: (a) the ferredoxin we used was entirely in the oxidized form at low potentials (Table IV); and (b) the methylated C/Fe-SP (24) was not pure since the reaction mixture contained CODH, CO, ferredoxin, and methyltransferase and was subsequently chromatographed on Sephadex G-25 after the methylation. Thus, other proteins were present during the methylation of CODH.

6. Methyl Exchange Reaction between Methylated CODH and Methylated C/Fe-SP—This reaction is summarized in Equation 5.

\[
\text{C}^3\text{H}_3\text{--C/Fe-SP} + {^4}\text{CH}_3\text{--CODH} \rightleftharpoons \text{C}^3\text{H}_3\text{--CODH} + {^4}\text{CH}_3\text{--C/Fe-SP} \tag{5}
\]

It had never previously been reported or postulated. Even the occurrence of this reaction has wide implications in the mechanism of methyl transfer. Approximately 30-40% of the methyl groups were exchanged after incubation of [4C]CH3-CODH with [3H]CH3-C/Fe-SP at either ambient (~0 mV) or low potentials (Table IV). The calculated rate of this interprotein methyl exchange reaction was 0.08 nmol min⁻¹ (mg CODH)⁻¹. This was most likely not the maximal rate; however, our present methodology requires separation of the two proteins after the reaction, and 5 min is as fast as we were able to perform the reaction and the separation. After 5 min the reaction was at equilibrium (Table IV). Interestingly, the rate of methyl exchange reaction between the two methylated proteins is independent of the redox potential of the solution.

7. Conversion of CH3-CODH into [2-14C]Acetyl-CoA—This reaction is summarized in Equation 6.

\[
\text{CH}_3\text{--CODH} + \text{CO} + \text{CoASH} \rightarrow \text{CH}_3\text{--CO-SCoA} + \text{CODH} \tag{6}
\]

and evaluates the competence of the methyl group of the methyl-CODH intermediate to form acetyl-CoA. With 14C-methylated CODH as methyl donor, [2-14C]acetyl-CoA was synthesized from CO and CoA (Table V). During the reaction, ~50% of the methyl groups were converted to acetyl-CoA, ~20% of the methyl group remained bound to CODH, and another 20% was converted to acetate. The latter was detected as a radiolabeled peak by chromatography on HPLC immediately before the protein with a retention time of 2.5-3 min and coeluted with standard acetate. Thus the conversion of CODH-bound methyl groups to acetyl-CoA and acetate is higher than that (53%) reported earlier (24). In addition, ~20% of the methyl group remained bound to CODH. In a separate experiment, after incubation under the same conditions as in Table V for 20 or 40 min, ~15% of the methyl group remained on CODH, implying that the reaction had probably reached equilibrium within 20 min. In the presence of CO only, over 50% of the methyl groups were converted to acetyl-CoA, and another 20% was converted to acetate. The latter was detected as a radiolabeled peak by chromatography on HPLC immediately before the protein with a retention time of 2.5-3 min and coeluted with standard acetate. Thus the conversion of CODH-bound methyl groups to acetyl-CoA and acetate is higher than that (53%) reported earlier (24). In addition, ~20% of the methyl group remained bound to CODH. In a separate experiment, after incubation under the same conditions as in Table V for 20 or 40 min, ~15% of the methyl group remained on CODH, implying that the reaction had probably reached equilibrium within 20 min. In the presence of CO only, over 50% of the methyl groups were converted to acetate. Addition of [3H]CH3-C/Fe-SP to the reaction mixture containing CODH, CO, and CoA also formed H-labeled acetyl-CoA in a reaction strongly dependent on CODH.

