Reactivity of a Paramagnetic Enzyme−CO Adduct in Acetyl-CoA Synthesis and Cleavage†

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ABSTRACT: Partial reactions of acetyl-CoA cleavage by the *Methanosarcina barkeri* acetyl-CoA decarbonylase synthase enzyme complex were investigated by UV−visible and electron paramagnetic resonance (EPR) spectroscopy. Reaction of the enzyme complex with carbon monoxide generated an EPR-detectable adduct with principal *g* values of 2.089, 2.076, and 2.028, and line widths of 13.76, 16.65, and 5.41 G, respectively. The EPR signal intensity was dependent upon both enzyme and carbon monoxide concentration. A second signal with $g_{av} = 2.050$ was generated by storage of the CO-exposed enzyme for 17 months at −70 °C. Reaction of the enzyme complex with low levels of CO caused reduction of the enzyme complex, but did not result in immediate formation of the NiFeC signal (designated NiFeC based on isotopic substitution studies carried out by others in analogous systems from *Clostridium thermoaceticum* and *Methanosarcina thermophila*). Further addition of CO generated the NiFeC signal, and the signal amplitude then increased progressively with increasing CO concentration. UV−visible spectra showed that enzyme Fe-S and corrinoid centers were already fully reduced at levels of CO significantly lower than needed for maximal EPR signal intensity. This result indicated that the EPR signal is formed by reaction of the reduced enzyme with CO (or a reduced one-carbon species), rather than with a one-carbon unit at the oxidation level of CO_2. Addition of coenzyme A, acetyl-CoA, or tetrahydrosarcinapterin had no effect on the EPR signal. In contrast, addition of N^5-methyltetrahydrosarcinapterin (CH_3-H_4SPt) abolished the EPR signal. EPR spectra recorded at 20−21 K revealed that reaction with CH_3-H_4SPt affects only the enzyme NiFeC signal, and does not influence other EPR-detectable Fe-S center(s). The results suggest that the enzyme−CO adduct reacts with CH_3-H_4SPt to form an EPR-silent enzyme−acetyl species. Preincubation of the enzyme complex with CO and CH_3-H_4SPt, both of which were required, produced an approximately 44-fold increase in the turnover rate of acetyl-CoA synthesis. The relevance of these findings to mechanisms involving possible reductive methylation of the enzyme and/or acetyl-enzyme formation is discussed.

Acetyl-CoA synthesis and cleavage in methanogens are catalyzed by a multienzyme complex composed of five different subunits, possibly arranged in an $\alpha_6\beta_6\gamma_6\delta_6\epsilon_6$ structure (Grahame, 1991, 1993; Grahame & DeMoll, 1995). The complex, which has been designated as the acetyl-CoA decarbonylase synthase (ACDS)[1] complex (Grahame & DeMoll, 1995), possesses CO_2/CO oxidoreductase (Terlesky *et al.*, 1986; Grahame, 1991), Co-β-methylcobamide/tetrahydropteridine methyltransferase (Grahame, 1993), and acetyl-CoA synthase activities (Grahame, 1993). Previously the ACDS complex has been referred to as either the carbon monoxide dehydrogenase complex and/or the carbon monoxide dehydrogenase−corrinoid complex. Reactions involved in overall synthesis and decomposition of acetyl-CoA have been studied (Grahame, 1991; Grahame & DeMoll, 1995), and it was established that the purified enzyme complex catalyzes two, analogous overall reactions, as follows.

$$CO + CH_3\text{-}H_4SPt + HS\text{-}CoA \rightleftharpoons \text{acetyl-CoA} + H_4SPt \quad (1)$$

$$CO_2 + 2Fd_{red}(Fe^{2+}) + 2H^+ + CH_3\text{-}H_4SPt + HS\text{-}CoA \rightleftharpoons \text{acetyl-CoA} + H_4SPt + 2Fd_{ox}(Fe^{3+}) + H_2O \quad (2)$$

In the presence of carbon monoxide, redox centers on the isolated enzyme complex undergo time-dependent, sequential reduction. Iron−sulfur centers are reduced first, followed by reduction of the corrinoid cofactor (Grahame, 1993). The measured E_o of −426 mV ($Co^{2+/1+}$) indicates that the reactive Co^{1+} oxidation state of the enzyme-bound corrinoid cofactor is readily accessible under physiologically relevant redox potentials. Rapid methyl group transfer from N^5-methyltetrahydrosarcinapterin to the enzyme Co^{1+} corrinoid compo-

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[1] Abbreviations: ACDS, acetyl-CoA decarbonylase synthase; CH_3-H_4SPt, N^5-methyltetrahydrosarcinapterin; H_4SPt, tetrahydrosarcinapterin; Fd, ferredoxin; CODH, carbon monoxide dehydrogenase; EPR, electron paramagnetic resonance; HPLC, high-pressure liquid chromatography; MOPS, 3-(*N*-morpholino)propanesulfonic acid; E_o, standard reduction potential; EC_{50}, concentration effective to obtain 50% response.

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nent has been shown by UV−visible spectroscopy (Grahame, 1993). Evidence supports a role for the corrinoid component as an intermediate carrier of the methyl group in the overall process of acetyl-CoA synthesis and cleavage.

A similar enzymatic function in eubacterial anaerobes, particularly in microorganisms of the genus *Clostridium*, is carried out by a group of enzymes that do not appear to be tightly associated in a complex (Pezacka & Wood, 1984; Ragsdale & Wood, 1985). Interaction of CO with the clostridial CO_2/CO oxidoreductase (CO dehydrogenase) has been studied extensively by EPR methods (Ragsdale *et al.*, 1982, 1983, 1985; Ragsdale & Wood, 1985; Lindahl *et al.*, 1990; Gorst & Ragsdale, 1991; Fan *et al.*, 1991; Shin & Lindahl, 1992, 1993; Kumar *et al.*, 1993; Anderson & Lindahl, 1994). Carbon monoxide reaction with the methanogen enzyme complex from *Methanosarcina thermophila* has also been studied by EPR spectroscopy (Terlesky *et al.*, 1986, 1987, Lu *et al.*, 1994). In certain cases ambiguities arise when the results of EPR studies on the two systems are compared; however, a number of common properties have been discovered.

