The Role of an Iron-Sulfur Cluster in an Enzymatic Methylation Reaction

METHYLATION OF CO DEHYDROGENASE/ACETYL-CoA SYNTHASE BY THE METHYLATED CORRINOID IRON-SULFUR PROTEIN*

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This paper focuses on how a methyl group is transferred from a methyl-cobalt(III) species on one protein (the corrinoid iron-sulfur protein [CFeSP]) to a nickel iron-sulfur cluster on another protein (carbon monoxide dehydrogenase/acetetyl-CoA synthase). This is an essential step in the Wood-Ljungdahl pathway of anaerobic CO and CO₂ fixation. The results described here strongly indicate that the [4Fe-4S] cluster of the CFeSP is required to regenerate Co(I) and recruit the protein back into the catalytic cycle. Our results strongly indicate that the [4Fe-4S] cluster of the CFeSP does not participate directly in the methyl transfer step but provides a conduit for electron flow from physiological reductants to the cobalt center.

The Wood-Ljungdahl pathway allows anaerobic microbes to grow with CO or CO₂ as their sole carbon source (1–4). This pathway, which has been most extensively studied in the acetogenic bacterium, Clostridium thermoaceticum, allows microbes to generate three molecules of acetyl-CoA from a single molecule of glucose. A cobamide-dependent methyl carrier protein, the CFeSP, is involved in two methyl transfer steps in this pathway (Fig. 1). It accepts the N⁵ methyl group of methyltetrahydrofolate (CH₃-H4folate)¹ in a reaction catalyzed by a CH₃-H4folate/CFeSP methyltransferase (MeTr) to form methylcob(III)amide (5, 6). Co(I) is the nucleophilic species that attacks the methyl group of CH₃-H4folate. Then the methyl group is transferred from the CFeSP to CODH/ACS, where acetyl-CoA is assembled from the methyl group, CO, and CoA (7, 8). The catalytic cycles involving the CFeSP (Fig. 1) are thought to involve shuttling between the methyl-Co(III) and Co(I) states.

Two hypotheses guide the experiments described in this paper. The first hypothesis is that methylation of the CFeSP competes with oxidative inactivation of Co(I) to the Co(II) state. There is substantial evidence that oxidation of Co(I) to Co(II) competes with catalytic turnover and leads to inactivated enzyme (Fig. 1). The Co(II)/I) midpoint potential (E_m) is below −500 mV (9). Thus, when the CFeSP is purified, it is in the inactive Co(II) state (9, 10). CODH and CO₃, pyruvate ferredoxin oxidoreductase and pyruvate, and reduced ferredoxin can activate the CFeSP and convert it to the Co(I) state (10). The reductive activation hypothesis is consistent with the observations that (i) the rate of Co(I) decay equals the rate of methyl-Co(III) formation during MeTr-catalyzed methylation of the CFeSP by CH₃-H4folate and (ii) the rate of Co(I) formation equals the rate of methyl-Co(III) decay in the reverse reaction (methylation of H₄folate) (6). The reductive activation reaction is thought to be crucial for survival of the cells. This is because the Eₚ of the Co(II)/I) couple is low enough that, even under the anaerobic conditions required for growth of acetogens and methanogens, the CFeSP is expected to undergo oxidative inactivation sporadically. There is strong evidence that the cobalamin-dependent methionine synthase also requires the Co(I) state for catalytic turnover (11, 12). This protein does not contain an Fe-S cluster but uses an S-adenosyl-L-methionine-dependent reactivation mechanism (13–15).

