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Characterization of the Iron-Sulfur Clusters in Ferredoxin from Acetate-Grown *Methanosarcina thermophila*

ANDREW P. CLEMENTS,[1]† LATONYA KILPATRICK,[2] WEI-PING LU,[3] STEPHEN W. RAGSDALE,[3] AND JAMES G. FERRY[1]*

Department of Biochemistry and Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061-0305[1]; Department of Chemistry, Princeton University, Princeton, New Jersey 08544[2]; and Department of Biochemistry, University of Nebraska-Lincoln, Lincoln, Nebraska 68583[3]

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Ferredoxin from *Methanosarcina thermophila* is an electron acceptor for the CO dehydrogenase complex which decarbonylates acetyl-coenzyme A and oxidizes the carbonyl group to carbon dioxide in the pathway for conversion of the methyl group of acetate to methane (K. C. Terlesky and J. G. Ferry, J. Biol. Chem. 263:4080–4082, 1988). Resonance Raman spectroscopy and electron paramagnetic resonance spectroelectrochemistry indicated that the ferredoxin contained two [4Fe-4S] clusters per monomer of 6,790 Da, each with a midpoint potential of −407 mV. A [3Fe-4S] species, with a midpoint potential of +103 mV, was also detected in the protein at high redox potentials. Quantitation of the [3Fe-4S] and [4Fe-4S] centers revealed 0.4 and 2.1 spins per monomer, respectively. The iron-sulfur clusters were unstable in the presence of air, and the rate of cluster loss increased with increasing temperature. A ferredoxin preparation, with a low spin quantitation of [4Fe-4S] centers, was treated with Fe^{2+} and S^{2-}, which resulted in an increase in [4Fe-4S] and a decrease in [3Fe-4S] clusters. The results of these studies suggest the [3Fe-4S] species may be an artifact formed from degradation of [4Fe-4S] clusters.

The *Methanosarcina* are the most metabolically versatile of the methane-producing Archaea, using H_2/CO_2, acetate, methanol, or methylated amines as growth substrates. Although the one-carbon reactions involved in the conversion of acetate to methane are well known, less is understood of the components involved in electron transport and the mechanism of energy conservation. It has been shown that, during acetotrophic growth in *Methanosarcina thermophila* TM-1, an enzyme complex with CO dehydrogenase activity catalyzes cleavage of the C-C and C-S bonds of acetyl-coenzyme A (CoA) and oxidation of the carbonyl group to CO_2 (1). An electron acceptor for the CODH enzyme complex is ferredoxin (1). This electron carrier is required for the conversion of acetyl-CoA to CH_4 and CO_2 in ferredoxin-depleted extracts of *Methanosarcina barkeri* (12). In acetate-grown *M. thermophila* (33) and *M. barkeri* (12), ferredoxin is also necessary for CO-dependent H_2 production which is coupled to ATP synthesis in *M. barkeri* (4). Thus, ferredoxin appears to be essential for electron transport and energy conservation during methanogenesis from acetate in *Methanosarcina* species.

Presently, the iron-sulfur clusters of three ferredoxins from the methanogenic Archaea have been biochemically characterized; however, none of these are known to participate in the pathway of acetate conversion to methane. The ferredoxins from methanol-grown *M. barkeri* Fusaro (DSM 804) and H_2/CO_2-grown *Methanococcus thermolithotrophicus* contain two [4Fe-4S] centers (16, 17), while the protein isolated from methanol-grown *M. barkeri* MS (DSM 800) is reported to coordinate a single three-iron center (28). The amino acid sequence of the ferredoxin from acetate-grown *M. thermophila*, as deduced from the gene (7), has the highest identity (92%) to the sequence of the three-iron ferredoxin purified from methanol-grown *M. barkeri* MS (18). Additionally, the spacing of eight cysteines in the deduced sequence (59 amino acids) suggests that the *M. thermophila* ferredoxin has the potential to coordinate two clusters of either the [3Fe-4S] or the [4Fe-4S] type per monomer. Here we report on the structure and redox properties of the iron-sulfur clusters in the *M. thermophila* ferredoxin as determined by resonance Raman (RR) spectroscopy and electron paramagnetic resonance (EPR) spectroelectrochemistry.

