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Spectroelectrochemical Studies of the Corrinoid/Iron–Sulfur Protein Involved in Acetyl Coenzyme A Synthesis by *Clostridium thermoaceticum*†

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ABSTRACT: An 88-kDa corrinoid/iron–sulfur protein (C/Fe-SP) is the methyl carrier protein in the acetyl-CoA pathway of *Clostridium thermoaceticum*. In previous studies, it was found that this C/Fe-SP contains (5-methoxybenzimidazolyl)cobamide and a $[4Fe\text{-}4S]^{2+/1+}$ center, both of which undergo redox cycling during catalysis, and that the benzimidazole base is uncoordinated to the cobalt (base off) in all three redox states, 3+, 2+, and 1+ [Ragsdale, S. W., Lindahl, P. A., & Münck, E. (1987) *J. Biol. Chem. 262*, 14289–14297]. In this paper, we have determined the midpoint reduction potentials for the metal centers in this C/Fe-SP by electron paramagnetic resonance and UV–visible spectroelectrochemical methods. The midpoint reduction potentials for the $Co^{3+/2+}$ and the $Co^{2+/1}$ couples of the corrinoid were found to be 300–350 and −504 mV (±3 mV) in Tris-HCl at pH 7.6, respectively. We also removed the (5-methoxybenzimidazolyl)cobamide cofactor from the C/Fe-SP and determined that its $Co^{3+/2+}$ reduction potential is 207 mV at pH 7.6. The midpoint potential for the $[4Fe\text{-}4S]^{2+/1+}$ couple in the C/Fe-SP was determined to be −523 mV (±5 mV). Removal of this cluster totally inactivates the protein; however, there is little effect of cluster removal on the midpoint potential of the $Co^{2+/1+}$ couple. In addition, removal of the cobamide has an insignificant effect on the midpoint reduction potential of the [4Fe-4S] cluster. A 27-kDa corrinoid protein (CP) also was studied since it contains (5-methoxybenzimidazolyl)cobamide in the base-on form. The reduction potentials for the $Co^{3+/2+}$ and $Co^{2+/1+}$ couples of the 27-kDa CP were found to be 215 and −630 mV, respectively, at pH 7.6. Our work provides evidence that the C/Fe-SP stabilizes the active Co^{1+} state of the C/Fe-SP relative to that of the 27-kDa CP and free corrinoids by control of the state of ligation of the benzimidazole base to Co^{2+}. The implications of this increased stability in the context of the methyl-transfer reaction catalyzed by this enzyme are discussed. Our results are consistent with a mechanism of methyl transfer to CO dehydrogenase in which a nucleophile on CODH performs a heterolytic displacement of the methyl group of the methylCo^{3+} corrinoid, resulting in Co^{1+} and methylated CODH.

The Wood pathway of acetyl-CoA synthesis is an autotrophic pathway of anaerobic growth [see Wood et al. (1986a,b), Ljungdahl (1986), and Fuchs (1987) for recent reviews]. The pathway recently has been found to occur in a number of anaerobic organisms, from acetogenic and sulfate-reducing eubacteria to methanogenic archebacteria (Wood et al., 1986a,b; Fuchs, 1987). The acetogenic bacteria use the pathway for generation of cell carbon and energy from CO, CO_2/H_2, or organic substrates and synthesize acetate as the major fermentation product. The methanogens use the pathway for synthesis of cell carbon and also for acetoclastic synthesis of methane. The Wood pathway is a unidirectional synthesis of a two-carbon compound, acetate, from two one-carbon precursors. The pathway is outlined in Figure 1, which emphasizes the role of the C/Fe-SP in the pathway. First, methyl-H_4 folate is formed from CO_2 in reactions involving formate dehydrogenase and tetrahydrofolate- (H_4folate) dependent enzymes (Ljungdahl, 1986). Then, the methyl group of methyl-H_4folate is transferred to a corrinoid/iron–sulfur protein (C/Fe-SP), forming a methyl-Co cobamide, in a reaction catalyzed by methyltransferase (MeTr) (Drake et al., 1981; Hu et al., 1984; Ragsdale et al., 1987). Next, CO_2 (after reduction) or CO reacts with CO dehydrogenase (CODH) to form an organometallic mixed-metal center consisting of Ni, two to three Fe, and CO (Ragsdale et al., 1986). Then, the methyl group of the methylated C/Fe-SP is transferred to CODH, apparently forming a methylcysteine intermediate (Pezacka & Wood, 1988), CoA binds, and CODH catalyzes the formation of acetyl-CoA from the bound methyl, CO, and CoA groups (Ragsdale & Wood, 1985).

We are interested in understanding the methyl-transfer reactions to the C/Fe-SP from methyl-H_4folate and from the methylated C/Fe-SP to CODH. The C/Fe-SP is an 88-kDa protein which was isolated from *Clostridium thermoaceticum* and was shown to act as a methyl carrier in the acetyl-CoA pathway, and an enzyme-bound methylcobamide was identified (Hu et al., 1984). Subsequently, the C/Fe-SP was purified to homogeneity and the metal sites in the protein were studied by EPR, Mössbauer, and UV–visible spectroscopy (Ragsdale et al., 1987). The C/Fe-SP contains a corrinoid, (5-methoxybenzimidazolyl)cobamide, and a $[4Fe\text{-}4S]^{2+/1+}$ cluster, both of which undergo redox changes during the reaction cycle of

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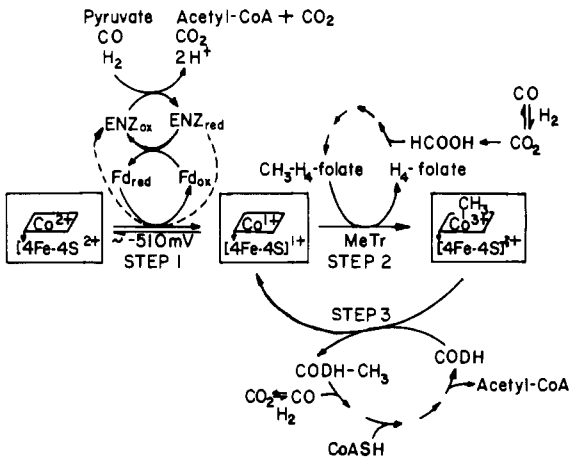

FIGURE 1: Pathway of acetyl CoA synthesis emphasizing the role of the C/Fe-SP.