8. Methyl Exchange Reaction between Methylated CODH
Acyetyl-CoA was formed after incubation of CODH with acetyl-CoA, clearly indicating that the methyl group bound to CODH is exchangeable with the methyl group on acetyl-CoA (Table VI). This exchange reaction occurred to a significant extent only at low redox potentials. However, as was found for the methylation of CODH (above), substantial exchange was observed at ~400 mV within a few minutes incubation. Overall 90% of the [14C]methyl group originally bound to CODH exchanged to acetyl-CoA during exchange at low potentials in the presence of much greater than a molar excess of acetyl-CoA (Table VI). An exchange rate of 3.7 nmol of methyl exchanged min⁻¹ (mg CODH)⁻¹ could be calculated from the data at ~500 mV. This value probably does not reflect a maximum rate since it may not be an initial velocity measurement and increasing the amount of CH₂-CODH (a substrate of the reaction) in the reaction mixture may enhance this rate. The maximum rate observed at ~0 mV was 130-fold lower. We also determined that addition of nonmethylated CODH or nonmethylated C/Fe-SP had no detectable effect on the rate (1.5-min reaction time) of this exchange reaction. Furthermore, addition of the nonmethylated proteins to the reaction did not effect the final amount of exchange; i.e. 100% of the label from the methyl group of CH₂-CODH still exchanged with the methyl of acetyl-CoA.

**DISCUSSION**

Several reactions studied here require reduction of a site on CODH or the C/Fe-SP to observe either maximal activity or, in some cases, to observe even detectable activity. We do not yet know the actual oxidation states of the metal centers on CODH involved in catalysis or substrate binding, therefore it is premature to attempt to assign oxidation states to the metal centers on CODH involved in catalysis or substrate binding, therefore it is premature to assign oxidation states to the metal sites. The reduced Fe⁺ state of globins is required for oxygen binding, and reduction of the heme sites of sulfite reductase (35), cytochrome P-450 (36), and nitrate reductase (37) increases the affinity of these enzymes for their substrate. Methyl-CoM reductase undergoes reductive activation, apparently to reduce the Ni²⁺ center to the active +1 state that can bind the methyl group of methyl-CoM (38). In addition, when purified aerobically, the Ni/Fe-S hydrogenase requires reductive activation (39) by a one-electron reduction of a redox center with an Eₐ of ~310 mV (40).

Based on our results, Fig. 4 is a postulated scheme for the pathway of acetyl-CoA synthesis. The methyl transfer reactions involved in acetyl-CoA synthesis can be broken down into three steps: methylation of the C/Fe-SP, methyl transfer to CODH, and methyl migration to form an acetyl-enzyme intermediate. Here we have studied the sequence of methyl transfer reactions in the pathway with methyl iodide as the methyl donor since this simpler system does not require methyltransferase, an enzyme purified earlier (41). All the reactions performed here were with an enzyme that lacks any protein with a subunit molecular weight of the CODH disulfide reductase. This is important since it was recently suggested that the active form of CODH contains three subunits (22). Thus, our results indicate that the active form of CODH is a two-subunit enzyme characterized earlier (42).

**Acetyl-CoA Synthesis from Methyl Iodide, CO, and CoA by CODH and the C/Fe-SP**—We found, as before (23), that acetate and acetyl-CoA are both formed during the synthesis from a methyl donor, CO, and CoA in reactions that require both the C/Fe-SP and CODH. Our reaction conditions are highly simplified in that methyl iodide, CO, CoA, C/Fe-SP, and CODH are the only reactants required. Disulfide reduc-

**TABLE VI**

| Potential (mV) | Reaction time (min) | [2-¹⁴C]Acetyl-CoA formed (nmol) | [¹⁴C]CH₃ still bound to CODH | Methyl exchanged (%) |
|---------------|---------------------|-------------------------------|---------------------------|---------------------|
| ~0            | 5                   | 0.05                          | ND                        | 10                  |
| ~0            | 10                  | 0.11                          | ND                        | 15                  |
| ~0            | 60                  | 0.25                          | ND                        | 17                  |
| ~0            | 20                  | 1.8                           | ND                        | 10                  |
| ~0            | 60                  | 1.73                          | ND                        | 15                  |
| ~350 (±10)    | 5                   | 1.8                           | ND                        | 72                  |
| ~430 (±10)    | 10                  | 2.15                          | ND                        | 65                  |
| ~450 (±10)    | 20                  | 0.56                          | ND                        | 70                  |
| ~500 (±5)     | 1                   | 1.38                          | ND                        | 55                  |
| ~570 (±20)    | 40                  | 2.3                           | ND                        | 92                  |

The reaction mixture in 300 μl contained 2.5 nmol of [¹⁴C]CH₃-CODH (100% methylated, 3800 dpm/nmol); acetyl-CoA, 0.3 nmol and 0.1 M Tris maleate, 0.2 M KCl buffer. pH 7.3. The reaction was initiated by adding acetyl-CoA. An electrochemical titrator was used for reactions at low poised potentials as described under "Experimental Procedures." Acetyl-CoA present in about 50 mL of reaction mixture was isolated by HPLC as described under "Experimental Procedures," and radioactivity in the isolated acetyl-CoA fraction was determined. Percent methyl exchanged was calculated from determination of the amount of [¹⁴C]acetyl-CoA formed.