The first EPR studies on the clostridial CO dehydrogenase were reported by Ragsdale *et al.* (1982). It was noted that a characteristic EPR signal ($g_\perp = 2.074$, $g_\parallel = 2.028$) was generated in the presence of CO. A nickel−carbon species was implicated based on the results of isotopic substitution experiments (Ragsdale *et al.*, 1983), and later it was established that iron is also an important component (Ragsdale *et al.*, 1985). In certain instances, CO-treated enzyme preparations were observed to exhibit two, overlapping signals (Ragsdale *et al.*, 1985). One of these NiFeC signals (Signal 1) was simulated computationally assuming axial symmetry of the *g*-tensor. This signal was obtained in the presence of saturating CO and 1 mM CoASH. However, it was mentioned by Ragsdale *et al.* (1985) that acetyl-CoA could substitute for CoASH in the formation of this signal. The second signal (Signal 2) with *g* values of 2.062, 2.047, and 2.028 ($g_{av} = 2.05$) was observed in the absence of CoASH. It was found that addition of CoASH converted Signal 2 into Signal 1. Moreover, different enzyme preparations yielded varying amounts of Signal 2. It was speculated that Signal 2 arises from an altered form of the enzyme (Ragsdale *et al.*, 1985; Lindahl *et al.*, 1990).

EPR studies on the methanogen enzyme also demonstrated the existence of the NiFeC spectrum upon addition of CO alone (Terlesky *et al.*, 1987). As in the case of the clostridial system, two overlapping signals (Signal I with *g* values of 2.089, 2.078, and 2.030, and Signal II with *g* values of 2.057, 2.049, 2.027) were also found with the methanogen enzyme complex. It was reported that addition of 20 mM acetyl-CoA to the CO-reduced enzyme followed by removal of CO in the headspace generated Signal II. However, without removal of CO, addition of acetyl-CoA was reported to have no effect on the EPR spectrum. Subtraction of Signal II from the spectrum generated by acetyl-CoA addition produced Signal I with nearly axial symmetry, and the overall spectrum of the CO-reacted enzyme could be computationally reproduced by addition of Signal I to Signal II. Additional similarities to the clostridial enzyme are that the $g_\parallel = 2.030$ portion of Signal I is broadened either by replacement of the iron in the enzyme with ^{57}Fe or by reaction of the natural-abundance Fe-enzyme with ^{13}CO, thus indicating that CO is interacting with Fe. This has recently been confirmed for the clostridial enzyme by Raman spectroscopy (Qiu *et al.*, 1994).

Integration of Signal 1 has produced between 0.2 and 0.4 spin/Ni-containing dimer in the clostridial system (Ragsdale *et al.*, 1982; Shin & Lindahl, 1993). The finding that the signal does not integrate to a larger number has been shown not to be caused by loss of the EPR active centers during purification procedures (Shin & Lindahl, 1993).

The rate at which the NiFeC signal forms in the clostridial system led Gorst and Ragsdale (1991) and later Kumar *et al.* (1993) to suggest that the NiFe−CO species may be involved in acetyl-CoA formation. Also, there is a body of indirect evidence suggesting that the NiFeC species may have physiological significance in the overall process of acetyl-CoA synthesis and decomposition.

Herein EPR and UV−visible spectroscopic methods are applied to study the reaction of CO with the ACDS complex from *Methanosarcina barkeri*. Direct evidence is provided that one of the physiologically relevant substrates of the ACDS complex, namely, CH_3-H_4SPt, is capable of specifically quenching the NiFeC signal. In addition, it is shown that a highly active form of the enzyme is produced in the absence of CoASH under conditions that may favor reductive methylation and/or the formation of an acetyl-enzyme intermediate.

MATERIALS AND METHODS

Reagents. Acetyl-CoA, trilithium salt (>95%, HPLC), and coenzyme A, disodium salt (>96%, HPLC), were purchased from Fluka Chemical Corp. Tetrahydrosarcinapterin (H_4-SPt) was purified from *M. barkeri* and used to prepare N^5-methyl-tetrahydrosarcinapterin (CH_3-H_4SPt) as described (Grahame, 1991). Carbon monoxide gas (research grade >99.99%) from Matheson Corp. was used as supplied. Carbon monoxide concentration in dilute aqueous solutions was estimated based on the α-value of 0.02208 for CO solubility at 23 °C (Lange, 1961). All other gases were humidified and further purified by washing with an aqueous solution of reduced methylviologen. Unless otherwise specified, all chemicals were commercial products of analytical reagent grade.

ACDS Complex. The acetyl-CoA decarbonylase synthase enzyme complex was isolated from acetate-grown cells of *M. barkeri* by anaerobic gel filtration chromatography as previously described (Grahame, 1991), and was further purified by stepwise elution from phenyl−Sepharose 4B (Grahame, 1993). Cobamide concentration was determined based on spectrophotometric measurement of dicyanocobamide formation in air-oxidized, detergent-treated samples of the enzyme as described previously (Grahame, 1991).

EPR Spectroscopy. All samples for EPR spectroscopy were prepared under strictly anaerobic conditions inside the NIH Anaerobic Laboratory (Poston *et al.*, 1971), or within a Coy-type anaerobic chamber. EPR spectra were recorded on samples from reaction mixtures containing the enzyme complex frozen in quartz EPR tubes (Wilmad Glass Co. product 707). Prior to use, EPR tubes were rinsed with anaerobic deionized water and dried with gaseous N_2. In order to allow UV−visible scanning of the sample contained in the EPR tube immediately prior to freezing, tubes were preselected for the ability to fit inside a standard semi-micro UV−visible spectrophotometer cuvette (4 mm inside width).