the C/Fe-SP (Ragsdale et al., 1987). The cobalt corrinoid may exist in three redox states, Co^{3+}, Co^{2+}, and Co^{1+}. In this protein, the 5-methoxybenzimidazole base is unligated to the cobalt, remaining in the base-off form in all redox states of the cobalt (Ragsdale et al., 1987). The as-isolated protein contains cobalt in the Co^{2+} form as a base-off corrinoid. In the reaction mechanism (Figure 1), the corrinoid is reduced to the Co^{1+} state, which is an extremely powerful nucleophile (Abeles & Dolphin, 1976). In a reaction catalyzed by MeTr, Co^{1+} then reacts with the methyl group of methyl-H_4folate to form a CH_3–Co^{3+} species. On the basis of studies of others on vitamin B_{12} and its analogues (Lexa & Saveant, 1976; Lexa et al., 1977, 1979), we proposed that the unusual base-off conformation is important for the mechanism of the methylation of the C/Fe-SP by methyl-H_4folate and in the transfer of the methyl from methylcobalt to CODH (Ragsdale et al., 1987).

The equilibrium redox potentials have been determined for vitamin B_{12} and a number of its analogues (Lexa & Saveant, 1976; Lexa et al., 1977, 1979). In aquocobalamin, the $E^{\circ\prime}$ of the $Co^{3+/2+}$ couple is −40 mV vs SCE (+202 mV vs NHE) and that of the $Co^{2+/1+}$ couple is −851 mV at pH 7.0 (−609 mV vs NHE) as determined by thin-layer spectroelectrochemical and cyclic voltametric studies, respectively (Lexa & Saveant, 1976; Lexa et al., 1977). For a base-off corrinoid, the potential for the $Co^{3+/2+}$ couple was approximated to be +280 mV vs SCE (+522 mV vs NHE at pH −2.4) (Lexa et al., 1977). The predicted midpoint potential for aquocobinamide at pH 7.6 is ca. +390 mV vs NHE [see Figure 10 of Lexa et al. (1977)]. The pH dependence of the $Co^{3+/2+}$ couple is +60 mV per pH unit below pH 2.9 and −60 mV per pH unit change above pH 7.8. In addition, formation of base-off Co^{2+} favors reduction to the 1+ state, and reduction of Co^{2+} to Co^{1+} in vitamin B_{12} apparently occurs through an intermediate base-off state (Lexa et al., 1977). We postulated that in the C/Fe-SP, the base-off configuration increases the stability of Co^{1+}, the active form of Co which performs the oxidative addition (also termed a nucleophilic attack) of the methyl of methyl-H_4folate to form the methyl-Co^{3+} corrinoid (Ragsdale et al., 1987).

In this paper, we have focused on the oxidation–reduction reactions involving the cobalt center of the C/Fe-SP and of a 27-kDa corrinoid protein (CP). We also have studied the redox chemistry of the [4Fe-4S] cluster in the C/Fe-SP which earlier was shown to be essential for the reactions involving the C/Fe-SP (Ragsdale et al., 1987). The 27-kDa CP from *C. thermoaceticum* has an uncertain function but, just as the C/Fe-SP, contains (5-methoxybenzimidazolyl)cobamide (Ljungdahl et al., 1973; Ragsdale et al., 1987). In the work reported in this paper, we have determined the equilibrium reduction potentials for the $Co^{2+/1+}$ and $Co^{3+/2+}$ couples of the C/Fe-SP, of the 27-kDa CP, and of aquocobalamin and (5-methoxybenzimidazolyl)cobamide. Our results strongly indicate that Co^{1+} in the C/Fe-SP is more stable than in free corrinoids or in the 27-kDa CP due to the base-off conformation of Co^{2+}. We determined that the midpoint equilibrium redox potential for the [4Fe-4S] cluster is only slightly more negative than that for the $Co^{2+/1+}$ couple in the C/Fe-SP. We also obtained results consistent with a heterolytic mechanism of displacement of the methyl group of the methylcobamide in the methyl transfer to CODH.

Materials and Methods

Growth of the Organism and Enzyme Purification. C. thermoaceticum was grown with glucose as the carbon source under a 100% CO_2 atmosphere (Ljungdahl & Andreesen, 1978). The 27-kDa CP and C/Fe-SP were purified under strictly anaerobic procedures as described (Ragsdale et al., 1987).

Analytical Techniques. UV–visible spectroelectrochemistry was performed as described earlier (Gustafson et al., 1986). In order to achieve the low potentials required for reduction of the C/Fe-SP and the 27-kDa corrinoid protein and to minimize the amount of protein solution required, an EPR spectroelectrochemical titrator was designed (Harder et al., 1989). The titration apparatus requires small volumes (ca. 0.6 mL) of protein and facilitates the transfer of the sample from an electrochemical pouch to an EPR tube. Thus, no oxygen is leaked into the system which would perturb ambient potentials and denature the oxygen-labile C/Fe-SP. The electrochemical cell utilizes a typical three-electrode circuit with Ag/AgCl reference and counter electrodes and a gold foil working electrode. Typically, a concentrated dye solution was added to the cell, which was degassed by cycling with vacuum and deoxygenated purified argon for at least 1 h. The oxygen was removed by passing the argon over a heated copper catalyst. Final concentrations of the dyes were from 0.5 to 1 mM. Under a high positive gas flow, 0.65 mL of protein in Tris-HCl/KCl (50 mM, pH 7.6/200 mM) was added to the cell via a 1-mL syringe (gas tight, Hamilton) which had been purged with argon. The cell was then cycled several additional times with vacuum/argon. When potentials were poised in the −450-mV range and below, the buffer solution also contained 5 mM dithiothreitol (DTT) for increased protein stability. A potentiostat (ECO 551) was used to initially poise the potential below the desired equilibrated potential. Subsequently, the potentiostat was turned off, and the cell was allowed to drift to equilibrium while a voltmeter (4.5-digit high impedance, Beckman Model 4410) across the working and reference electrodes monitored the ambient potential. When the potential no longer drifted, indicating that equilibrium had been achieved, the cell was tipped to move the solution from the electrochemical pouch to the EPR tube. The sample then was frozen in liquid nitrogen for EPR spectroscopy.