Reactions conditions were the same as described above, except that 1.96 nmol of [¹⁴C]CH₃-CODH was used. The spin column technique was used to separate the protein from acetyl-CoA at the end of reaction and ¹⁴C remaining in the protein fraction was determined. The percent methyl exchanged was calculated from the amount of the [¹⁴C]methyl group dissociated from CODH.

**FIG. 4.** A proposed scheme for the synthesis of acetyl-CoA emphasizing the methyl transfer reactions involving CODH and the corrinoid/iron-sulfur protein ([Co]prot).
Methyl Transfers in Acetyl-CoA Synthesis

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tase and/or reducing agents are not required. CoA has no
effect on the rate or the total amount of acetate formed when
the methyl and carbonyl donors are in excess. Formation of
acetate is strong evidence for an enzyme-bound acetyl inter-
mediate which we propose is hydrolyzed to acetate.

Formation of methylated C/Fe-SP appears to occur slower
than transfer of the methyl from methylated C/Fe-SP to
CODH (see Fig. 3). Formation of methyl-cob(I)amide from
methyl iodide and Co²⁺ apparently is irreversible since at
longer times at these positive potentials the C/Fe-SP becomes
completely methylated. In addition, the Co center is methylat-
ed at a potential ~200 mV more positive than its midpoint
potential. These results are highly significant with respect to
methylation of the C/Fe-SP under physiological conditions.

One can calculate, based on the midpoint potential (~504
mV; Ref. 6) of the Co²⁺+/+ couple of the C/Fe-SP, that in the
absence of methyl iodide 0.27% of the C/Fe-SP would be
expected to be reduced from Co²⁺ to the active methyl-ac-
cepting Co²⁺ form at ~350 mV; however, 30% of the C/Fe-SP
is methylated within 6 min. Thus, under physiological condi-
tions in which the reduced Co²⁺ species is immediately trapped
by the methylation, the cell does not need to deliver electrons
at very low potentials to generate methyl-cob(I)amide on
the C/Fe-SP. Recently, Banerjee et al. (9) have studied the
methylation of methionine synthase in which the Co²⁺+/+ redox
system is coupled to the irreversible methylation by S-adeno-
syl-L-methionine and found that the apparent midpoint
potential of the redox couple was increased by several hundred
mV. It will be interesting to determine the dependence on
redox potential of methylation of the C/Fe-SP with CH₃-
H₂folate as methyl donor.

Methylation of CODH at Controlled Redox Potentials—
These results are interpreted according to Steps 2 and 3 of
Fig. 4. The C/Fe-SP is required for acetyl-CoA synthesis from
CH₃-H₂folate (6, 43) and, thus, for methylation of CODH by
CH₃-H₂folate. Pezacka and Wood (24) proposed that the
active site of CODH is directly methylated by methyl iodide
without the mediation of the C/Fe-SP. Here, we have shown
that the C/Fe-SP is required for both methylation of CODH
and acetyl-CoA synthesis with methyl iodide as methyl donor.

Very little C/Fe-SP is required for this methylation; e.g., less
than a 1% contaminant of C/Fe-SP in CODH preparations is
enough to catalyze significant methylation of CODH (Table
II).