MATERIALS AND METHODS

Materials. All chemicals used were reagent grade.

Organism and culture conditions. *M. thermophila* TM-1 (35) was grown with sodium acetate as the carbon and energy source as described before (31) and was stored in liquid N_2.

Protein purification and manipulation. All steps involving cells or protein fractions were performed at 25 to 30°C in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) under a N_2:H_2 (19:1) atmosphere. Cell lysate was prepared as previously described (34), except that 2-mercaptoethanol was omitted from the cell breakage buffer. Ferredoxin was isolated as described before (34), without the pressure dialysis step, and collected in buffer A (50 mM TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid], pH 6.8, 1 mM $MgCl_2$, 10% [vol/vol] ethylene glycol) which contained 0.6 to 1.0 M KCl. Purified ferredoxin was stored under N_2 at −20°C.

Treatment of ferredoxin with excess iron and sulfide was performed by adding dithiothreitol, $Fe(NH_4)_2(SO_4)_2$, and Na_2S (stock solutions in anaerobic 50 mM TES buffer, pH 6.8) to the purified protein to yield approximately 10, 8, and 8 moles of each, respectively, per mole of ferredoxin; the final protein concentration was 0.4 mg/ml. The mixture was incubated at 27°C for 6 h and was then applied to a Mono-Q HR10/10 column (Pharmacia, Piscataway, N.J.) equilibrated with buffer A. The column was washed with 20 ml of buffer A

* Corresponding author. Phone: (703) 231-7121.
† Present address: Laboratory of Biochemistry, National Cancer Institute, Building 37, Room 4A-01, Bethesda, Maryland 20892.

prior to elution of ferredoxin with buffer A containing 0.7 M KCl.

Analytical methods. Protein concentrations were determined by the micro method with bicinchoninic acid reagent (Pierce Chemical Co., Rockford, Ill). Dialyzed and lyophilized ferredoxin from *M. thermophila* was the standard. Homogeneity of ferredoxin preparations was determined by denaturing polyacrylamide gel electrophoresis (12% acrylamide) using the Laemmli buffer system (23); protein was visualized with silver stain (27). Iron and acid-labile sulfur were estimated as previously described (2,13); each determination was the average of at least four replicates.

RR spectroscopy. Purified ferredoxin was concentrated to 4.7 mg/ml by incubating the sample at 35 to 40°C under an N_2 gas flow. Concentrated ferredoxin, determined to be electron-transfer competent by the metronidazole reduction assay, was stored at −70°C or in liquid N_2 until analyzed. RR spectra of the frozen protein solution held in vacuo (9) were obtained by collecting scattering 135° from the incident beam by using an ORTEC model 9315 photon counter and an IBM computer. Excitation lines were provided by a Spectra Physics 170 Ar^+ laser with the laser radiation dispersed by a Spex 1401 double monochromator fitted with a cooled RCA 31034A photomultiplier. Data analysis was performed with the spectral analysis program Lab Calc.

EPR spectroscopy and potentiometric titrations. Ferredoxin samples were frozen in liquid N_2 prior to EPR analyses. The iron-sulfur clusters in ferredoxin were titrated by using an electrochemical EPR cell as described previously (15). All redox titrations were readily reversible. Spectra were recorded on a Varian model E115 spectrometer equipped with a frequency counter (E1P model 548A) and a liquid helium cryogenic system (Heli-Tran model LTD-3-110, APD Cryogenics). Double integration of EPR signals was performed with 1 mM copper perchlorate as the standard. The intensity of the g (g factor) = 1.94 signal at potentials between −300 mV and −500 mV versus the normal hydrogen electrode was used to monitor the redox state of [4Fe-4S] clusters in the presence of methyl viologen (E'_0 = −430 mV) and benzyl viologen (E'_0 = −350 mV) (approximately 100 μM each). Disodium anthraquinone-1,5-disulfonic acid (E'_0 = −174 mV), potassium indigotetrasulfonate (E'_0 = −46 mV), methylene blue (E'_0 = +11 mV), and phenazine methosulfate (E'_0 = +55 mV) (100 μM each) were used in titrating the g = 2.02 signal of [3Fe-4S] clusters at potentials higher than −300 mV. The standard equilibrium reduction potential (E'_0) of the [4Fe-4S] and [3Fe-4S] clusters at pH 7.0 and the number of electrons (n) involved in the reaction were calculated from the Nernst equation: $E_h = E'_0 + 59/n \times \log([\text{oxidized}]/[\text{reduced}])$, where E_h is the poised potential measured at equilibrium.