EPR spectroscopy was performed on a Varian Model E115 spectrometer with an automatic frequency counter (EIP, Model 548A). The temperature was maintained at 100 K with a variable-temperature controller (Varian) for spectroscopy of the corrinoid and at 9 K with a liquid helium cryogenic system (Heli-Tran Model LTD-3-110, APD Cryogenics) for titration of the [4Fe-4S] cluster. The digital temperature readout on the Air Products unit had been calibrated with a calibrated gold thermocouple. Spectroscopic parameters are stated in the figure legends. The spin concentrations were

determined by double integration of the spectra with reference to a solution of 1 mM copper perchlorate as described by Fee (1978). In each of the spectra shown in this paper, there was an intense peak at $g = 2.0$ due to the reduced dye radical. This peak was off scale and was subtracted from the corresponding spectra. The remnant of the dye peak can be observed in the figures by a horizontal line at ca. 3320 G. Subtraction of this signal allows accurate integration of the spectra to yield precise determination of the concentrations of Co^{2+}. The subtraction procedure did not compromise the quality of our analysis since the relative ratios of [ox]/[red] were identical when determined from either the integrated spectra or from one of the intense lines in the EPR spectra in a region not affected by the dye signal.

Data were analyzed by a Nernst plot with eq 1.

$$E = E^{\circ\prime} + (0.059/n)[\log ([ox]/[red])] \quad (1)$$

Electrochemical Studies of Methylated C/Fe-SP. The C/Fe-SP was methylated with methyl iodide. The anaerobic titrator was made anaerobic, and 0.03 mL each of TRIQUAT and 4,4′-dimethyl-*N*,*N*′-trimethylene-2,2′-dipyridinium dibromide, each at 3 mg/mL, was added. After the titrator cell was thoroughly degassed, C/Fe-SP (18 mg in 0.6 mL of 50 mM Tris-HCl and 0.2 M KCl) was added, and the reduction potential was posed to −590 mV vs NHE. The system was incubated for 1 h before addition of 0.06 mL of 0.2 M [^{14}C]methyl iodide (245 dpm/nmol) in methanol. The potential increased to −400 mV after the addition, and the reaction was incubated for 1 h at this potential. After this time, the EPR signal due to Co^{2+} was totally lost. Then the sample was desalted with the spun-column technique (Penefsky, 1977). In brief, the sample was applied to a 3-mL Sephadex G-25 column that had been equilibrated with 50 mM Tris-HCl and centrifuged at 4000 rpm, and the protein-containing eluate was applied to a second column similarly prepared and centrifuged. Then, 0.3 mL of the eluate from this second column was transferred to an EPR tube. Aliquots (0.02 mL) of the initial sample, of the sample after poising the potential, and of the sample after the "spun column" were injected into 4 mL of scintillation fluid and counted in a liquid scintillation counter (Beckman). Next, this solution of methyated C/Fe-SP was added to the anaerobic titrator in the presence of the two dyes (described above) and incubated for 1 h at a stable potential of −565 mV vs NHE. Then the EPR spectrum was recorded, and the solution was applied to a spun-column. Finally, the radioactivity of 0.2 mL of the sample was determined in a scintillation counter, and the EPR spectrum was recorded.

Removal of Metal Centers from the C/Fe-SP. The cobamide was removed from the C/Fe-SP by incubation of the protein (18 mg) with 600 mg of urea and 3 mg of dithiothreitol in a total volume of 0.81 mL of 50 mM Tris-HCl, pH 8.6, for 16 h in the anaerobic chamber at 16 °C. The reaction mixture was then chromatographed on Sephadex G-50 equilibrated with 50 mM-Tris-HCl, pH 8.6, plus 6 M urea by the spun-column technique (Penefsky, 1977). The protein fraction was then chromatographed on a Sephadex G-25 spun column equilibrated with 50 mM Tris-HCl, pH 8.6, to remove the urea.

The [4Fe-4S] cluster was removed from the C/Fe-SP by incubation with a 50-fold molar excess of mersalyl acid in 1 mL of 50 mM Tris-HCl, pH 8.6, for 12 h in the anaerobic chamber. The protein solution was then chromatographed on a Sephadex G-25 column by the spun-column technique.

Chemicals. Dyes used for the titrations were as follows: 4,4′-dimethyl-*N*,*N*′-trimethylene-2,2′-dipyridinium dibromide, $E^{\circ\prime}$ of −680 mV; *N*,*N*′-trimethylene-2,2′-dipyridinium dibromide (TRIQUAT), $E^{\circ\prime}$ of −540 mV; methyl viologen, $E^{\circ\prime}$ of −440 mV; dichlorophenolindophenol (DCPIP), $E^{\circ\prime}$ of 220 mV; *N*,*N*,*N*′,*N*′-tetramethylphenylenediamine (TMPD), $E^{\circ\prime}$ of 270 mV. The first two dyes were synthesized as described by Salmon and Hawkridge (1980). All other dyes were purchased from Aldrich Chemical Co. (Milwaukee, WI) and used without further purification.

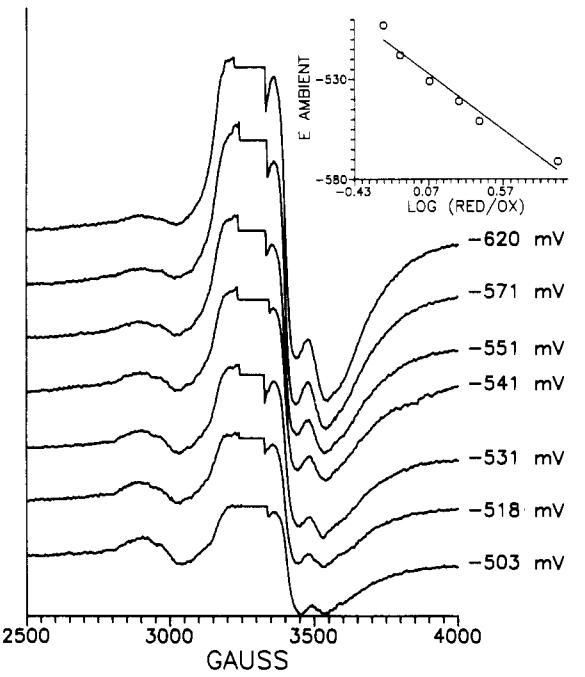

FIGURE 2: Determination of the midpoint potential for the [4Fe-4S]$^{2+/1+}$ couple by EPR spectroelectrochemical titration. The C/Fe-SP (150 μM) was in Tris-HCl/KCl (50 mM, pH 7.6/200 mM). EPR conditions were as follows: temperature, 9 K; field set, 3300 G; gain, 5 × 10^3; power, 0.5 mW; scan rate, 500 G/min; frequency, 9.300 GHz. Ambient potentials in order of decending intensity are shown. An intense peak which occurred at $g = 2.0$ (3320 G in this figure) due to the dye radical was subtracted from the spectra. (Inset) Nernst plot of the EPR titration. The signal intensity was calculated by double integration of the spectrum.