The second hypothesis is that reductive reactivation of the Co(II) center requires the FeS cluster of the CFeSP to communicate with physiological reductants. The large 55-kDa subunit of the CFeSP contains a [4Fe-4S] cluster (10). This was the first “B₁₂” protein identified that also contains an iron-sulfur cluster. Subsequently, a similar CFeSP was purified from Methanosarcina thermophila, where it is involved in the conversion of acetyl-CoA to methane (16). Analogos have been identified in the genome sequences of Methanobacterium thermoautotrophicum (17), Methanococcus janashii (18), and Archaeoglobus fulgidus (19). What is the role of the cluster? Several years ago, we proposed that the [4Fe-4S] cluster facilitates the conversion of Co(II) to Co(I) (9, 10). This proposal is supported by recent studies of a C20A variant of the CFeSP in which the 4Fe cluster (Eₚ = −532 mV) is converted to a 3Fe cluster (Eₚ = −31 mV) (20). This 500-mV increase in the midpoint potential severely cripples the CFeSP’s ability to be activated by physiological electron donors like CODH/ACS or reduced ferredoxin. These studies led to the hypothesis that the low potential 4Fe cluster relays electrons from the CODH subunit of CODH/ACS to the cob(II)amide CFeSP, thereby facilitating its reactivation to Co(I).

Optimal conditions for studying acetyl-CoA synthesis from CO, CH₃-H₄folate, and CoA have been established (21). The

¹ The abbreviations used are: H₄folate, tetrahydrofolate; MeTr, methyltransferase; CFeSP, corrinoid iron-sulfur protein; CODH, carbon monoxide dehydrogenase; ACS, acetyl-CoA synthase.
reaction requires MeTr, the CFeSP, and CODH/ACS. Ferredoxin stimulates the reaction 4-fold. Under these conditions, the Co(II)-CFeSP must be reduced to Co(I) before it can enter the catalytic cycle. In the studies described here, we monitored the synthesis of acetyl-CoA from CO, CH$_3$H$_4$folate, CoA, and the methylated form of either the wild type or C20A variant CFeSP. If the two hypotheses described above are correct, then, when the C20A variant of the methylated CFeSP is used, the rate of acetyl-CoA synthesis will slowly decrease at the rate of oxidative inactivation of the cobalt center. Furthermore, we can measure the UV-visible spectra of the C20A variant during and after the reaction to directly determine if Co(I) forms during the linear phase of the reaction and if Co(II) forms when the reaction has undergone inactivation. These results can be compared with those for the wild type protein. According to our hypotheses, when the wild type protein is used, this inactivation will not be observed because CODH and CO$_2$ will rapidly reduce the Co(III) protein, recruiting the inactive protein back into the catalytic cycle.

EXPERIMENTAL PROCEDURES

Materials—N$_2$ (99.98%) and CO (99.99%) were obtained from Lin- weld (Lincoln, NE). N$_2$ was deoxygenated by passing through a heated column containing BASF catalyst. Reagents were of the highest purity available. CH$_3$H$_4$folate was purchased from Amersham Pharmacia Biotech.

Organism and Enzyme Purification—Construction of the C20A CFeSP variant, cloning the gene into Escherichia coli and reconstituting the protein with B$_{12}$ and the FeS cluster was described earlier (20). The wild type (10) and variant (20) CFeSPs were purified as described under strictly anaerobic conditions as described. CODH/ACS (22), ferredoxin II (23), and MeTr (6) were purified as described under strictly anaerobic conditions at 17 °C in a Vacuum Atmospheres chamber maintained below 1 ppm oxygen. Protein concentrations were determined by the Rose Bengal method (24).

Enzyme Assays—The wild type and variant CFeSPs were methylated with methyl iodide essentially as described earlier (8). The as-isolated protein was first reduced by reacting with 10 mM titanium(III) citrate.

The solution was then incubated for 15–30 min at 13 °C with 20-fold excess $^4$CH$_3$I and centrifuged through a Sephadex G-50 column (26) to remove the unreacted methyl iodide and the titanium citrate. Therefore, methyl iodide, which inhibits CODH/ACS, was absent from reactions involving the methylated CFeSP.

The reaction of CH$_3$H$_4$folate, CO, and CoA to form acetyl-CoA was performed as described previously (21). Under these conditions, the concentration of CODH/ACS is rate-limiting. The reaction was performed in the dark in a glass V-shaped reaction vial capped with a red rubber serum stopper. Details of the reaction mixture are given in the Fig. 2 legend. The reaction was quenched at various times by removing 5-μL aliquots into 5 μL of 2.2 n perchloric acid. The amount of acetyl-CoA formed was measured by Dowex 50W-X8 chromatography as described (27). UV-visible spectra were obtained on an OLIS-modified Cary 14 spectrometer. The data were then analyzed using the program KINSIM (28, 29) and modified by Gary Xin Hua and Dr. Bryce Plapp of the University of Iowa. The input rate constants are given under “Results.” The simulated data were plotted using SigmaPlot (Jandel Scientific, San Rafael, CA).

