Electrochemical and Spectroscopic Properties of the Iron-Sulfur Flavoprotein from *Methanosarcina thermophila* 

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An iron-sulfur flavoprotein (Isf) from the methanoarchaeon *Methanosarcina thermophila*, which participates in electron transfer reactions required for the fermentation of acetate to methane, was characterized by electrophoresis and EPR and Mössbauer spectroscopy. The midpoint potential ($E_m$) of the FMN/FMNH$_2$ couple was $-0.277$ V. No flavin semiquinone was observed during potentiometric titrations; however, low amounts of the radical were observed when Isf was quickly frozen after reaction with CO and the CO dehydrogenase/acytly-CoA synthase complex from *M. thermophila*. Isf contained a $[4Fe-4S]^{2+/3+}$ cluster with g values of 2.06 and 1.93 and an unusual split signal with g values at 1.86 and 1.82. The unusual morphology was attributed to microheterogeneity among Isf molecules. The $E_m$ value for the 2+/1+ redox couple of the cluster was $-0.394$ V. Extracts from H$_2$-CO$_2$-grown *Methanobacterium thermoautotrophicum* cells catalyzed either the H$_2$- or CO-dependent reduction of *M. thermophila* Isf. In addition, Isf homologs were found in the genomic sequences of the CO$_2$-reducing methanoarchaea *M. thermoautotrophicum* and *Methanococcus jannaschii*. These results support a general role for Isf in electron transfer reactions of both acetate-fermenting and CO$_2$-reducing methanoarchaea. It is suggested that Isf functions to couple electron transfer from ferredoxin to membrane-bound electron carriers, such as methanophenazine and/or b-type cytochromes.

The methanoarchaea are strictly anaerobic microbes that evolve methane as a product of their energy-yielding metabolism and are classified within the Archaea domain (1). These microbes utilize two distinct pathways for most of the methane produced in the biosphere. In the acetate fermentation pathway (Reaction 1), the methyl group is reduced to methane with electrons derived from oxidation of the carbonyl group to CO$_2$. In the CO$_2$ reduction pathway, CO$_2$ is reduced to methane at the expense of electrons provided by either hydrogen (Reaction 2) or formate (Reaction 3).

$$\text{CH}_3\text{COO}^- + \text{H}^+ \rightarrow \text{CH}_4 + \text{CO}_2$$

$$\text{CO}_2 + 4 \text{H}_2 \rightarrow \text{CH}_4 + 2 \text{H}_2\text{O}$$

$$4 \text{HCO}_2\text{H} \rightarrow 3 \text{CO}_2 + \text{CH}_4 + 2 \text{H}_2\text{O}$$

**REACTIONS 1–3**

Energy is conserved in both pathways by an electron transport-coupled phosphorylation of ADP. Although the understanding of electron transport in both of these pathways is incomplete, flavoproteins are of major importance. A flavoprotein has been described that appears to function in electron transport pathways of CO$_2$-reducing methanoarchaea (2, 3). The oxidations of H$_2$ and formate are catalyzed by the Fe-S flavoproteins, hydrogenase, and formate dehydrogenase (4–7). The final electron transfer step for both pathways (Reaction 4) is catalyzed by heterodisulfide reductase (HDR), which is a FAD/Fe-S protein in the CO$_2$ reducer, *Methanobacterium thermoautotrophicum* (8), and a heme/Fe-S in the acetate fermenters, *Methanosarcina thermophila* (9) and *Methanosarcina Barkeri* (10). This reaction regenerates the active sulfhydryl forms of the coenzymes, CoB and CoM (8). The substrate for HDR, the heterodisulfide CoB-S-S-CoM, is the product of the methane-forming reaction (Reaction 5), which is also common to both pathways and catalyzed by methyl-CoM reductase.

$$\text{CoB-S-S-CoM} + 2e^- + 2\text{H}^+ \rightarrow \text{HS-CoM} + \text{HS-CoB}$$

$$\text{CH}_3\text{S-CoM} + \text{HS-CoB} \rightarrow \text{CH}_4 + \text{CoB-S-S-CoM}$$