Results

Titration of the [4Fe-4S] Cluster in the C/Fe-SP. Titration of the [4Fe-4S] center in the C/Fe-SP protein was performed with both methyl viologen and TRIQUAT as redox mediators, each at 1 mM concentration, in a buffer consisting of Tris-HCl/KCl (50 mM/200 mM) at pH 7.6. In each titration, the cluster first was fully reduced and then oxidatively titrated. The reduced cluster is of the [4Fe-4S]$^{1+}$ type and exhibits a complicated EPR spectrum (Figure 2) which is identical with that described earlier (Ragsdale et al., 1987). The only EPR-active form of this cluster is the reduced 1+ state; thus, the concentration of the reduced cluster can be calculated by measuring the EPR signal intensity. The potential was equilibrated between −500 and −620 mV, the sample frozen, and the EPR spectrum recorded for each potential. The signal intensity was obtained both by double integration of the spectra and by measuring the intensity of the spectral feature at $g = 1.94$ (3425 G in Figure 2). Double integration of the spectrum of the fully reduced cluster yielded a spin intensity of one spin per mole. The shape of the EPR signal did not change throughout the experiment, indicating that the cluster remained intact and was not perturbed during the titration. When the data were plotted in the form of a Nernst plot (Figure 2, inset), the formal equilibrium reduction potential of the [4Fe-4S]$^{2+/1+}$ couple was calculated to be −523 mV (±5

mV) vs NHE. The slope of the Nernst plot was 56 mV. The theoretical slope of the Nernst plot for a one-electron transfer is 59 mV at 25 °C; therefore, our results indicate that the reduced cluster was oxidized by a reversible one-electron (per mole of enzyme) transfer. After oxidation, the sample was rereduced. Approximately the same formal potential, −525 mV (±5 mV), was calculated, demonstrating that the cluster can be reversibly oxidized and reduced.

Since the initial titration was performed in Tris-HCl, we examined the redox chemistry of the cluster in Bicine buffer. The pH of solutions in Tris-HCl buffers are known to increase significantly (~3.3 pH units) upon freezing to liquid nitrogen temperatures, whereas the pH in Bicine solutions decreases slightly by 0.4 pH unit. By performing a redox titration in K-Bicine buffer, pH 7.6, the calculated midpoint reduction potential of the [4Fe-4S] cluster was −532 mV (±7 mV) with a slope of 56 mV (±6 mV).

We also removed the cobamide factor from the C/Fe-SP and titrated the [4Fe-4S] cluster with EPR spectroelectrochemistry. The EPR spectra of the cluster from the cobamide-free enzyme was identical with that of the native protein both in line shape and in g values. The Nernst plot derived from the titration of the cluster yielded a cluster midpoint reduction potential of −517 mV, very similar to that of the native enzyme showing that removal of the corrin ring does not have a major effect on the reduction potential of the cluster.

Titration of the Cobalt Center in the C/Fe-SP: $Co^{2+/1+}$ Couple. Methyl viologen and TRIQUAT were used as redox mediators in the titration of the $Co^{2+/1+}$ couple of the C/Fe-SP. Co^{2+} is the only EPR-detectable form of Co; thus, the amount of Co^{2+} can be quantitated by determining the intensity of the Co^{2+} EPR signal. The EPR spectrum of the Co^{2+} form of the corrinoid in the C/Fe-SP (Figure 3) is identical with that reported earlier (Ragsdale et al., 1987) and is characteristic of a corrinoid in which the lower axial ligand, 5-methoxy-benzimidazole, remains uncoordinated to the cobalt[1] (base off). Data were analyzed by measuring the height of the 2526-G peak of the Co^{2+} signal. Analysis by the Nernst equation (Figure 3, inset) yielded an $E^{\circ\prime}$ of −504 mV (±5 mV) and a slope of 60 mV, indicating that one electron is transferred per mole.

The $Co^{2+/1+}$ redox couple of the C/Fe-SP was titrated in three different pH buffers in addition to the titration in Tris-HCl just described. The effect of temperature on the pH of different buffers was studied previously (Williams-Smith et al., 1977). The low temperatures required for EPR spectroscopy result in huge changes in the pH of some buffer solutions. The pH of solutions of Bicine (−0.4 pH unit) and Hepes (−0.4 pH unit) changes only slightly upon freezing at liquid N_2 temperatures; however, the pH of Tris-HCl solutions increases by 3.3 pH units, and the pH of potassium phosphate buffer solutions decreases by 1.1 pH unit. In K-Bicine buffer at pH 7.6, the midpoint reduction potential was determined to be −495 mV (±6 mV) vs NHE with a slope of 17 mV (±2 mV). In Bicine at pH 8.8, the formal potential was calculated to be −496 mV (±2 mV) with a slope of 17 mV (±1 mV), showing that the E° of the $Co^{2+/1+}$ is pH independent at least

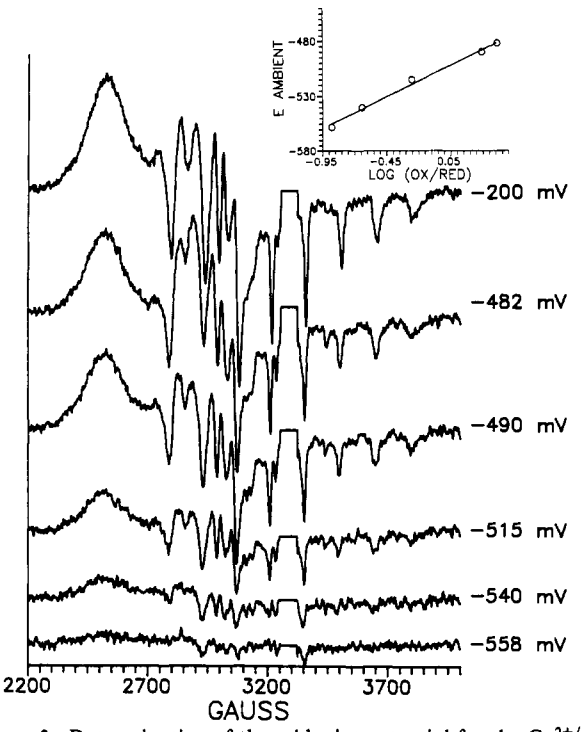

FIGURE 3: Determination of the midpoint potential for the $Co^{2+/1+}$ couple of the C/Fe-SP by EPR spectroelectrochemical titration. The C/Fe-SP (400 μM) was in 50 mM Tris-HCl and 200 mM KCl, pH 7.6. EPR conditions were as follows: temperature, 100 K; field set, 3100 G; gain, 2×10^4; power, 10 mW; scan rate, 500 G/min; frequency, 9.279 GHz. The poised ambient potentials are shown. The horizontal lines at ca. 3315 are from the remnant of the peak of the reduced dye which was subtracted from the spectra. (Inset) Nernst plot of the EPR titration. The intensity of the spectral feature at 2526 G was measured.