RESULTS

Synthesis of Acetyl-CoA from the Methylated CFeSP, CH$_3$H$_4$folate, CO, and CoA—We measured the synthesis of acetyl-CoA from CH$_3$H$_4$folate, CO, and CoA using the methylated CFeSP as the methyl carrier protein. If the variant CFeSP is used, this reaction is very slow, since the protein is in the Co(II) state and cannot be activated by physiological reductants (20). In the reaction studied here, the first turnover generates cob(I)amide, which undergoes remethylation by CH$_3$H$_4$folate or oxidation to cob(II)amide. When the wild type methylated CFeSP is used, acetyl-CoA synthesis continues linearly with time until the limiting reagent is depleted. The concentration of CO (1 mM) is limiting in these reactions. With the C20A variant, the rate of acetyl-CoA synthesis initially is the same as with the wild type protein and then slowly decreases and becomes negligible after ~100 turnovers (Figs. 2 and 3A, inset). This reaction was performed using three different concentrations of methylated CFeSP. The data with the variant proteins were fit to single exponential equations to estimate the inactivation rate constants (Fig. 2, solid lines; Table I). These rate constants are 0.0001 M$^{-1}$ s$^{-1}$, 0.0001 M$^{-1}$ s$^{-1}$, and 0.0001 M$^{-1}$ s$^{-1}$, respectively.

In these studies, we methylated the CFeSP with methyl iodide. This procedure was criticized by others who erroneously stated that our samples contained methyl iodide at concentrations sufficient to inhibit CO/acetyl-CoA exchange and CO oxidation activities (see Footnote 27 of Ref. 7). However, in our studies (25), we followed a protocol established after extensive studies to assure that the methyl-CODH is “viable” (8). After reacting the CFeSP with methyl iodide, the reaction mixture is chromatographed to remove methyl iodide and isolate the methylated CFeSP. Therefore, methyl iodide is absent from all enzymatic reactions in which the methylated CFeSP is used as a methyl donor. The catalytic competence of the methyl-CODH intermediate was demonstrated in three different reactions. First, in an exchange reaction between methylated CFeSP and methyl-CODH, 40% of the methyl groups underwent exchange. Second, in an exchange reaction between methyl-CODH and the methyl group of acetyl-CoA, 90% underwent exchange. Third, by measuring the conversion of methyl-CODH, CO, and CoA to acetyl-CoA, 80% of the methyl groups were converted to acetate or acetyl-CoA. Therefore, the methylation protocol is sound, and the published criticisms relating to this methodology are unjustified.
were inversely proportional to the CFeSP concentration. The rate constant for reactivation of the mutant protein ($k_{\text{cat}} = 0.0015 \text{ s}^{-1}$) has been measured earlier (20). It is 30–90-fold lower than these rates of inactivation. The UV-visible spectrum of the variant CFeSP after 40 min, when the reaction is fully inhibited and acetyl-CoA is no longer being formed, is characteristic of the cob(II)amide state (Fig. 3A).

These results demonstrate that reductive activation is required to regenerate the active form of the CFeSP. After it converts to the Co(II) state, the C20A variant cannot be reactivated at significant rates by its physiological electron donors (CODH and CO), because its high potential 3Fe-4S cluster cannot provide the driving force to reduce Co(II) to Co(I). The wild type protein undergoes reactivation over 4000-fold faster ($k_{\text{cat}} = 0.88 \text{ s}^{-1}$) than the mutant, so the reaction continues unabated until the substrates are depleted. Therefore, a low potential Fe-S cluster in the CFeSP is required for reductive activation of the CFeSP by physiological electron donors.