**REACTIONS 4 and 5**

Recently, a homodimeric Fe-S flavoprotein Isf (iron-sulfur flavoprotein) was identified from the acetate-fermenting methanoarchaeon *M. thermophila* (11). The isf gene was cloned and sequenced, and Isf was produced in *Escherichia coli* and partially characterized. Comparisons of the deduced Isf sequence with sequences in the available protein data bases suggest that Isf is a novel Fe-S flavoprotein. Reconstitution experiments suggest Isf is a required component of the electron transport chain in the pathway for the fermentation of acetate (11). The UV-visible absorption spectrum and cofactor and elemental analyses indicate that Isf contains one FMN and either one [4Fe-4S] or one [3Fe-4S] center per monomer. The sequence of...
Isf contains several cysteine residues; however, none of the typical cysteine motifs known to accommodate [4Fe-4S] or [3Fe-4S] centers are recognizable. Thus, the identity of the Fe-S center is uncertain. Here we report on EPR and Mössbauer studies of Isf from M. thermophila, which demonstrate that the Fe-S center is of the [4Fe-4S] type. Potentiometric titrations of the [4Fe-4S] and FMN centers predict a role for Isf in the electron transport pathway leading to the formation of CH₄ in acetate-fermenting methanoarchaea. Additional results are presented that indicate Isf homologs also have a role in electron transport pathways of CO₂-reducing methanoarchaea.

**EXPERIMENTAL PROCEDURES**

**Materials**—M. thermophila Isf was produced in E. coli strain BL21(DE3) and purified as described previously (11). Isf was stored at 80 °C in 50 mM potassium phosphate buffer at pH 7.0. For production of ⁵⁷Fe-labeled Isf, E. coli cells were in medium containing 20 μM ⁵⁷Fe (Pentwood Chemicals, Inc.), which was prepared by dissolving the iron metal in 2 N H₂SO₄ at 60 °C for 1 week. M. thermophila Fd (12) was purified as described. M. thermoaerotrophicum strain Marburg was grown as described (13). The following redox dyes were used: phenosafrin (Sigma), benzyl viologen (Sigma), methyl viologen (Sigma), 1,1′-trimethylenepyridyl-2″,2″-bipyridil, and 4,4′-dimethylenepyridyl (Aldrich). All experiments utilized NANOpure deionized water, N₂ (99.98%) and helium (99.999%) were obtained from Linweld (Lincoln, NE). Helium was deoxygenated by passing through an Oxisorb column and a heated column containing BASF catalyst.

**Methods**—Protein manipulations were performed under strictly anaerobic conditions between 1 and 5 ppm oxygen. The concentrations of the oxidized Isf were determined spectrophotometrically with the extinction coefficients previously determined (11) and by the Rose Bengal assay (14).

**UV-visible Spectroscopy and Potentiometric Titrations**—Potentiometric measurements were performed as described previously (15, 16). All electrochemical potentials are reported relative to the standard hydrogen electrode. Isf (3–4 μM) was titrated at 20 °C in 50 mM potassium phosphate buffer (pH 7.0–7.85) in a solution containing methyl viologen (0.1 mM) as the mediator dye with phenosafrin (Eₚ = − 0.252 V, pH 7.0) (5 μM) as the indicator dyes. The pH measured after the experiment was recorded as the pH for the titration. The visible spectra in each experiment were obtained and stored on an Olis-14 interfaced Cary spectrophotometer. The absorbance at 480 nm was used to monitor the amount of oxidized and reduced FMN after correcting the spectra for turbidity. The reduction potentials reported were determined by potentiometric measurements in the reductive direction. After each potentiometric titration of the FMN chromophore, the iron-sulfur flavoprotein was measured to determine the redox state of the [4Fe-4S] cluster during the titration. Potentiometric measurements were performed in the reductive and oxidative directions. The system was considered to have reached equilibrium when the measured potential drift was less than 1 mV in 2 min.

Mössbauer Spectroscopy—Mössbauer spectra were recorded on a constant acceleration spectrometer, model MS-1200D from Ranger Scientific, using a Janis SuperVareTemp cryostat (model 8DT), a Lakeshore temperature controller (model 340), and a ⁵⁷Co source in a rhodium foil purchased from Isotope Products Laboratory. All isomer shifts are quoted relative to iron metal at room temperature.

**RESULTS**

**Sequence Analysis**—Resequencing of M. thermophila genomic DNA revealed an error in the previously reported isf sequence (11). The corrected isf and deduced Isf sequences are shown in Fig. 1. The corrected Isf contains 191 residues, 81 fewer than previously reported. Additionally, the C-terminal residues 177KLCVDYLIQKRNKRED191 in the corrected Isf sequence replace 177NSVMSWNFLRKIEIN191 in the previously reported Isf. Resequencing revealed the corrected isf sequence in pML701 used for the heterologous production of Isf reported here and previously (11).