A Varian E-9 X-band spectrometer was used to record EPR spectra on samples maintained at 95 or 105 K. Temperature was maintained by controlled evaporation of liquid N_2 and transfer of the gas through a flow-through quartz insert dewar. Magnetic field calibration was verified with 2,2-diphenyl-1-picrylhydrazyl as a *g*-value marker. Spectra at 55 and 20−21 K were recorded on a Bruker X-band ESP-300 spectrometer equipped with a Hewlett-Packard 5352B frequency counter. Temperature control was achieved by means of an Oxford Instruments ER-910 continuous-flow liquid helium cryostat. In general, EPR samples were stored frozen in a liquid N_2 storage dewar. However, as discussed under Results, the spectrum of one sample was recorded before and after storage for 17 months in a −70 °C freezer capped with a serum stopper but otherwise not protected from air. Additional details of the conditions used for individual EPR measurements are described in the figure legends.

UV−Visible Spectroscopy. Spectrophotometric measurements were made with a Hewlett-Packard 8452A diode array spectrophotometer operating inside the NIH Anaerobic Laboratory, or inside a Coy-type anaerobic chamber. Spectral measurements on enzyme samples contained in EPR tubes were performed by holding the EPR tubes vertically in the light path of the spectrophotometer, inside and at one end of a water-filled semi-micro quartz cuvette. Each tube was scanned 4 times (in orientations produced by successive 90° rotations), and the average spectrum was calculated. Reference scans were recorded on EPR tubes that contained only water, and were subtracted accordingly.

Synthesis of Acetyl-CoA by a Highly Active Form of the Enzyme. Synthesis of acetyl-CoA from CoASH, CO, and CH_3-H_4SPt was carried out under anaerobic conditions at 37 °C in reaction mixtures prepared in a similar fashion to those described previously (Grahame, 1993). Reaction mixtures, 600 μL, contained the following components at the indicated final concentrations: CH_3-H_4SPt (0.12_6 mM), coenzyme A (0.17 mM), CO-saturated water (one-third by volume), ACDS complex (0.47 nmol of cobamide), and MOPS buffer sodium salt (50 mM) at pH 7.2. All components except coenzyme A were combined in a stoppered semi-micro cuvette and preincubated for 10 min at 37 °C. Acetyl-CoA synthesis was then initiated by addition of CoASH (10 μL of a 10 mM anaerobic stock solution). Initial rates observed after preincubation for 15 min were not significantly different from those found after 10 min; however, preincubation for 5 min [e.g., Figure 5 in Grahame (1993)] was inefficient at producing rapid initial velocities. Aliquots were removed and frozen approximately 2 min after initiation of the reactions. Subsequent analysis by reversed phase HPLC verified that acetyl-CoA was produced, and that conversion of CH_3-H_4SPt to H_4SPt was complete.

Acetyl-CoA synthesis was assayed based on spectrophotometric detection of the concomitant conversion of CH_3-H_4SPt to H_4SPt, as described previously (Grahame, 1991; Grahame & DeMoll, 1995). A reevaluation of the molar absorptivity coefficients of H_4SPt and CH_3-H_4SPt at pH 7.2 was performed. The analysis was based on quantification by reversed phase HPLC (Grahame & DeMoll, 1995) of the amount of H_4SPt and CoASH remaining after synthesis of acetyl-CoA from CO, CH_3-H_4SPt, and CoASH had reached completion in a series of reaction mixtures containing a constant level of CoASH with increasing amounts of CH_3-H_4SPt. Graphical analysis of the HPLC peak areas of CoASH and H_4SPt as a function of the amount of CH_3-H_4-SPt added to the reaction mixtures was used to determine the point at which CH_3-H_4SPt ceased to be the limiting reagent. Above this point, chromatograms showed that CoASH had been consumed completely, and that residual CH_3-H_4SPt was now present. The absolute concentration of CH_3-H_4SPt was calculated from the CoASH concentration at the equivalence point. The stock CoASH concentration was determined separately based on thiol analysis by use of Ellman's reagent with the molar absorptivity of the 2-nitro-5-thiobenzoate dianion taken as 13 600 M^{-1} cm^{-1} (Habeeb, 1973). The relative concentration of H_4SPt was obtained by comparison of the difference spectra produced during conversion of CH_3-H_4SPt to H_4SPt to the difference spectra calculated by subtracting individual spectra recorded separately on solutions of the respective compounds at the same pH. A value of 10 700 M^{-1} cm^{-1} at 304 nm (λ_{max}) was obtained for H_4SPt. At the peak of 298 nm for CH_3-H_4SPt, the value 8900 M^{-1} cm^{-1} was determined. Tests at different pH showed significant variation in the absorptivity of both compounds. A single phosphodiester group is present in the structure of H_4SPt (van Beelen *et al.*, 1984a,b). Therefore, analysis of organic and inorganic phosphate was conducted on the stock H_4SPt preparation by use of the ascorbate−molybdate procedure with or without ashing (Ames & Dubin, 1960). Based on the value of the molar absorptivity determined herein and the phosphate assay results, it was found that 0.97 (±0.02) mol of organic phosphorus was present per mole of H_4SPt. Inorganic phosphate was not detected in the H_4SPt preparation.

The molar absorptivity value for CH_3-H_4SPt at pH 7.2 of 8900 M^{-1} cm^{-1} at 298 nm was found to be comparable with the value published for N^5-methyltetrahydromethanopterin at pH 7.0 of 8600 M^{-1} cm^{-1} at 295 nm (Escalante-Semerena *et al.*, 1984). However, the value at pH 7.2 of 10 700 M^{-1} cm^{-1} at 304 nm obtained for H_4SPt does not agree with previous data on tetrahydromethanopterin at pH 7.0 of 15 200 M^{-1} cm^{-1} at 302 nm (Escalante-Semerena *et al.*, 1984). The difference absorptivity between H_4SPt and CH_3-H_4SPt at the maximum of 312 nm at pH 7.2 was found to be 2500 M^{-1} cm^{-1}. This value is used hereafter in all calculations of rates of acetyl-CoA synthesis. This value differs significantly from the published value of $\Delta\epsilon = 3600$ M^{-1} cm^{-1} (Grahame, 1991). This is because absolute concentration data previously had relied on the value of 15 200 M^{-1} cm^{-1} at 302 nm as the primary determination of H_4SPt concentration and as the basis from which the value of $\Delta\epsilon = 3600$ M^{-1} cm^{-1} at 312 nm was originally determined. Therefore, previous data on absolute concentrations of H_4SPt and CH_3-H_4SPt must be corrected accordingly. However, because the relative concentrations of CH_3-H_4SPt and H_4SPt are known from spectral differences produced by interconversion of CH_3-H_4SPt and H_4SPt in reactions of acetyl-CoA synthesis and cleavage, no correction is required for previous data pertaining to relative concentrations of CH_3-H_4SPt and H_4-SPt.