RESULTS

UV–visible-light spectroscopy and elemental analyses. The UV–visible-light spectrum of the as-purified ferredoxin is shown in Fig. 1. The $A_{388/300}$ of three different preparations ranged from 0.77 to 0.82. The extinction coefficients of the monomer at 388 and 300 nm were 26.7 ± 2.5 and 33.9 ± 3.4 mM^{-1} cm^{-1}, respectively. The protein contained 7.4 ± 0.8 atoms of iron and 6.7 ± 0.2 atoms of acid-labile sulfur per monomer. These results suggested that the monomer contained two iron-sulfur centers of either the [3Fe-4S] or [4Fe-4S] type.

Exposure of the protein to air did not increase either the A_{300} or the A_{388}; instead, there was a time-dependent decrease in absorbance that was substantially greater than when incubation took place under anaerobic conditions (Fig. 2). Additionally, the stability of the clusters in the presence of air decreased as the incubation temperature was increased. These results suggested that the iron-sulfur clusters were sensitive to either oxygen or high redox potentials.

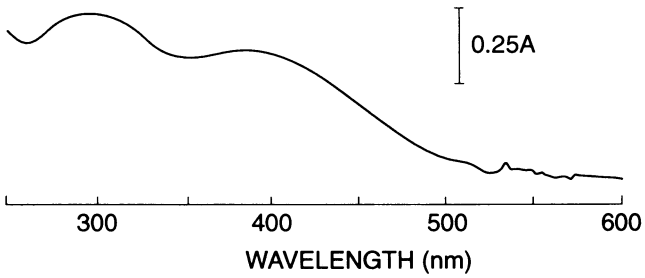

FIG. 1. UV–visible-light spectrum. As-purified ferredoxin (0.13 mg/ml) was in buffer A containing 0.7 M KCl.

RR and EPR spectroscopy. RR analyses of as-purified ferredoxin produced spectral bands at both 337 cm^{-1} and 348 cm^{-1} (Fig. 3). RR bands at 337 cm^{-1} have been previously assigned to the A_1 breathing modes of [4Fe-4S] clusters (10), while [3Fe-4S] centers have a characteristic band at 348 cm^{-1} (20). Thus, the RR results indicated that ferredoxin contained both [3Fe-4S] and [4Fe-4S] species. The intensity of the 337 cm^{-1} band was significantly greater than that of the band at 348 cm^{-1}, regardless of whether the excitation wavelength was set at 488.0 or 457.9 nm (Fig. 3). Bands at 348 cm^{-1} are often maximally enhanced at 488.0 nm, but analysis of the ferredoxin revealed only a slight increase in the band intensity when it was compared with spectra recorded at 457.9 nm. Although RR data are not quantitative, the results suggested that [4Fe-4S] clusters were the predominant species.

EPR spectroscopic analyses of as-purified ferredoxin at high redox potentials detected a paramagnetic iron species with an apparent g value of 2.02 (Fig. 4). The signal, which is characteristic of [3Fe-4S] clusters, was detected at potentials between +47 and +139 mV. Additionally, a weak signal at g = 4.3 was observed at +80 mV, possibly resulting from adventitiously bound Fe^{3+} (not shown). This signal disappeared at potentials lower than −300 mV. A minor feature at approximately g = 2.09 was detected at +105 mV (Fig. 4), but the significance of this signal is not known. The excited (3+) state of [4Fe-4S]