**Single Turnover Kinetics of the Variant Methylated CFeSP**—When the wild type methylated CFeSP is reacted with CODH/ACS, a low potential metal center (cluster A) on ACS undergoes methylation as the CFeSP is converted to the Co(I) state (25). It remained a possibility that there is some degree of radical chemistry in this reaction (see below for discussion). We considered that disabling the electron transfer pathway to the cobalt center might uncover or shift the predominantly SN2 mechanism to a radical pathway. If so, the initial cobamide product of the methyl transfer reaction would be cob(II)amide, instead of cob(I)amide. We mixed the methylated C20A variant CFeSP and CODH/ACS in the presence of CoASH and CO and followed the spectrum of the reaction mixture. The broad absorption band from methyl-Co(III) at 450 nm decreases as the intensity of the 390-nm peak from cob(I)amide increases (Fig. 3B). The reaction yields clean isosbestic points, clearly demonstrating that demethylation of the variant methyl-CFeSP (like the wild type protein) by CODH/ACS is accompanied by conversion of CH₃-Co(III) to Co(I). The final absorption changes at 390 nm yield a difference extinction coefficient ($\Delta\varepsilon$) of $17 \text{ mM}^{-1} \text{ cm}^{-1}$, which indicates complete conversion of methyl-Co(III) to Co(I). The rate of Co(I) formation is $0.21 \mu\text{M} \text{ min}^{-1}$, which yields a $k_{\text{cat}}$ for CODH/ACS under these conditions of $1 \text{ min}^{-1}$.

**FIG. 2. Acetyl-CoA synthesis from methyl-Co(III)-CFeSP.** The acetyl-CoA synthesis assays were performed in a total volume of 0.1 ml at 55°C. The reaction mixtures contained 50 mM Tris-maleate buffer (pH 5.8); 0.0275 nmol of MeTr; 200 nmol of $^{14}\text{CH}_3\text{H}_4\text{folate}$ (1000 dpm/nmol); 0.083 nmol of ferredoxin; 250 nmol of CoASH; and 0.8 (A), 0.575 (B), or 0.4 nmol (C) of the methylated wild type (□) or C20A variant (□) CFeSP. The reactions were initiated with 0.0275 nmol of CODH. The solid lines are from linear fits (wild type) or exponential fits (mutant) of the data. The dotted lines are based on kinetic simulations of the data according to the mechanism shown in Scheme 1 using the parameters given in Table I.

**FIG. 3. Formation of Co(I) followed by oxidation to Co(II) after demethylation of the Co(III)-CFeSP.** A, UV-visible spectrum of the cob(II)amide state of the variant protein produced after 40 min of the reaction described in the legend to Fig. 2A. The number of turnovers (□) and nmol of acetyl-CoA (□) produced with three different amounts (0.4, 0.575, and 0.8 nmol) of the methylated C20A variant CFeSP are shown. Inset, bottom, the reaction mixture contained $3.5 \mu\text{M}$ C20A variant CFeSP, 200 nM CODH, 1 mM CoASH, and 1 atmosphere of CO in 50 mM Tris-HCl, pH 7.6, in a final reaction volume of 1 ml. Demethylation of the CFeSP was followed by monitoring the appearance of the 390-nm peak of cob(I)amide. Scans were taken every 2 min. The reaction was complete after 17 min.
is Co(II)-CFeSP. Since the concentrations of CoA and CH₃-H₄folate were saturating and 4-fold higher than the concentration of CO, they were
by an SN2 mechanism (6, 20, 30). The results described here
methionine synthase strongly indicate that this reaction occurs
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38, in press.

for the wild type and C20A CFeSP (20). However, the full set of
rate constants for acetyl-CoA synthesis from the methylated
CFeSP, CO, and CoA have not yet been measured. A full
simulation that included all of these steps would contain about
40 rate constants. We made several simplifying assumptions to
wrap all of these reactions into three steps (Scheme 1). The first
step is the MeTr-catalyzed methylation of the CFeSP (B + E →
C + E). The CFeSP and methyl-CFeSP concentrations are
below their K_m values, and the CH₃-H₄folate concentration is
much higher than its K_m value. Therefore, the rate constants
for the methylation of the CFeSP and demethylation of the methyl-
CFeSP by MeTr can be substituted by the k_cat/K_m values
for the CFeSP and the methylated CFeSP, respectively. Since the
CH₃-H₄folate concentration (2.5 mM) is much higher than its
K_m (10 μM) value in these reactions, we also omitted this term
from the simulation. In addition, we simplified the acetyl-CoA
synthesis steps by summarizing them into a single reaction (C
+ F + G → B + F + H [acetly-CoA])

for the wild type and 0.0015 s⁻¹ for the
C20A variant (20) CFeSP. These
simplifying assumptions allowed us to focus attention on
the oxidative inactivation and reductive activation reactions
and quantitatively test the reductive activation hypothesis.