Reaction of the ACDS Complex with CO and CH_3-H_4SPt. Samples of the enzyme complex were thawed under an atmosphere of 100% argon. In order to produce concentrations of CO in the range of 5−100% saturation, aliquots of the enzyme (100 μL) were mixed in 12 × 75 mm tubes with 50 μL of argon-saturated 0.2 M MOPS buffer (pH 7.2) and different volumes of argon-saturated water. The reactions

(final volume 280 μL) were then initiated by addition of CO-saturated water. When present, other components (dithiothreitol, CoASH, acetyl-CoA, H_4SPt, CH_3-H_4SPt) were added to the solution prior to mixing with the enzyme. The reaction mixtures were then maintained under a N_2/H_2 atmosphere (approximately 1% H_2) for approximately 5 min at 24 °C, whereupon an aliquot (220 μL) was transferred to an EPR tube. Each sample was frozen in liquid N_2 immediately after recording its UV−visible spectrum (approximately 5 min after CO addition). Samples with 100% CO were prepared slightly differently, in that 180 μL of solution containing MOPS buffer was saturated with CO prior to addition of the enzyme, and the reaction mixture was subsequently agitated gently for 6−7 min under CO gas prior to transfer to the EPR tube. Transfer to EPR tubes was made by means of 1.67 mm i.d. polyethylene tubing attached to a syringe, and the technique was developed to prevent excessive suction in order to avoid degassing of samples. Control experiments showed that the signal intensity was proportional to the amount of enzyme present. Reactions that were initiated at 0.4 mM CO and maintained for 10 min prior to freezing exhibited approximately 92% of the signal intensity of samples that were frozen after 5 min.

Additional tests were conducted to quantify the amount of CO that was lost by diffusion prior to transfer of the samples to the EPR tubes. Approximately 40% of the total added CO was lost within 30 s during the initial mixing of CO−saturated water in the 12 × 75 mm tubes. Thereafter, the remaining amount of CO declined slowly. It was estimated that approximately 50% of the total CO remained in solution at the time samples were transferred to the EPR tubes for freezing. All concentrations of CO shown in figures refer to the initial level of CO to which the enzyme was exposed, and have not been corrected for the losses indicated.

In tests conducted under a defined atmosphere of CO in N_2, incubation was carried out with periodic, gentle agitation of the enzyme in a 12 × 75 mm test tube capped with a serum stopper. Both N_2 and CO were separately purified and humidified by washing with solutions of reduced methylviologen. The gas mixture was prepared in a manner similar to that described previously (Grahame, 1993), and was allowed to flow continuously above the sample at approximately 6 cm^3/min by means of syringe needles inserted through the stopper to permit gas entry and exit. Prior to introduction of the enzyme, an equal volume of buffer was preequilibrated in the tube by bubbling with the gas mixture. The enzyme was introduced through the stopper with a syringe, and immediately thereafter an aliquot of CO-saturated water was injected sufficient to increase the CO concentration to the final level of approximately 67 μM. Aliquots were removed at different times through a port constructed in the serum stopper, transferred to EPR sample tubes, and frozen in liquid N_2.

RESULTS

NiFeC EPR Spectrum. Reaction of the *M. barkeri* ACDS complex with carbon monoxide resulted in the formation of a paramagnetic enzyme−CO adduct with an EPR spectrum similar to the major NiFeC signal (Signal 1) observed by others in certain CODH preparations from sources such as *M. thermophila* and *C. thermoaceticum*. As shown in Figure 1 (panel A), formation of the signal was strictly dependent on CO. Computer simulation of the spectrum (Figure 1, panel B) demonstrated that the spectrum could be attributed