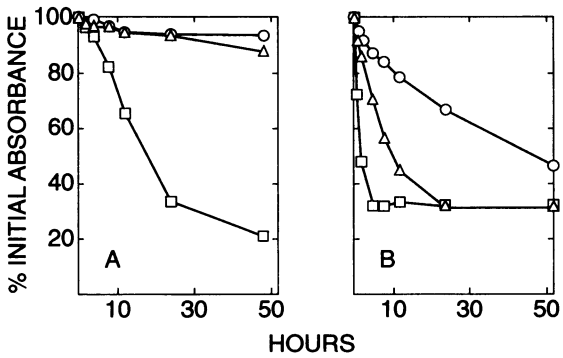

FIG. 2. Stability of the iron-sulfur clusters. Ferredoxin (0.13 mg/ml) was incubated anaerobically under N_2 (A) or aerobically (B) in buffer A containing 0.34 M KCl. The incubations were at 4°C (○), 23°C (△), or 50°C (□). The decrease in A_{388} was monitored and compared with the initial absorbance of 0.48.

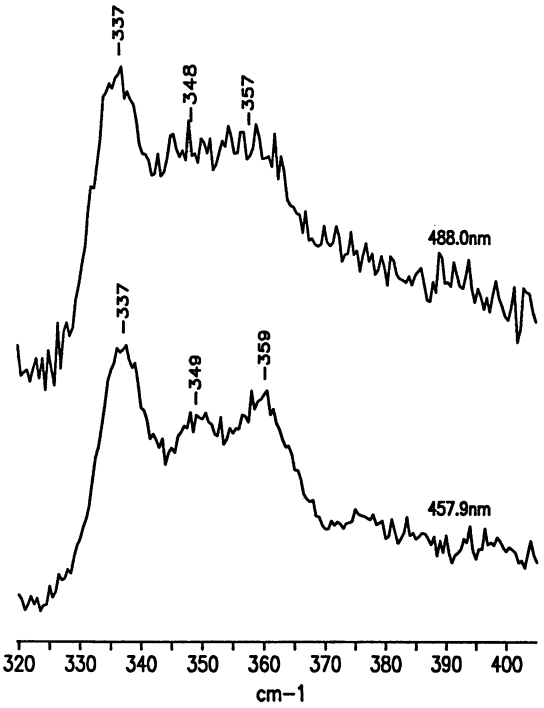

FIG. 3. RR spectroscopy. Protein (4.7 mg/ml) was thawed and loaded immediately into the sample cell of the spectrophotometer (5 min air exposure). Experimental conditions: laser power, 200 mW; slit width, 6 cm^{-1}; wavelength, 488.0 nm or 457.9 nm.

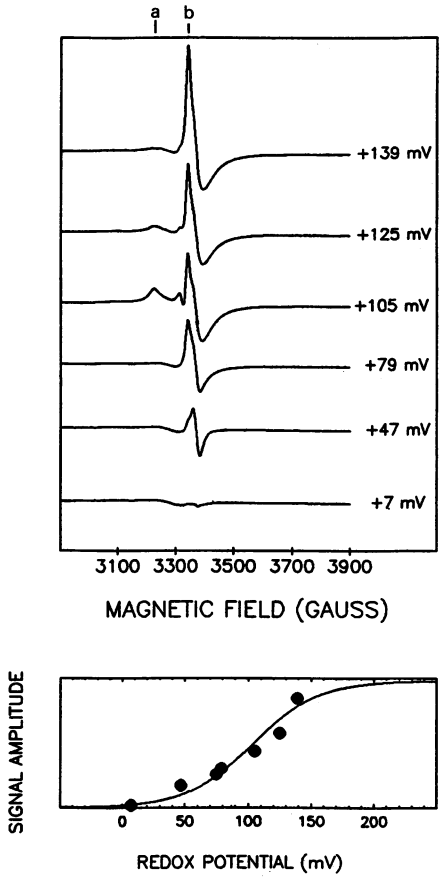

FIG. 4. EPR spectroscopy of the [3Fe-4S] clusters at different redox potentials. (A) Spectra. Sample: 700 μl of 50 mM TES buffer (pH 7.0) contained 100 μM ferredoxin (7.0 Fe per monomer), 1 mM MgCl, 8.5% (vol/vol) ethylene glycol, and 0.6 M KCl. Experimental conditions: temperature, 9 K; microwave power, 2 mW; microwave frequency, 9.451 GHz; receiver gain, 2×10^4, modulation amplitude, 10 G; modulation frequency, 100 kHz. The g values at the top of the figure: a, 2.09; b, 2.02. (B) Titrations. The amplitude of the $g = 2.02$ signal was monitored as the redox potential was varied. The best fit lines were based on a one-electron transfer and the E'_0 was determined by a Nernst analysis.