There is a greater than 10-fold acceleration of the rate of
methylation of CODH by methyl iodide at low redox poten-
tials. That the rates of methylation of CODH and of the C/
Fe-SP are nearly identical (Fig. 3) suggests that the rate-
limiting step in the methylation of CODH is methylation of
the C/Fe-SP. Then, after formation of the methylated C/Fe-
SP, this group is transferred to CODH. Based on this exper-
iment alone, it is not clear whether CODH or the C/Fe-SP or
both proteins require reduction since we cannot distinguish
two separate reductions. Clearly the C/Fe-SP must be reduced
from the Co²⁺ to Co⁺ state to activate it for methylation (6,
7). That the methylation of CODH from the methylated C/
Fe-SP is redox potential-sensitive is strong evidence that
redox chemistry is not only required in the methylation of the
Co center of the C/Fe-SP, but also in reductive activation of
a nucleophile site on CODH. Methylation of this site on
CODH from the methylated C/Fe-SP never reaches a full
equivalent/mol of CODH; however, with methyl iodide as
methyl donor, CODH is fully methylated. In addition, methy-
lation via methylated C/Fe-SP exhibits a peculiar depend-
ence on redox potential. It requires a potential of ~350 mV,
however at even lower potentials, the reaction appears to be
less complete. Our explanation for these findings involves
reactions 1a, 1b, 2, and 3 (Fig. 4). When excess methyl iodide
is the methyl donor, both the C/Fe-SP and CODH can be
stoichiometrically methylated; however, with approximately
equimolar amounts of methyl-C/Fe-SP and CODH, the extent
of methylation of CODH is determined by the equilibrium
ratio of [methyl-C/Fe-SP] [CODH]/[C/Fe-SP] [methyl-
CODH] as well as the redox equilibria between the Co²⁺+/+
and CODH/CODH⁺ couples. At lower potentials, the C/Fe-SP
is more extensively reduced and once the methyl group is
transferred to CODH, at low potentials, the ratio of Co⁺/Co²⁺
becomes higher, thus the equilibrium could favor methylation
of the C/Fe-SP. However, at higher potentials (in the ~350
mV range), the equilibrium ratio of Co⁺/Co²⁺ is lower, thus,
once the methyl transfer to CODH occurs, the initially formed
Co⁺ product would equilibrate with Co²⁺ which cannot accept
a methyl group from CODH. This analysis implies that the
methyl acceptor site on CODH is less nucleophilic than the
Co⁺ C/Fe-SP. It may also indicate that the redox couple of
the methyl acceptor site on CODH has a more positive poten-
tial than the Co²⁺+/+ couple (as shown in Fig. 3); however,
there is not a strict relationship between nucleophilicity and
reduction potential.

Pezacka and Wood (24) proposed that the methyl binding
site on CODH is a cysteine. They incubated CODH with
radiolabeled methyl iodide or the methylated corrinoid pro-
tein, acid-hydrolyzed the peptide bonds, chromatographed the
amino acid residues, and identified a radiolabeled methyl-
cysteine residue. When methylated CODH was incubated with
CO and CoA and the same analysis repeated, this methyl-
cysteine peak was absent. We now consider that the cysteinyl
thiol may not be the essential methyl acceptor group on
CODH for acetyl-CoA synthesis.

Based on our results, there are compelling reasons to pro-
pose a metal center rather than the cysteinyl thiol as the
initial nucleophile. First, there appears to be more than one
methyl binding site on CODH based on three exchange reac-
tions conducted with methylated CODH which was prepared
by our standard conditions (at low potentials for short reac-
tion times). The occurrence of the exchange between the
methyl of the methylated methylated CODH with the methyl
group of acetyl-CoA (discussed below) and with the methyl of
the methylated C/Fe-SP requires two methyl acceptor sites.
In addition, methylated CODH is fully active in catalysis of
the exchange reaction between acetyl-CoA and CO. Second,
methylation of CODH occurs over a wide pH range from b.5 to 7.3
(24). This pH profile is not consistent with the normal profile
for cysteine ionization and could reflect changes in coordina-
tion state around a metal center although there are examples
in which cysteine residues in proteins have low pKₐ values.