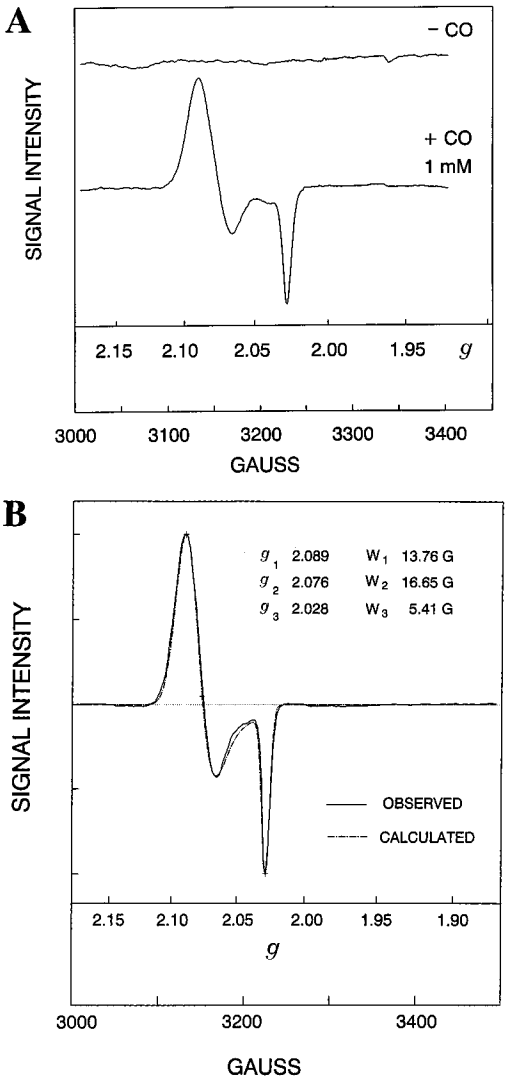


FIGURE 1: CO-dependent NiFeC EPR signal in the *M. barkeri* ACDS complex. EPR analysis was conducted on samples of the ACDS enzyme complex (18 μM cobamide) prepared at 24 °C in the presence or absence of 1 mM carbon monoxide (panel A), and in the presence of 0.4 mM CO (panel B). Samples were frozen in liquid N_2 following transfer to quartz EPR tubes under strictly anaerobic conditions as described under Materials and Methods. The total time of exposure to CO at 24 °C was 5 min. Spectra were recorded at 105 K with identical receiver gain (8×10^3) at 9.163 GHz, 40 mW microwave power, with field modulation of 8 G at 100 kHz. For computer simulation (panel B), solid-phase EPR powder spectra were computed by use of the program XPOW [Belford & Nilges, 1979; see also Lindahl *et al.* (1990) and references cited therein] generously provided by Dr. Steve Ragsdale (University of Nebraska, Lincoln, NE). A separate program, EPRANAL, was used to generate repeatedly new input files for use in automatic execution of the XPOW program as a subprocess. The spectrum calculated after each execution of XPOW was judged for closeness of fit to the experimental spectrum based on a least-squares criterion. An iterative routine was implemented to obtain global optimization of six parameters (g_1, g_2, g_3, W_1, W_2, W_3) contained in each new input file. The program EPRANAL was written in C language (Borland, Inc.) by Mr. Kenneth Gable at the USUHS Department of Biochemistry, Bethesda, MD, and run on an MS-DOS-based computer with an Intel 80486 microprocessor. The best-fit values were $g_{1,2,3}$ = 2.089, 2.076, and 2.028, and $W_{1,2,3}$ = 13.76, 16.65, and 5.408 G (40.22, 48.38, and 15.35 MHz, respectively).

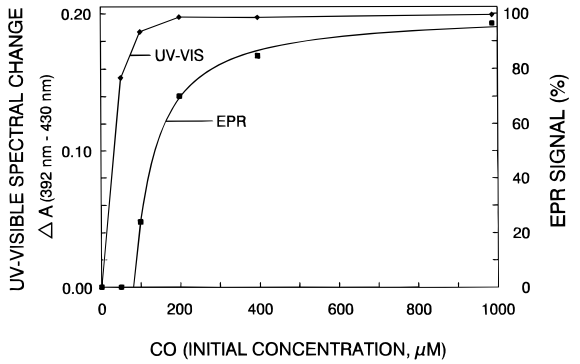


FIGURE 2: CO concentration dependence of the NiFeC signal and corresponding UV−visible spectral changes. Samples of the ACDS complex (18 μM cobamide final concentration) were reacted at 23 °C with CO at the concentrations indicated. As described under Materials and Methods, the concentrations indicated are initial levels of CO added to the enzyme, and have not been corrected for subsequent losses due to diffusion. UV−visible spectra were recorded directly on the samples contained in quartz EPR tubes as described under Materials and Methods. The samples were subsequently frozen in liquid N_2, and EPR spectra were measured as described in the legend to Figure 1. The total amount of CO remaining in solution at the time of freezing was determined to be approximately 50% of that added initially, as mentioned under Materials and Methods. The EPR signal percentage was obtained from the amplitude of the peak at $g = 2.089$. The UV−visible parameter $\Delta(A_{392} - A_{430})$ was obtained by subtracting the absorbance at 430 nm from the absorbance at 392 nm in scans on the samples containing CO after subtraction of the absorbance difference at the same wavelengths observed in the sample without CO. This parameter is sensitive to changes in the corrinoid cofactor $Co^{2+/1+}$ redox state, but also contains a minor contribution from redox changes in Fe-S centers (Grahame, 1993).

to a single paramagnetic species with rhombic symmetry of the *g*-tensor. When the iterative fitting procedure described in Figure 1 was constrained to yield only axial approximations ($g_1 = g_2$, $W_1 = W_2$) the best-fit spectrum (not shown) did not match the experimental spectrum as well as when three different principal *g* and *W* values were employed. The values obtained for the three *g* components were 2.089, 2.076, and 2.028 with corresponding line widths of 13.76, 16.65, and 5.41 G, respectively.

The area around *g* 2.05 is a region in which a second signal has been observed previously (Ragsdale *et al.*, 1985; Tersleky *et al.*, 1987; Lu *et al.*, 1994). Also, in this region the largest deviations from the calculated spectrum were observed (Figure 1B). Furthermore, the EPR spectrum observed after storage of the sample for 17 months at −70 °C, as described under Materials and Methods, showed a dramatic increase in the amplitude of the *g* 2.05 signal. The spectrum observed after storage contained two additional, resolved peaks in this region (data not shown). The spectral component produced during storage was resolved by subtraction of the spectrum observed in a freshly prepared sample from the spectrum of the sample stored for 17 months, and exhibited *g* values of 2.078, 2.046, and 2.026 ($g_{av} = 2.050$). Based on results from double integration, the $g_{av} = 2.050$ signal constituted approximately 30% of the overall signal in the sample stored at −70 °C.

Effects of CO Concentration on UV−Visible and EPR Spectra. The effect of varying CO concentration on the EPR signal intensity is shown in Figure 2. The NiFeC signal was not detected until the initial level of added CO was approximately 4-fold in excess of enzyme (cobamide). At this threshold level of CO, the enzyme corrinoid cofactor

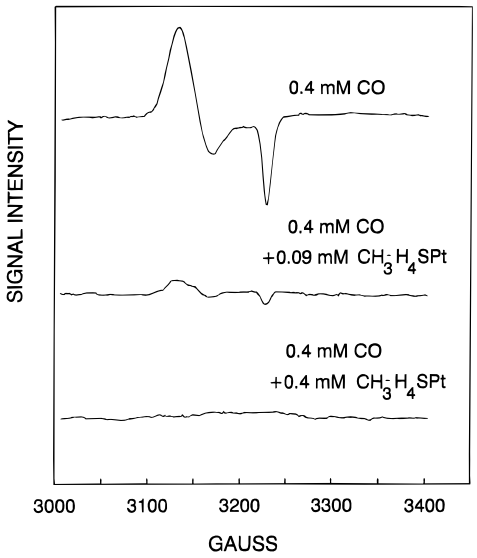


FIGURE 3: Effect of CH_3-H_4SPt on the EPR NiFeC signal. Reaction of the ACDS enzyme complex (18 μM cobamide) with 0.4 mM CO was carried out in the absence and in the presence of CH_3-H_4-SPt (0.09 and 0.4 mM) as described under Materials and Methods. The procedures for sample preparation were as described under Materials and Methods. Spectra were acquired at 95 K with all other instrument parameters as described in the legend to Figure 1.