over this range. In phosphate at pH 7.6, the formal potential was calculated to be −498 ± 20 mV with a slope of 57 ± 31 mV. In Hepes, the formal potential was −500 mV (±2 mV), and the slope was 17 mV (±1 mV). The slopes for the titrations performed in Bicine and Hepes buffers are unusual and would correspond to an approximately three-electron transfer. Obviously, reduction of Co^{2+} to Co^{1+} is a one-electron process, so the unusual slopes relate to some as yet unknown aspect of the redox system. Unusual slopes in a Nernst plot often related to nonreversibility of the oxidation–reduction process. However, all our titrations were performed in both oxidative and reductive directions and are clearly reversible. We should note that titration of the [4Fe-4S] cluster in Bicine yielded a one-electron slope, just as the titration in Tris (above); thus, the unusual slope is not due solely to properties of the buffer. It is possible that the buffer coordinates to the Co center; however, we do not yet understand how these buffers are influencing the slopes of the titrations.

Because of the importance of the cluster in methylation of CODH (Ragsdale et al., 1987), we thought it was possible that one role of the cluster could be to stabilize the Co^{1+} state, thereby raising the reduction potential of the $Co^{2+/1+}$ couple. We found that the EPR spectrum of the Co^{2+} cobamide in the cluster-disrupted C/Fe-SP was identical with that of the native protein. The formal reduction potential was calculated was −495 mV (±7 mV) with a slope of 50 mV (±6 mV).

Titration of the Cobalt Center in the C/Fe-SP: $Co^{3+/2+}$ Couple. Several methods were attempted to determine the potential of the $Co^{3+/2+}$ couple of the C/Fe-SP, including microcoulometry, EPR, and UV–visible spectroelectrochemistry, by poising the potential between 200 and 400 mV with Fe(III)/Fe(II) hexacyanide and TMPD (both at a final

[1] When the C/Fe-SP is in a solution containing 0.3 M ammonium sulfate, we observed complicated EPR spectra apparently due to a mixture of ammonium-ligated and H_2O-ligated Co^{2+}. Ligation of the ammonium nitrogen (for nitrogen $I = 1$) would result in a three-line superhyperfine splitting of the Co^{2+} peak. Removal of the ammonium sulfate resulted in the regeneration of the clean spectra as shown in Figure 3.

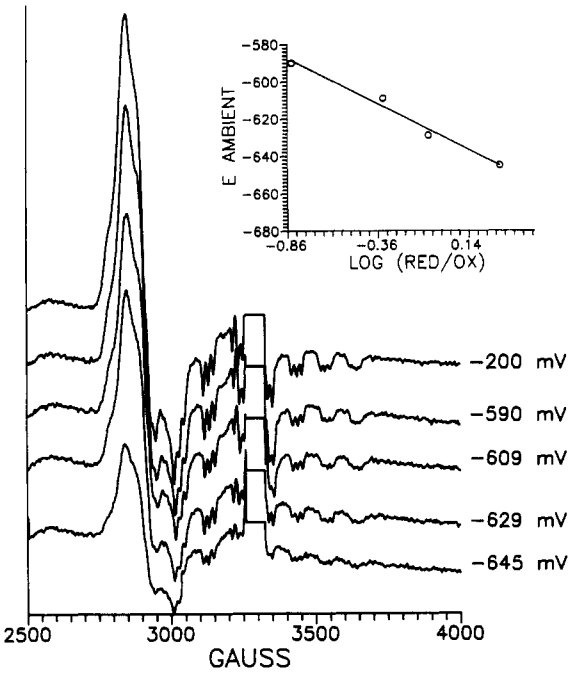

FIGURE 4: Determination of the midpoint potential of the $Co^{2+/1+}$ couple of the 27-kDa CP. The 27-kDa CP (750 μM) was in 50 mM Tris-HCl and 200 mM KCl, pH 8.0. EPR conditions were as follows: temperature, 100 K; field set, 3300 G; gain, 1×10^4; power, 10 mW; scan rate, 500 G/min; frequency, 9.280 GHz. Ambient potentials are shown. (Inset) Nernst plot of EPR titration by following the intensity of the peak at 2855 G.

concentration of 5 mM) as mediators. Apparently, the mediator, once oxidized, was rereduced by the protein, even when the mediator was in high concentration. At these high potentials, we also noted denaturation of the protein during the experiment. We observed that the much of the corrinoid had dissociated from the protein during the titration as determined by gel filtration chromatography after the experiment. We were only able to quantitatively oxidize ca. 50% of the Co^{2+}, on the basis of the decrease in the EPR signal intensity. On the basis of these EPR experiments and similar titrations with UV–visible spectroelectrochemistry, we approximate the $E^{\circ\prime}$ of the couple as between 330 and 370 mV. In other experiments, we chemically poised the potential at 350 mV by adding an appropriate ratio of Fe(III)/Fe(II) hexacyanide or TMPD and determined that the EPR signal was 50% reduced, demonstrating that the $E^{\circ\prime}$ was approximately 350 mV (±20 mV).

Redox Studies of the Methylated C/Fe-SP. The C/Fe-SP was methylated with methyl iodide after electrochemical reduction. Following the procedures described above, the C/Fe-SP was 100% methylated as judged by EPR spectroscopy and by liquid scintillation counting of the methylated protein solution. After 1-h incubation at −565 mV, we were unable to detect the formation of any CH_3-Co^{2+} by EPR spectroscopy. In addition, there was no loss of the methyl group from the C/Fe-SP as detected by liquid scintillation counting of the protein solution after chromatography on Sephadex G-25.