centers in high-potential iron proteins has axial values at 2.03 and 2.08 to 2.11 (3). The $g = 2.02$ signal corresponded to 0.4 spins per monomer when the redox potential was poised at +142 mV. The E'_0 (pH 7) of the [3Fe-4S] cluster was +103 ± 10 mV (slope = 60 ± 2.5 mV; $R^2 = 0.96$) (Fig. 4).

At potentials lower than −300 mV, a signal with g values at 2.08, 1.92, and 1.88 developed (Fig. 5). The spectrum became more complex as the redox potential was poised below −350 mV, a phenomenon which has been attributed to spin-spin coupling between two paramagnetic $[4Fe\text{-}4S]^{1+}$ clusters (24). Spectra recorded at potentials between −335 and −480 mV showed a mixture of the singly- and fully-reduced forms of ferredoxin (Fig. 5). These spectra had line shape and g values almost identical to those recorded for the *Clostridium pasteurianum* ferredoxin (24, 30), although a feature at $g = 2.12$ (3097 G) in the fully-reduced *C. pasteurianum* ferredoxin (30) was not observed in the protein from *M. thermophila*. The feature at $g = 2.08$ in the *M. thermophila* ferredoxin increased in intensity as the potential was decreased from −335 to −404 mV, but it then appeared to decrease as the redox potential was lowered from −404 to −480 mV (Fig. 5). A similar feature at $g = 2.06$ was previously observed in the *C. pasteurianum* ferredoxin (30) at potentials between −377 and −463 mV. Double integration of the spectrum of the fully-reduced *M. thermophila* ferredoxin (E_h = −480 mV) corresponded to 2.1 spins per monomer.

The E'_0 of the [4Fe-4S] centers at pH 7 was −407 ± 8 mV (slope = −62 ± 3 mV; $R^2 = 0.97$). This value was obtained by plotting the redox potential versus the signal intensity from double integration of the EPR signals at each potential (Fig. 5). An E'_0 value of −414 ± 4 mV (slope = −60 ± 2 mV; $R^2 = 0.99$) was calculated when the signal amplitude at $g = 1.94$ was plotted versus redox potential. The monophasic slope of approximately −60 mV, when either method was used, indicated that both [4Fe-4S] clusters participate in one-electron transfers and have a similar E'_0.

Reconstitution of [4Fe-4S] clusters. Although anaerobic precautions were taken during the purification of ferredoxin, one preparation (8.5 Fe per monomer) contained 0.24 spins attributable to [3Fe-4S] centers and 1.02 spins attributable to [4Fe-4S] centers. The result suggested that the [4Fe-4S] centers may have been altered during purification, possibly by conversion to the [3Fe-4S] type. Therefore, we attempted to reconstitute the [4Fe-4S] centers by treating the ferredoxin with excess Fe^{2+} and S^{2-} in the presence of dithiothreitol. The treatment did not alter the amount of iron (8.2 Fe per monomer) and the EPR spectra had identical line shapes and g values when compared with those of the as-purified protein (data not shown). However, the treatment decreased the intensity of the [3Fe-4S] signal to 0.16 spins per monomer and increased the [4Fe-4S] signal to 2.06 spins per monomer. The increase in spins associated with the treatment suggested that