was approximately 90% reduced, as determined by UV−visible spectral measurements. At an initial concentration of CO equal to approximately 200 μM, complete reduction of Fe-S and corrinoid centers was observed. At higher levels of CO, no further changes in the UV−visible spectrum were detected. However, further addition of CO in excess of 200 μM continued to produce an increase in the NiFeC signal intensity, as shown in Figure 2. Although the data obtained above the threshold level suggested an apparent EC_{50} of around 50 μM (25 μM after accounting for CO lost due to diffusion), additional experiments indicated that the actual EC_{50} may be considerably lower. The signal intensity was monitored as a function of time of incubation with 67 μM CO under a defined atmosphere containing 6.8% CO in 93.2% N_2, as described under Materials and Methods, and was compared with the signal intensity produced after 6 min of incubation under an atmosphere of 100% CO (saturating level). No signal was detected after 5-min incubation with 67 μM CO (a level below the threshold shown in Figure 2). However, after 15 min the signal was observed at approximately 90% of the saturating level. At 35 min, the signal intensity decreased to about 70%. The results showed that extensive development of the signal occurred after sustained incubation at a level of CO considerably lower than that required for reaction within 5 min in an open system.

NiFeC Species Reactivity with CH_3-H_4SPt. Separate addition of dithiothreitol (5 mM), coenzyme A (4 mM), acetyl-CoA (1 mM), or tetrahydrosarcinapterin (240 μM) had no effect on either the shape or the intensity of the NiFeC EPR spectrum. However, as shown in Figure 3, addition of 90 μM N^5-methyltetrahydrosarcinapterin (CH_3-H_4SPt) markedly attenuated the signal. At higher levels of CH_3-H_4SPt (400 μM), the NiFeC signal disappeared completely. No additional signals were detected. The effect of CH_3-H_4SPt was specific to the NiFeC signal, as shown by spectra recorded at 20−21 K (Figure 4). The spectra shown in Figure 4 also indicate that addition of CH_3-H_4SPt had no

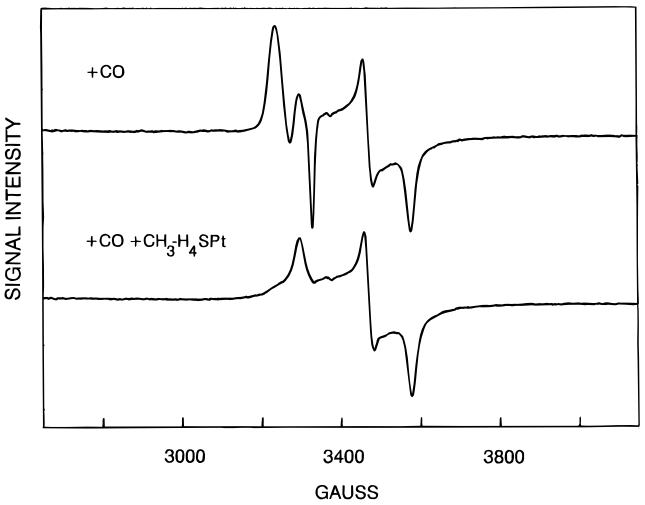


FIGURE 4: Effect of CH_3-H_4SPt on the low temperature EPR signals of the enzyme complex reacted with CO. Reaction of the ACDS enzyme complex with CO was carried out as described under Materials and Methods. The spectrum at the top was from a sample containing 0.40 mM CO (upper spectrum). The same concentration of CO was present in a separate sample that contained in addition 270 μM CH_3-H_4SPt (lower spectrum). Each spectrum consisted of an average of eight scans at 9.447 GHz, with a field modulation amplitude of 5 G at 100 kHz, obtained with 3.86 mW microwave power. Temperature was maintained at 20−21 K as described under Materials and Methods.

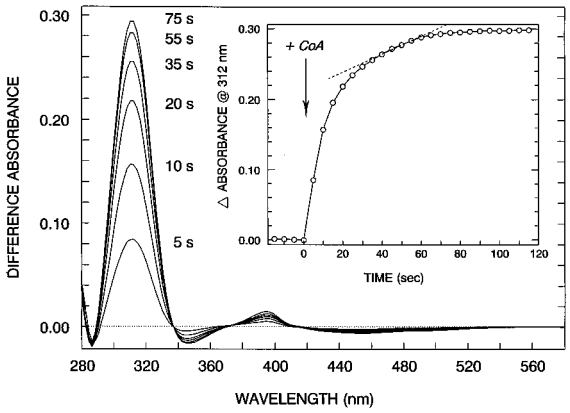


FIGURE 5: Acetyl-CoA synthesis from CO, CH_3-H_4SPt, and CoASH by a highly active form of the enzyme. Synthesis of acetyl-CoA from CO, CH_3-H_4SPt, and CoASH was carried out as previously described (Grahame, 1993), except that the enzyme complex was pre-incubated at 37 °C for 10 min in the presence of CH_3-H_4SPt and CO before the reaction was initiated by addition of CoASH. The progress of the reaction was monitored by the change in absorbance at 312 nm, as described under Materials and Methods. The procedures used for preincubation and subsequent reaction to form acetyl-CoA are described under Materials and Methods. The inset shows the progress curve as the total absorbance change at 312 nm plotted as a function of time prior to and after initiation of the reaction. Product concentration is directly related to the absorbance change by the difference molar absorptivity coefficient ($\Delta\epsilon$ = 2500 M^{-1} cm^{-1}), as given under Materials and Methods.

effect on the redox state of Fe-S centers. The data suggest the possibility that an EPR-silent acetyl-enzyme intermediate may have formed from carbon monoxide and the methyl group of CH_3-H_4SPt.