Titration of the Cobalt Center in the 27-kDa CP: $Co^{2+/1+}$ and $Co^{3+/2+}$ Couples. By EPR spectroelectrochemistry, we determined the midpoint potentials for both the $Co^{2+/1+}$ and $Co^{3+/2+}$ couples of the 27-kDa CP. In initial experiments at pH 7.6, we were unable to obtain adequate Nernst plots due to the low midpoint potential, the instability of reduced TRIQUAT, and the hydrogen overpotential; however, we estimated that the potential was between −600 and −650 mV at pH 7.6. At pH 8.0, generation of hydrogen at the gold electrode occurs at lower potentials; thus, we were successful

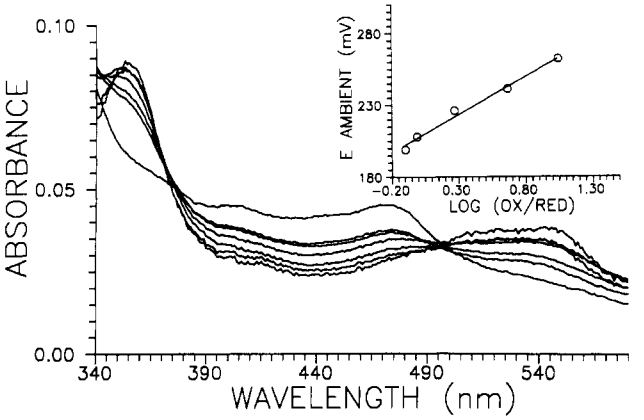

FIGURE 5: Determination of the midpoint potential for the $Co^{3+/2+}$ couple of the 27-kDa CP. Shown are the Nernst plots of EPR titrations by following the signal intensities at 2855 G at pH 7.6 (squares) and pH 8.0 (circles).

in poising stable potentials between −200 and −650 mV. The EPR spectrum of the Co^{2+} form of this protein (Figure 4) is identical with that seen previously and is characteristic of a base-on corrinoid (Ragsdale et al., 1987). The $Co^{2+/1+}$ couple of this CP, at pH 8.0, was found to have a midpoint potential of −630 mV (±4 mV) and a slope of 50 mV (±5 mV) (Figure 4, inset).

We also followed the oxidation of Co^{2+} to Co^{3+} by EPR spectroelectrochemistry. On the basis of a Nernst plot, the $Co^{3+/2+}$ oxidation–reduction potential for the 27-kDa protein at pH 7.6 was calculated to be 215 mV (±4 mV) with a slope of 71 mV (±5 mV) (Figure 5, squares). At pH 8.0, the midpoint potential shifted to 260 mV (±1 mV), with a slope of 75 mV (±2 mV) (Figure 5, circles). This shift in potential from pH 7.6 to pH 8.0 for the 27-kDa CP is opposite to that of the aquocobalamin, which shifts to lower potential with higher pH (Lexa & Saveant, 1976; Lexa et al., 1977). This shift is due to the pK of water which is the upper axial ligand in aquocobalamin. Hydroxide is a stronger ligand resulting in a lower potential for the $Co^{3+/2+}$.

Titration of the Cobalt Center of Vitamin B_{12} and in Free (5-Methoxybenzimidazolyl)cobamide: $Co^{3+/2+}$ Couples. The redox potentials for aquocobalamin and free (5-methoxybenzimidazolyl)cobamide isolated from the C/Fe-SP were determined by UV–visible spectroelectrochemistry. Aquocobalamin was generated from cyanocobalamin. Cyanocobalamin was electrochemically reduced to the Co^{2+} state, extruding the CN^- ligand from the Co. We then separated the CN^- ligand with Sephadex G-10, yielding aquocobalamin. The UV–visible spectra of the isolated corrinoid from the C/Fe-SP are shown in Figure 6. The spectra of the dye have been subtracted, which results in poor spectral resolution from 340 to 360 nm. TMPD absorbs heavily from the UV to 400 nm and from 550 to 650 nm but has little absorption at 447 nm, where the analytical data were taken for the Nernst plot. The isolated corrin from the C/Fe-SP is base on, which was determined by the presence of a shoulder at 440 nm of the UV–visible spectra in the Co^{2+} form (Lexa et al., 1977). The midpoint redox potential of the free (5-methoxybenzimidazolyl)cobamide calculated from the Nernst plot is 207 mV (±3 mV) with a slope of 53 mV (±4 mV). The midpoint potential calculated for the aquocobalamin, 200 mV, is close to that determined earlier, 202 mV, by Lexa et al. (1977). On the basis of the comparison between the 27-kDa CP and the C/Fe-SP, the potential of the $Co^{2+/1+}$ couple for the free (5-methoxybenzimidazolyl)cobamide is estimated to be −610 mV vs NHE.[2]

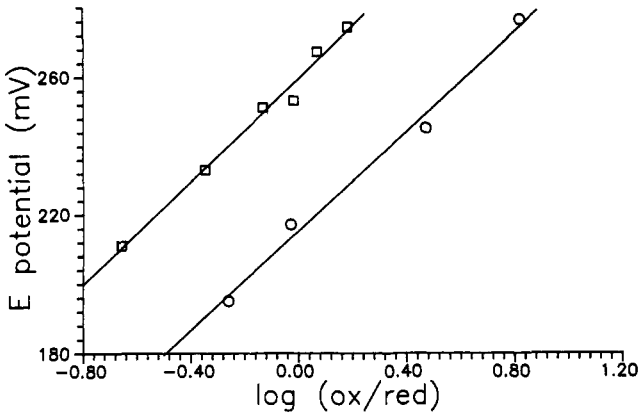

FIGURE 6: Determination of the midpoint potential for the $Co^{3+/2+}$ couple of the corrin isolated from the C/Fe-SP. The corrin (4 μM) was titrated with 20 μM TMPD in 50 mM Tris-HCl and 200 mM KCl, pH 7.6. Ambient potentials were 43, 199, 208, 226, 241, 262, and 295 mV. Potentials correspond respectively to the decrease in absorbance at 447 nm where analytical data were taken.