After supplying the above rate constants, we input rate
constants for inactivation of Co(I) (beginning with those derived
from the exponential fits as a starting point) into the KINSIM
program. The dashed lines in Fig. 3 are the simulated progress
curves for the reactions performed at three concentrations of

We observe a striking decay in the rate of acetyl-CoA
synthesis when the C20A variant CFeSP is used as the methyl
carrier protein (Fig. 2). We can estimate the inactivation rate
constant by fitting the data to an exponential equation. To
analyze the data more quantitatively, we simulated the progress
curve. This procedure requires inputting the relevant
rate constants and concentrations of substrates and enzymes
for each reaction in this multistep process. We have determined
all of the elementary reaction rates and the steady-state kinetic
parameters for the MeTr⁵ and the CODH (32) reactions. We
also have determined the rate of formation of Co(I) from Co(II)

4 S. W. Ragsdale, unpublished data.
5 Seravilli, J., Zhao, S. Y., and Ragsdale, S. W. (1999) Biochemistry
38, in press.
the methylated CFeSP. The simulated progress curves fit the data well, indicating that our simplifying assumptions are reasonable. To better understand the factors that control whether the CFeSP will undergo oxidative inactivation, reductive reactivation, or methylation, we used KINSIM to output the concentrations of the three states of the CFeSP, Co(I), Co(II), and methyl-Co(III) for the mutant and wild type proteins. Fig. 4 shows these species for the reaction shown in Fig. 2A with 8 \micro M CFeSP. The pre-steady state reaction is identical for the mutant and the wild type proteins. With the wild type protein, the concentration of Co(II) quickly reaches a value that is about 2% of the total CFeSP present and slowly decays to about 0.4%. The predominant species at the end of the reaction is methyl-Co(III). However, with the C20A mutant, Co(II) increases in an exponential fashion as Co(I) decays. At the end of the reaction, there is a mixture of methyl-Co(III) (32% of the total CFeSP) and Co(II) (58% of the total). In both reactions, there is a significant amount of methyl-Co(III) at the end of the reaction, because the CH$_3$-H$_4$folate concentration is higher than that of CO. The essential parameters are the relative rates of inactivation relative to the rates of reactivation and Co(I) methylation. With an inactivation rate constant of about 0.1 min$^{-1}$, the reaction profile begins to deviate from linearity even when the reactivation rate is as high as the inactivation rate constant. Interestingly, increasing the reactivation rate constant above the measured value has minimal effect on the progress curve. For example, increasing the reactivation rate 10-fold (to 8.8 min$^{-1}$) increases the slope of the reaction’s progress curve by only 3%. This indicates that the environment and location that nature has selected for the FeS cluster in the CFeSP is nearly optimal for its role in reductive activation.

The redox potential for the [4Fe-4S]$^{2+/-1+}$ couple is about 20 mV more negative than that of the Co(II)/Co(I) couple and is nearly equal to that of the CO$_2$/CO couple. The design of a low potential cluster is clearly important; however, based on a purely electrochemical argument, one would expect CO to reduce the Co(II) state about as easily as the cluster 2+ state. However, the requirement for the cluster in reductive activation of Co(II)-CFeSP by physiological electron donors is nearly absolute. The cluster is located in a separate subunit from the cobamide. Solving the crystal structures of the CFeSP, MeTr, and CODH/ACS would greatly enhance our understanding of how these proteins interact in electron and methyl transfer reactions.