Third, the dependence on redox potential of the methylation
of CODH is unlike that which would be expected for reduction
of a dithiol to an active thiolate. Most disulfide/dithiol redox
reactions occur in the range of -200 to -300 mV; yet, as
discussed above the redox center which is methylated has a
redox potential of ~350 mV. Fourth, methylation of CODH
at low potentials is not inhibited by pHMB which is a potent
thiol reagent. In addition, acetyl-CoA is synthesized from the
pHMB-treated and methylated CODH in the presence of CO
and CoA. It, however, is possible that the active thiol may
not be accessible to pHMB. Fifth, based on model chemistry,
formation of an acetyl intermediate would be expected to
involve a methyl migration (CO insertion) reaction. There
is ample precedence for carbonyl insertions occurring on metal
centers (see Refs. 14 and 44, for example), but we are not
aware of such reactions occurring at a thioether. Raybuck et
component groups and the Ni-Fe-C signal is elicited by the
change reaction is independent of redox potential and the
that had never previously been proposed, we found that there
this site by methylation would preclude the ability of CODH
to accept the methyl group from acetyl-CoA. If, however,
metal acceptor since addition of the nonmethylated proteins
anism for this exchange. Either binding or cleavage of acetyl-
the hydrolysis (for the amino acid analysis) of methylated
enzyme because we would not expect the acidic condition of
light of our findings that suggest that a metal center is the
site which is the methyl acceptor (M\,\text{CH}) on CODH. We
reversible and help explain our results that indicate there are two methyl
binding sites on CODH.

Which metal center on CODH would be most likely to act
as a methyl acceptor? One such candidate is the Ni-Fe-C
center since it appears to be the binding site for the CO which
will become the carbonyl of acetyl-CoA. The midpoint reduc-
tional potential for this center is \(-560\,\text{mV}\) which is lower
the Co\(^{2+}/^+\) couple. There are other metal centers in
CODH,\(^3\) however, and further studies will be required to
determine which one of these could be the methyl binding
site.

Pezaaka and Wood (24) considered both a reductive cleav-
age and a nucleophilic mechanism to explain the methyl
transfer from the methyl cob(III)amide to CODH. The reduce-
tive cleavage would generate a radical methyl intermediate.
That the rate of the methyl-CODH/methyl-C/Fe-SP exchange
reaction is independent of redox potential and the
inability to reduce methyl-cob(III)amide to methyl-
cob(II)amide at redox potentials of \(-600\,\text{mV}\) or greater (7)
are inconsistent with the reductive cleavage mechanism and
strongly supports a heterolytic cleavage of the methyl-cobalt
bond. Methylation of CODH is proposed (reaction 3, Fig. 4)
to occur by a nucleophilic methyl displacement reaction simi-
lar to the reaction of the methyl of CH\(_2\)H\(_2\)olate w/ Co\(^{2+}\) to
form methyl-Co\(^{3+}\).

Reactions Performed by Methylated CODH—By performing
an exchange reaction between methylated CODH and the
methyl group of acetyl-CoA (Equation 6, above), a reaction
that had never previously been proposed, we found that there
is more than one methyl binding site on CODH. If there were
only a single methyl binding site on CODH, then blockage of
this site by methylation would preclude the ability of CODH
to accept the methyl group from acetyl-CoA. If, however,
there is a second methyl acceptor site on CODH, then this
reaction would be possible. Interestingly, this reaction re-
quires reductive activation as do the methylation reactions
described above. We are assured that small amounts of non-
methylated C/Fe-SP or CODH which could remain as con-
taminants in our reaction mixtures were not involved in
catalysis of the exchange by serving as the proposed second
methyl acceptor since addition of the nonmethylated proteins
had no effect on the rate of the reaction.

Further studies will be required to propose a possible mecha-
nism for this exchange. Either binding or cleavage of acetyl-
CoA requires reductive activation of CODH. Recent experi-
ments\(^8\) indicate that the Ni Fe C EPR signal is generated
upon reaction of CODH with acetyl-CoA at low potentials,
indicating that reduced CODH can cleave acetyl-CoA into its
component groups and the Ni-Fe-C signal is elicited by the

\(^{7}\) C. M. Gorst, and S. W. Ragsdale, manuscript in preparation.

\(^{8}\) C. M. Gorst, and S. W. Ragsdale, manuscript in preparation.
scheme. We must emphasize that we do not yet know the actual oxidation state of this metal center.

Step 5 involves the methyl migration (CO insertion) to form the acetyl intermediate which, in the absence of CoA, is hydrolyzed to acetate. This indicates that acetyl-enzyme formation precedes CoA binding. It is interesting that even in the presence of CoA, some acetate is formed.