Rapid Synthesis of Acetyl-CoA. In order to test for an influence on the rate of acetyl-CoA synthesis, preincubation of the enzyme complex at 37 °C was carried out in the presence of the substrates CH_3-H_4SPt and CO, both individually and in combination. After the incubation, acetyl-CoA synthesis was initiated by addition of CoASH. Preincubation for 10 min in the presence of both CH_3-H_4SPt and CO, as described under Materials and Methods, resulted in a very high initial rate of acetyl-CoA synthesis, as shown in Figure 5. The reaction exhibited two phases. A very rapid, initial phase was observed during the first 10 s of reaction. In the reaction shown in Figure 5, slightly more than half of the CH_3-H_4SPt substrate was consumed during this rapid phase. The second, much slower phase (shown in the Figure 5 inset by the short dashed line over the interval of approximately 40−60s) corresponded to an approximately constant rate of reaction. In reactions carried out with 0.50 and 0.25 times the amount of enzyme, the duration of the initial phase was unchanged. However, as a result, the second phase in these reactions exhibited much longer, distinctly linear segments that accounted for conversion of a larger proportion of the available substrate. With lower amounts of CH_3-H_4SPt or higher amounts of enzyme, however, the reaction reached completion within the time period of the rapid initial phase, and the second phase of the reaction was not observed. The rate of reaction in both phases was found to be proportional to the amount of enzyme added. The rate of acetyl-CoA synthesis in the first phase was 2.0 μmol min^{-1} mg^{-1}. Reaction mixtures that were preincubated for either 10 or 15 min exhibited subsequent initial rates of synthesis that were approximately 44 times higher than in otherwise identical reaction mixtures that either were not pre-incubated or were preincubated for only 5 min. Separate preincubation for 10 min with CH_3-H_4SPt or CO individually did not produce the marked, rapid initial rate. However, preincubation with CO alone did cause an approximately 2-fold increase in the rate as compared with reaction mixtures that were preincubated with CH_3-H_4SPt alone, or preincubated with both CO and CH_3-H_4SPt for 5 min. A lag phase was exhibited in a reaction that was initiated by addition of enzyme and CoASH to a mixture of substrates CO and CH_3-H_4SPt that had been preincubated for 10 min.

DISCUSSION

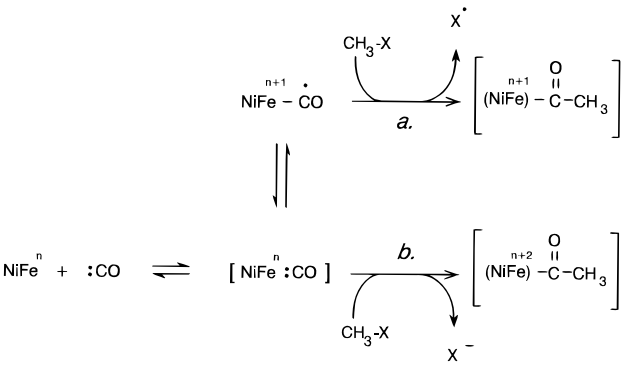
UV−visible and EPR spectroscopic techniques were used to characterize the reactivity of the *M. barkeri* ACDS complex with substrates involved in synthesis and cleavage of acetyl-CoA. Reaction of the ACDS complex with carbon monoxide resulted in the formation of a paramagnetic enzyme−CO adduct that exhibited an EPR spectrum similar to the NiFeC Signal 1 reported from both the clostridial (Ragsdale *et al.*, 1982) and the *M. thermophila* (Terlesky *et al.*, 1987) enzymes. Previous studies on the reaction of carbon monoxide with the *M. barkeri* enzyme complex indicated that CO causes sequential reduction of the Fe-S centers and cobamide groups on the enzyme (Grahame, 1993). In the present study, the analysis of CO reactivity was extended by measurement of the effect of CO concentration on the EPR properties of the enzyme. The results presented in Figure 2 showed that the NiFeC EPR signal was not formed until a threshold amount of CO had been added. At the minimum level of CO needed to form the NiFeC species, UV−visible measurements indicated that Fe-S centers on the enzyme were completely reduced, and that approximately 90% of the corrinoid cofactor was reduced to the Co^{1+} state. Formation of the NiFeC signal then resulted upon further increase in the amount of CO added. Although the dependence of the signal amplitude on CO

concentration indicated that half-maximal signal formation occurred at approximately 25 μM CO, separate experiments conducted under a CO/N_2 atmosphere in which CO was maintained at 6.8% indicated that half-maximal signal formation may occur at levels of CO significantly lower than was apparent with the open system employed in Figure 2. The finding of high signal intensity at low levels of CO is in agreement with the results observed with CODH from *C. thermoaceticum* in which signal intensity in the range of 60−80% of maximum was produced at a level of added CO equal to the concentration of enzyme [Figure 3 in Shin and Lindahl (1992)]. The results are in accord with the previous findings of Shin and Lindahl (1992) that reaction of CO to form the NiFeC adduct proceeds by interaction of CO with the reduced form of the enzyme, and that formation of the paramagnetic adduct may result from direct binding and interaction of the fully reduced enzyme with CO itself rather than with CO_2 formed during initial reduction of the enzyme. The earlier data of Gorst and Ragsdale (1991) demonstrated that prereduction was also required in order to detect the signal generated by interaction with acetyl-CoA.

Experiments were conducted in order to test for reaction of the enzyme−CO adduct with physiologically important substrates involved in the formation and decomposition of acetyl-CoA. No evidence was found for reaction with acetyl-CoA, CoASH, or H_4SPt. However, addition of low concentrations of CH_3-H_4SPt resulted in the disappearance of the NiFeC signal. Although it was mentioned by Gorst and Ragsdale (1991) that the NiFeC signal disappears when CO-treated clostridial CODH is reacted with the corrinoid/Fe-S protein and methyl iodide, we question whether or not methyl iodide adequately mimics a physiologically relevant methyl group donor for the enzyme complex from *M. barkeri*. Nonetheless, reaction of the methylated, clostridial corrinoid/iron-sulfur protein (obtained by reaction with CH_3I) was recently shown to transfer methyl to CO-reduced CODH, forming a methyl−Ni adduct (Kumar *et al.*, 1995). Our results indicate that CH_3-H_4SPt is capable of reaction (either directly or indirectly) with the enzyme−CO adduct, and further suggest the formation of an enzyme−acetyl intermediate. Since the resulting product was not detectable by EPR spectroscopy, the bonding arrangement of the nonparamagnetic, putative acetyl-enzyme would be distinct from that of the paramagnetic CO adduct. Reaction of the enzyme−CO adduct with a physiologically relevant substrate of the ACDS complex indicates that the NiFeCO species could be involved in the catalytic process of acetyl-CoA synthesis and/or cleavage. However, we are uncertain about whether or not the NiFeC species is a true intermediate along the reaction pathway, or instead might represent an addition product formed in a side reaction.