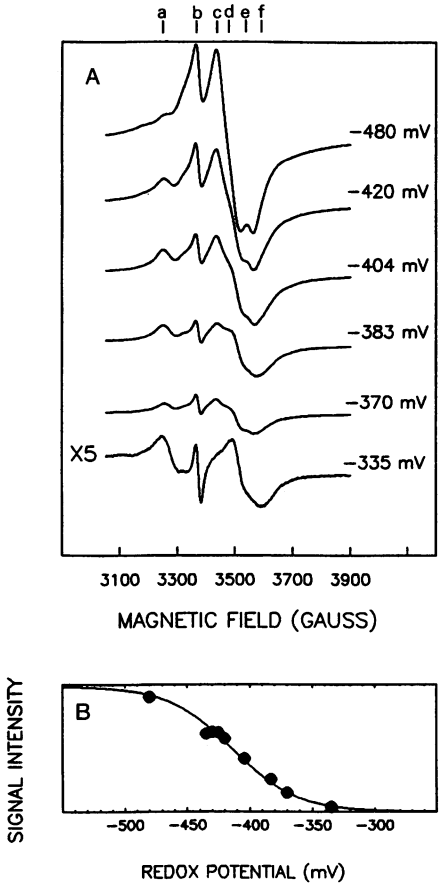

FIG. 5. EPR spectroscopy of the [4Fe-4S] clusters at different redox potentials. (A) Spectra. The *g* values at the top of the figure: a, 2.08; b, 2.01; c, 1.97; d, 1.94; e, 1.92; f, 1.88. (B) Titrations. The intensity of the $g = 1.94$ signal was monitored as the redox potential was varied. Experimental conditions and analyses were the same as those described in the legend to Fig. 4.

some of the [3Fe-4S] centers were converted to the [4Fe-4S] type in vitro.

DISCUSSION

RR and EPR spectroscopic studies suggest that the native ferredoxin from acetate-grown *M. thermophila* contains two interacting [4Fe-4S] clusters per monomer. Based on these results and the deduced amino acid sequence (7), the calculated monomer mass of ferredoxin, including iron and sulfur, is approximately 6,790 Da. The E'_0 of the [4Fe-4S] centers is −407 mV, which is within the range of −150 to −500 mV reported for low-potential [4Fe-4S] clusters (6, 25). Ferredoxins of similar size with similar cluster compositions and E'_0 have been isolated from H_2/CO_2-grown *M. thermolithotrophicus* (5, 17) and *C. pasteurianum* (24, 30, 32); however, the amino acid sequence of the *M. thermophila* ferredoxin shares only 34 and 42% identity, respectively, with these ferredoxins (7). It is surprising that the ferredoxin from *M. barkeri* MS (18), with an amino acid sequence 92% identical to the *M. thermophila* ferredoxin, is reported to contain a single three-iron center (28). Reconstitution of one preparation of *M. thermophila* ferredoxin resulted in an increase in intensity of the EPR signal from [4Fe-4S] clusters and a decrease in the intensity of the signal from [3Fe-4S] clusters. These effects suggest that the [4Fe-4S] centers were degraded. The lability of the iron-sulfur clusters of the ferredoxin from *M. thermophila*, even when purified under anaerobic conditions, suggests that some clusters could also have been damaged during the isolation of the homologous ferredoxin from *M. barkeri* MS. It has been reported that the 2 × [4Fe-4S] ferredoxins isolated from *Thermodesulfobacterium commune* (14), *M. thermolithotrophicus* (17), and *C. pasteurianum* (21) contain a small amount of [3Fe-4S] clusters. In the case of the *T. commune* ferredoxin, it was proposed that the [3Fe-4S] species resulted from oxidative damage during purification. Ferredoxin II from *Desulfovibrio gigas* contains a single [3Fe-4S] cluster with an E'_0 of −130 mV (29).

The final step in electron transport in methanogenesis from all substrates is CoM-S-S-HTP reduction to HS-CoM and HS-HTP ($E'_0 = -200$ mV) (11, 19, 22). CO-dependent CoM-S-S-HTP reduction has been detected in extracts of both *M. thermophila* (8) and *M. barkeri* MS (12) grown on acetate; the reaction in the latter organism requires ferredoxin. Low-potential [4Fe-4S] clusters would be expected in the *M. thermophila* ferredoxin which functions in accepting electrons from the CO dehydrogenase complex following oxidation of the carbonyl group of acetyl-CoA, or CO, to CO_2 ($E'_0 = -540$ mV) (26). Thus, a redox center with a E'_0 as high as +103 mV most likely would not participate in electron transfer during methanogenesis from acetate. On the basis of the results reported here, it appears that the [3Fe-4S] cluster in the *M. thermophila* ferredoxin is an artifact of oxidative damage and is not physiologically relevant.

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