Step 6 involves addition of CoA to CODH. We have provided little information on this step in the present study. Earlier work (12, 20, 21) indicates that CoA binds to a site on CODH near the Ni-Fe center and involves arginine and thryptophan residues. The reasons for placing this step after formation of the acetyl-CODH are that (a) acetate is formed in the absence of CoA from methylated CODH and CO and (b) the exchange reaction between CoA and acetyl-CoA occurs at a rate approximately six times faster than the exchange between CO and the carbonyl of acetyl-CoA (22). That the CO/acetyl-CoA exchange reaction occurs in the absence of CoA suggests that CoA binds very tightly to CODH and there is minimal dissociation of CoA (reverse of Step 6 in Fig. 4). The binding constant, determined by quenching of intrinsic tryptophan fluorescence is ~100 nM (20).

Step 7 involves the formation of acetyl-CoA, possibly by the thiolic cleavage of the acetylmethyl intermediate with the SH of CoA as the nucleophile.

Since several of the individual steps in the synthesis are redox potential dependent, it is likely that the overall synthesis is dependent on the redox potential. We are unable to test this postulate since the donors of the carbonyl of acetyl-CoA so far studied (CO, pyruvate) are electron donors. For example, addition of CO to an enzyme solution containing redox potentials from -0 mV to --600 mV.

The proposed mechanism shown in Fig. 4 is in accord with stereochemical analyses of the formation of acetyl-CoA. The questions of the stereochemical correlation and the possibility that the major factor controlling the stereochemical outcome of these steps may be the redox potential

Acknowledgments—We thank Professor M. Mahmun Hossain (University of Wisconsin, Milwaukee) and Dr. Ruma Banerjee (University of Michigan) for valuable discussions and Professor Benjamin Feinberg (University of Wisconsin, Milwaukee) for the use of his potentiotstat.

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Controlled potential enzymology of methyl transfer reactions involved in acetyl-CoA synthesis by CO dehydrogenase and the corrinoid/iron-sulfur protein from Clostridium thermoaceticum.

W P Lu, S R Harder and S W Ragsdale

*J. Biol. Chem.* 1990, 265:3124-3133.

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Additions and Corrections

Vol. 265 (1990) 3124–3133

Controlled potential enzymology of methyl transfer reactions involved in acetyl-CoA synthesis by CO dehydrogenase and the corrinoid/iron-sulfur protein from Clostridium thermoaceticum.

Wei-Ping Lu, Scott R. Harder, and Stephen W. Ragsdale

Page 3133, last paragraph: The second and third sentences should read:

"Transfer of the methyl from CH₃H⁺folate to the C/Fe-SP would result in inversion of configuration. The transfer of the methyl group to CODH would also be expected to result in inversion, leading to net retention."

Vol. 265 (1990) 5345–5348

Human platelets form 3-phosphorylated phosphoinositides in response to α-thrombin, U46619, or GTPγS.

Gregory L. Kucera and Susan E. Rittenhouse

While this paper was in press, a paper by R. D. Nolan and E. G. Lapetina was published ("Thrombin stimulates the production of a novel polyphosphoinositide in human platelets," 1990 J. Biol. Chem. 265, 2441-2445) describing a similar event.

Vol. 265 (1990) 6713–6725

Biosynthesis of Fusarium culmorum trichothecenes. The roles of isotrichodermin and 12,13-epoxytrichothec-9-ene.

Lolita O. Zamir, Kenneth A. Dvor, Anastasia Nikolakakis, and Françoise Sauriol

Page 6713: The first sentence of the footnote should read:

This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada (to L. O. Z.) and by a graduate fellowship (to A. N.).

Vol. 265 (1990) 7959–7966

Molecular species analysis of mitogen-stimulated 1,2-diglycerides in fibroblasts. Comparison of α-thrombin, epidermal growth factor, and platelet-derived growth factor.

Melissa S. Pessin, Joseph J. Baldassare, and Daniel M. Raben

Page 7960, under “Analysis of Diglyceride Linkage”:

Dimethylpyridine/benzene should be “4-dimethylaminopyridine/benzene.”

Page 7961, Table I: Species 7, 8, 19, and 25 were incorrectly identified. These species should be: 7 = 16:1ω7(9)-18:1ω9(7); 8 = 16:1ω7-18:1ω7; 19 = 16:0-20:5ω6; and 25 = 18:1ω7-20:5ω3.

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.

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