Discussion

The C/Fe-SP is important in the methylation reactions leading to the synthesis of acetyl-CoA by acetogenic bacteria (Hu et al., 1984; Ragsdale et al., 1987; Pezacka & Wood, 1988). It accepts the methyl group from methyl-H_4folate and then transfers this group to CODH for the final steps in the synthesis. In earlier studies (Ragsdale et al., 1987), it was found that both [4Fe-4S] cluster and the corrinoid undergo redox changes during the reaction cycle of the C/Fe-SP (see Figure 1). Both cobalt and [4Fe-4S] centers in the as-isolated protein are reduced from the 2+ to the active 1+ state before the C/Fe-SP can be methylated (Ragsdale et al., 1987). Interestingly, this reduction can be catalyzed by reduced CODH in the absence of additional electron carriers (Ragsdale et al., 1987). Thus, understanding the redox chemistry of the metal centers is preeminent in understanding the mechanisms of the methylation reactions of the C/Fe-SP. In the work reported here, we have determined the midpoint reduction potentials of the metal centers of the C/Fe-SP, and in order to assess the importance of the base-off versus the base-on conformation, we also determined the midpoint potentials of a 27-kDa CP and of the free cobamide. Recently, Schulz et al (1988) reported the properties of a membrane-associated corrinoid protein from *Methanobacterium thermoautotrophicum*. Calculated redox potentials for the $Co^{2+/1+}$ couple of this protein were reported to be quite positive (−350 mV); however, these authors also obtained approximately −350 mV for the potential of hydroxycobalamin, which has been determined by others to be −600 mV (Saveant et al., 1979), a value similar to that of other cobalamins. Further studies are required to confirm or negate the value for this membrane protein which may play a role in electron transfer in the metabolism of methanogens (Schulz et al., 1988).

We found that Co^{1+} is much more stable in the C/Fe-SP than in the 27-kDa CP or other model corrinoids, including the cofactor isolated from the C/Fe-SP. The $Co^{2+/1+}$ couple of the C/Fe-SP is 125 mV more positive than that of vitamin B_{12} or the same corrinoid factor [(5-methoxybenzimidazolyl)cobamide] bound to the 27-kDa CP. The two most striking differences between the corrinoid environments in the two proteins are that the C/Fe-SP contains no metal centers other than the corrinoid which is in the base-on form. We considered both possibilities: that the cluster played a major role in stability of the Co^{1+} state and that the base-off conformation was of key importance. The stability of the 1+ state is only slightly affected by the [4Fe-4S] cluster since disruption of the cluster with mercurial reagents had no detectable effect on the reduction potential of the $Co^{2+/1+}$ couple. This treatment, however, results in total loss of activity of the C/Fe-SP in acetyl-CoA synthesis. In addition, removal of the corrin had little effect on the reduction potential of the $[4Fe\text{-}4S]^{2+/1+}$ couple. Furthermore, the corrinoid center and the [4Fe-4S] cluster are distant enough in the protein that dipolar or exchange interactions are not detected by either Mössbauer or EPR spectroscopy (Ragsdale et al., 1987). Apparently, the major reason for the increased stability of the nucleophilic Co^{1+} state is that the benzimidazole base is uncoordinated to the cobalt center. This hypothesis is consistent with electrochemical studies of model corrinoids and cobamides which lack a heterocyclic base (Hogenkamp & Holmes, 1970; Saveant et al., 1979; Lexa & Saveant, 1976).

The finding that the $Co^{2+/1+}$ couple in the C/Fe-SP has a more positive midpoint potential than most other corrinoid complexes allows the base-off corrinoid center to be more easily reduced to the active 1+ state by physiological electron donors (CO, pyruvate, H_2). Comparing the redox potential of the protein-bound versus the free corrinid, we calculate that when (5-methoxybenzimidazolyl)cobamide binds the C/Fe-SP, it becomes base off and the midpoint potential increases by 125 mV. This would correspond to 12.1 kJ/mol or 2.9 kcal/mol of stabilization energy provided to the Co^{1+} state by binding to the protein. Interestingly, methionine synthase was reported to contain a Co^{2+} corrinoid in the base-off form (Frasca et al., 1988). Further studies will be required to determine the molecular mechanism of maintaining the cobamide of the C/Fe-SP in the base-off form. Studies of model complexes have shown that steric effects and degrees of hydration can alter the equilibrium between the base-on and the base-off conformations (Pratt, 1982). Steric interference by amino acid residues in the vicinity of the lower axial position of the corrinoid would select against the base-on form.

The midpoint redox potentials reported in this paper are expected to be the same as the values that one would obtain by performing spectroscopy and electrochemistry at room temperature and appear to be pH independent between pH ~7 and ~9. Several different buffers at varying pH values were used in order to study the possible shift in the formal potential due to the apparent pH shift in the buffer due to the cryogenic temperature of the EPR measurement and in order to observe any pH dependence of the reduction potential. In Bicine (pH 7.6 and 8.8), Tris, Hepes, and potassium phosphate buffers, even though in some cases the slopes of the Nernst plots are unusual, the formal reduction potentials for both the $Co^{2+/1+}$ couples are approximately the same.

The [4Fe-4S] cluster appears to be essential for the catalytic activity of the C/Fe-SP (Ragsdale et al., 1987; W.-P Lu and S. W. Ragsdale, unpublished results) although the function of the cluster is still unknown. Our results show that the $[4Fe\text{-}4S]^{2+/1+}$ couple is isopotential (within 17 mV) with that of the $Co^{2+/1+}$ cobamide. One possible function of the cluster may be to act as the initial acceptor of electrons from reduced CODH which then would provide reducing equivalents to reduce the Co^{2+} center to the active Co^{1+} state. The ambient

[2] The difference in the potentials of the 3+/2+ couple of the C/Fe-SP and the 27-kDa CP is 135 mV; between the 2+/1+ couple, it is 125 mV. In addition, the potentials of the 3+/2+ and the 2+/1+ couples in aquocobalamin are similar to those of the corrinoid in the 27-kDa CP. Therefore, we estimate the potential of the 2+/1+ couple of the isolated corrinoid to be approximately −610 mV, which is 125 mV lower than that of the C/Fe-SP. This potential is similar to that determined by Lexa et al. (1977) for aquocobalamin (−609 mV).

intracellular redox potential during growth of *C. thermoaceticum* is not known, yet it is most likely in the −200 to −400 mV range since the major redox carriers (ferredoxin, flavins) and electron donors (pyruvate, H_2) have midpoint potentials in this range. On the basis of the potential of the $Co^{2+/1+}$ couple, in vivo the majority of the cobamide in the C/Fe-SP is expected to be in the 2+ state. Stabilization of the Co^{1+} state by 125 mV relative to base-on corrinoids thus makes reduction to the Co^{1+} state and, thus, the methylation reaction faster. However, if the kinetics are fast or if the methyl donor is strongly electrophilic, then even a partial reduction of the C/Fe-SP would allow for a rapid methylation.