The studies described here also provide insight into the mechanism of methyl group transfer from the methylated CFeSP to CODH/ACS. There are three possible mechanisms (33). The first is heterolytic cleavage of the Co-C bond by an S$_2$2-type mechanism involving attack by a nucleophilic center of ACS (Mechanism I). This nucleophilic center has not been unambiguously identified. Evidence from our laboratory, summarized in recent reviews (1, 2, 34, 35), and from Lindahl’s laboratory (7) indicates that the nucleophile is the nickel site of cluster A. We favor a mechanism involving a Ni(I) nucleophile; however, Barondeau and Lindahl (7) favor a Ni(II) nucleophile. A second possibility (Mechanism II) is a homolytic mechanism in which electron transfer from the reduced Fe-S cluster of the CFeSP would form a methyl-Co(II) species that would disproportionate to form Co(I) and methyl-nickel. A third possibility (Mechanism III), that the CH$_3$-Co(III) species is cleaved homolytically to form a CH$_3$-Ni species and Co(II), is inconsistent with stopped flow studies of the wild type CFeSP. Co(I) forms at the same rate that methyl-Co(III) decays, apparently ruling out a Co(II) intermediate (36). Mechanisms II and III invoke transfer of a methyl radical to CODH/ACS.

Methylation of CODH/ACS has been modeled using compounds such as CH$_3$-Co(dmgBF$_2$)$_2$py (where dmgBF$_2$ represents (difluoroboryl)dimethylglyoximato and py represents pyridine) as the methyl group donor complex and Ni(tme) (where tme represents 1,4,8,11-tetramethyl-1,4,8,1-tetraazacyclotetradecane) for physiological electron donors. In the case of model compounds, Co-C bond homolysis is possible, since the Ni(tmc)(II/I) complex represents 1,4,8,11-tetramethyl-1,4,8,1-tetraazacyclotetradecane as the methyl acceptor (37, 38). Two equivalents of the Ni(I) complex were required, one to reduce methyl-Co (III) to methyl-Co(II) and the other to capture the methyl radical generated upon cleavage of the methyl-Co(II) species. Thus, studies of the inorganic model system support Mechanism II.

On the other hand, several criteria favor Mechanism I for the analogous enzymatic methyl transfer. Martin and Finke (39) noted the thermodynamic problem with mechanism II: reduction of CH$_3$-Co(III) requires redox potentials that are too low for physiological electron donors. In the case of model compounds, Co-C bond homolysis is possible, since the Ni(tmc)(II/I) couple, which has a reduction potential of $-1.18$ V (versus SHE), is capable of reducing CH$_3$-Co(III), with an $E_m$ of $-1.2$ V. However, neither CO nor any of the redox centers present in CODH/ACS from C. thermoaceticum have $E_m$ values below $-550$ mV (8, 40–43). Thus, CODH is too weak as a reductant to reduce methyl-cob(III)amide. We were unable to detect any reduction or cleavage of the methyl-Co(III) state of the CFeSP after several hours of incubation in the absence of CO and CODH/ACS (9). Mechanism I is also supported by studies of acetyl-CoA synthase using chiral CH$_3$-H$_4$folate, which is converted to acetyl-CoA with retention of configuration (44). The most straightforward interpretation of these studies is that transfer of the methyl group to CODH occurs with inversion of configuration, as expected for an S$_2$2 type displacement. Although reactions exist in which the radical is transferred before it has the chance to rotate (and thus randomize), if the methyl group is transferred as a radical species, racemization would be the most likely outcome.

Further support for Mechanism I comes from recent studies...
using the variant C20A CFeSP (20). The methylated C20A variant, which cannot accept electrons from CODH and, therefore, cannot reduce methyl-Co(III) to methyl-Co(II), generates acetyl-CoA as rapidly as the wild type protein. This result appears to be inconsistent with Mechanism II. However, this study did not evaluate the redox state of the CFeSP during the reaction. The finding that electron transfer to the cobalt center is crippled in the variant offers the possibility of uncovering reaction intermediates that would be masked by the rapid reduction by CODH of the wild type protein. Furthermore, it is possible that a radical pathway (such as Mechanism III), which may be a minor component of the reaction with the wild type protein, could become the dominant pathway used by the variant form of the CFeSP. However, just as with the wild type protein, demethylation of methyl-Co(III) is accompanied by radical chemistry. An important question remains. What conditions determine whether a methyl transfer reaction will occur through a homolytic or heterolytic mechanism?

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