Analysis of the NiFeC signal intensity during CO titration of dithionite-reduced clostridial CODH led Shin and Lindahl (1992) to conclude that after binding of CO a subsequent electron transfer step must occur in order to generate the NiFeC signal. Shown in Scheme 1 are two alternate routes for the formation of EPR-silent enzyme−acetyl intermediates from reaction of a paramagnetic enzyme−carbonyl adduct with a methyl group (ultimately derived from CH_3-H_4SPt). In pathway *a*, direct reaction of a methyl donor species with the NiFeC species would result in an unpaired electron remaining on the enzyme group acting as methyl group donor [indicated to be a nickel site in the clostridial CODH (Kumar *et al.*, 1995)]. As a result of reaction by path *a*, a new paramagnetic species might be expected to form. However, no new EPR signals were observed following addition of CH_3-H_4SPt (Figures 3 and 4). The route designated *b* depicts methyl group donation to an enzyme−CO species postulated to be related to, but distinct from, the NiFeCO adduct. Reaction by route *b* would also result in loss of the NiFeC signal through depopulation of the [NiFe:CO] form without formation of a new paramagnetic center. One may postulate that the role of the NiFe center would be to stabilize positively charged intermediates along the reaction pathway leading to acetyl-CoA formation. Production of the reduced NiFeC center might occur only as a side-reaction resulting from high concentrations of the powerful, nonphysiological reducing agent carbon monoxide. In view of the carbene character of CO (Hine, 1964), it is tempting to speculate that the NiFe center may function to promote spin inversion of an isolated p-orbital electron, as would be required for subsequent bond formation with an electron-deficient methyl group.

Scheme 1: Hypothetical Routes for Formation and Destruction of a Paramagnetic ACDS Enzyme−CO Adduct in *Methanosarcina barkeri*[a]



[a] The enzyme center involved in binding CO is designated as NiFe. The paramagnetic NiFeC species (indicated as $NiFe^{n+1}$−CO) is depicted as being formed by electron transfer from bound CO. The group X represents the proximal site on the enzyme from which the methyl group is transferred, resulting in the formation of an EPR-silent acetyl-enzyme intermediate. Information available for the clostridial system suggests X as a nickel site (Kumar *et al.*, 1995). In designation of the acetyl adduct, it is not intended to specify bonding to a particular atom (nickel, iron, or otherwise), since the site of acetyl interaction has not yet been identified. Relative formal oxidation states are indicated. Two potential pathways for acetyl-enzyme formation (designated as *a* or *b*) are drawn for comparison.

Widely varying levels of a second form of the NiFeC signal (with g_{av} 2.05) have been observed in different preparations of the *C. thermoaceticum* enzyme (Ragsdale *et al.*, 1985; Lindahl *et al.*, 1990), and at various times also with the *M. thermophila* enzyme complex (Terlesky *et al.*, 1987; Lu *et al.*, 1994). It was shown earlier that treatment of the *M. thermophila* enzyme with 20 mM acetyl-CoA followed by manipulations to remove CO in the headspace resulted in the complete loss of features due to NiFeC Signal 1, with residual levels of the *g* 2.05 signal still detectable (Terlesky *et al.*, 1987). In our studies, acetyl-CoA had no effect on the signal shape or intensity. Furthermore, an EPR sample that contained 2 mM acetyl-CoA and saturating levels of carbon monoxide was thawed, vacuum-degassed, and refrozen, without effect on the spectrum; however, when the procedure was repeated a second time the signal was lost entirely. We have observed conditions under which the g_{av}

2.05 signal is produced as an artifact of storage at −70 °C, possibly due to oxidation. This finding is consistent with the previously mentioned conclusions that the signal is caused by an altered form of the enzyme (Ragsdale *et al.*, 1985; Lindahl *et al.*, 1990). That the *g* 2.05 signal might arise from inadvertent oxidation of the enzyme is consistent with the results found here and with reports that various preparations from different organisms have widely divergent levels of this signal.

Acetyl-CoA synthesis activity of the ACDS enzyme complex was increased approximately 44-fold by preincubation with CH_3-H_4SPt plus CO. Whereas methylation of the corrinoid coenzyme is complete in less than 1−4 s after addition of CH_3-H_4SPt (Grahame, 1993), incubation for 10 min was required to produce the highly active form of the enzyme. The conditions required to generate the highly active enzyme species are considered to promote reductive methylation at a site separate from the corrinoid coenzyme and/or the formation of an acetyl-enzyme intermediate. At present, it is not possible to distinguish between reductive methylation or acetyl formation as being directly responsible for increasing the activity of the enzyme. However, our finding that the enzyme−CO species is reactive in the presence of a methyl donor suggests the latter.

The "activated" form of the enzyme shown in Figure 5 is ultimately converted to a less active form during the reaction of acetyl-CoA synthesis. In the highly active form, the enzyme undergoes multiple turnovers. During the first 10 s of reaction in Figure 5, approximately 80 mol of acetyl-CoA is synthesized per mole of enzyme cobamide. In each catalytic event, the acetyl group would be transferred to CoASH, and then reformation of the acetyl-enzyme would be required. During the time in which the acetyl is reformed, one might consider that oxidation of the enzyme could account for progressive losses of the "activated" form. Although one could envision a similar mechanism for the loss of an enzyme intermediate that would be formed by reductive methylation, we suspect that oxidative loss of an acetyl-enzyme intermediate is likely for the reasons mentioned previously. Direct demonstration of the formation of an acetyl-enzyme is the subject of future studies to investigate the identity and stoichiometry of enzyme species formed in reactions with CH_3-H_4SPt.

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