Figure 1 is a proposed mechanism for the C/Fe-SP, based on the results reported here and elsewhere (Ragsdale et al., 1987; Pezacka & Wood, 1988).

Step 1. The as-isolated protein contains the cobamide in the Co^{2+} state and the cluster also in the 2+ state. Reduction of the cluster and the cobamide to the 1+ states occurs at fairly low potentials (ca. −520 mV). This reduction is most likely performed directly by the reduced form of CODH since both complex formation (Hu et al., 1984) and electron transfer (Ragsdale et al., 1987) between the two proteins have been observed. In addition, metal centers on CODH have midpoint reduction potentials in the range of the $Co^{2+/1+}$ and $[4Fe\text{-}4S]^{2+/1+}$ couples of the C/Fe-SP (Lindahl, Münck, and Ragsdale, submitted for publication). We have observed stimulation by ferredoxin of the rate of methylation of the cobamide, so apparently reduced ferredoxin can also couple to the C/Fe-SP. Ferredoxin can be reversibly oxidized and reduced in acetogens by a number of redox systems, including CODH (Ragsdale et al., 1981), hydrogenase (Ragsdale & Ljungdahl, 1984), and pyruvate–ferredoxin oxidoreductase (Drake et al., 1981).

Step 2. Once the active Co^{1+} cobamide has been formed, we postulate that MeTr catalyzes a nucleophilic attack of Co^{1+} on the *N*-methyl group of methyl-H_4folate. Co^{1+} is an extremely strong nucleophile (Abeles & Dolphin, 1976), and we have observed the formation of Co^{1+} followed by its rapid disappearance upon formation of methyl-Co^{3+} by UV–visible spectroscopy (Ragsdale et al., 1987). The [4Fe-4S] cluster was found to be oxidized after methylation of the cobamide; however, further studies are required in order to determine if oxidation of the cluster is required in the reaction cycle.

Step 3. Once methylcobamide is formed, the methyl group is transferred to CODH. The mechanism of this methyl transfer is still under investigation. Cleavage of the methyl group of methylcobamide can occur by a number of mechanisms which have been classed into three categories depending on the intermediate state of the methyl group: (a) formation of a methyl anion, (b) formation of a methyl radical, and (c) formation of a methylcarbonium ion. On the basis of our and other studies of vitamin B_{12} and analogues, we favor a mechanism involving a nucleophilic attack on the methylcobamide, generating (formally) Co^{1+} and $[CH_3^+]$, even though the reductive formation of a methyl radical intermediate before formation of the methylcysteine on CODH was recently proposed by others (Pezacka & Wood, 1988). The base-off conformation, as found in the C/Fe-SP, stabilizes methyl-Co^{3+} against homolysis (Pratt, 1982). However, Kräutler (1987) proposed that the stability of the CH_3–Co bond toward homolysis is not greatly affected by coordination of the nucleotide base. In addition, reductive cleavage of the methylcobalt bond occurs at only very low redox potentials (less than −1.0 V) (Lexa et al., 1979; Hogenkamp & Holmes, 1970).

We decided to test the possibility that the mechanism of methyl transfer from the methylated C/Fe-SP to CODH could involve a reductive cleavage mechanism. In this mechanism, the methylcob(III)alamin is first reduced to methylcob(II)alamin, followed by homolysis to yield a methyl radical and Co^{1+}. We reduced the methylated C/Fe-SP to −0.56 V and determined if there was any loss of the methyl group from the protein and if there was any reduction to the CH_3-Co^{2+} state. We were unable to detect any loss of methyl group from the methylated C/Fe-SP after incubation at these low potentials, and no EPR-active CH_3-Co^{2+} was seen. Our analysis was quantitative enough that we would have been able to detect a 5% reduction to methyl-Co^{2+} or a 5% loss of methyl groups from the protein. Thus, it appears that that reductive cleavage of CH_3-Co^{3+} does not occur at potentials above −620 mV (1 log unit more negative than the applied potential). This potential is very negative relative to physiological substrates and electron carriers which generally have redox potentials more positive than −500 mV. It is possible that at very low reduction potentials (approximately −1.0 V) this one-electron reduction of CH_3-Co^{3+} to CH_3-Co^{2+} could occur; however, this potential is much lower than could be physiologically significant.

The reactions of methylcobamide with thiol groups have been studied by Hogenkamp and co-workers (Fanchiang et al., 1984; Hogenkamp et al., 1985). Their studies indicated that thiols react by a nucleophilic displacement of the methyl group from methylcobamide, yielding the thioether and Co^{1+}. Even though these model reactions are most favorable at moderately alkaline pH values, there are well-studied examples in which the microenvironment of cysteinyl residues in proteins lowers the pK_a of the thiol proton in the pH 6–7 range [see Kallis and Holmgren (1980), for example] where the synthesis of acetate (Drake et al., 1981) and methylation of CODH (Pezacka & Wood, 1988) are most active. The reaction of methionine synthase may be analogous to that of the methylation of CODH. In this case, it is felt that there is a nucleophilic attack by the thiol of homocysteine on the methyl of the methyl-Co cobamide, producing methionine. Other studies (W.-P. Lu and S. W Ragsdale, submitted for publication) also are consistent with a nucleophilic attack by a redox-active center on a CODH on the methyl group of methyl-Co^{3+}. Thus, on the basis of the combined evidence reported above, we favor a mechanism for methylation of CODH which involves a nucleophilic displacement by some group on CODH, causing heterolysis of the methylcobalt bond, which would yield Co^{1+} and the methylated CODH intermediate. Whether this nucleophile on CODH would be an ionized thiol of cysteine (Pezacka & Wood, 1988) or possibly a metal center on CODH has yet to be determined. Clearly, CODH can form a methylcysteine intermediate which can donate the methyl to form the C-2 of acetate (Pezacka & Wood, 1988). Co^{1+} is proposed to be the initial product of the transfer of the methyl group to CODH. Co^{1+} is in equilibrium with Co^{2+}, and which form predominates in vivo depends on the equilibrium redox potential to which the cobamide is exposed and how quickly and efficiently the Co^{1+} is trapped by MeTr and methyl-H_4folate for the next reaction cycle.

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