**Evidence for Isf Homologs in Phylogenetically and Physiologically Diverse Methanoarchaea**—M. thermophila is physiologically distinct from Methanococcus jannaschii and M. thermoautotrophicum, which are unable to ferment acetate to CH₄ and instead oxidize H₂ and reduce CO₂ to CH₄. The genome of M. jannaschii (19, 20) contains two ORFs (MJ1083 and MJ0731) encoding predicted proteins with 194 and 192 residues that have high identity to the corrected M. thermophila Isf sequence (Fig. 2). Inspection of the genomic sequence of M. thermoautotrophicum (21) identified three ORFs that also share significant identity to Isf (Fig. 2). An N-terminal cysteine motif in Isf from M. thermophila is strictly conserved in the M. jannaschii and M. thermoautotrophicum sequences. These results suggest a previously unrecognized electron transfer role for Isf homologs in electron transport pathways of CO₂-reducing methanoarchaea. M. jannaschii, M. thermoautotrophicum, and M. thermophila represent all three currently described families of methanoarchaea, suggesting that Isf homologs are present in phylogenetically diverse methanoarchaea, which further supports a general function for this electron carrier.

**Extraction of H₂-CO₂-grown M. thermophila cells**—Extracts of H₂-CO₂-grown M. thermophila cells catalyzed the reduction of FMN thermophila Isf with either H₂ or CO as the electron donor (Fig. 3). These results suggest that Isf homologs are components of electron transport chains in CO₂-reducing methanoarchaea initiating with either hydrogenase or CO dehydrogenase (CODH). The rate of Isf reduction with CO as the electron donor was greater than with H₂, indicating that CO-dependent reduction of Isf does not require prior con-
Fig. 1. Nucleic acid sequence and predicted amino acid sequence of Isf from M. thermohalina. The DNA is presented in the 5'→3' direction. The predicted amino acid sequence of Isf is shown in single-letter code directly below the first base of each codon. *, initial base of translation stop codon.

version to H2 and CO2. The addition of ferredoxin stimulated the rate of Isf reduction 10-fold by CO and 1-fold by H2. These results suggest that 8Fe ferredoxins are able to couple the oxidation of either H2 or CO to the reduction of Isf; indeed, the sequence of the M. thermoaerotrophicum genome reveals several ORFs encoding putative 8Fe ferredoxins (21). The ability of H2 or CO to serve as electron donors for the reduction of M. thermohalina Isf in a CO2-reducing species is further indicative of a function for Isf homologs in electron transport pathways of phylogenetically and physiologically diverse methanoarchaea. Additional research with purified proteins is necessary to determine the precise role of Isf homologs in electron transport pathways of CO2-reducing methanoarchaea.

EPR Spectroscopy of the Fe-S Center—The EPR spectra of the reduced Fe-S center (Fig. 4) exhibited g values of 2.06 and 1.93 typical of [4Fe-4S] clusters; however, the signal in the region of gmin shows two, instead of one, negative absorption features with g values at 1.86 and 1.82. The relaxation properties are also indicative of a [4Fe-4S] cluster, since the g = 1.93 signal was not observable above 25 K (22). An average of 1.3 spins/molecule of dimeric Isf was determined from different samples by double integration of the EPR signal at 10 K referenced to a copper perchlorate standard. The existence of two negative features in the spectrum of the cluster is unexpected, since rhombic spectra should exhibit only three g values. The extra features did not derive from the violagen dyes, since dithionite reduction of Isf with sodium dithionite alone yielded an EPR spectrum identical to that shown in Fig. 4. In some Fe-S proteins, complex spectral features can be simplified by incubation with low concentrations of urea; however, dithionite reduction of Isf in the presence of 2 mM urea had no effect on the spectrum. The possibility that the two negative absorption features derived from strong hyperfine interaction between the unpaired electron on the cluster and a strongly coupled proton (would produce a two-line splitting because it has a nuclear spin of 1/2) also was ruled out. When Isf was extensively exchanged with D2O and reduced with dithionite, the spectrum was identical to the one possible deconvolution: a single broad quadrupole doublet with an average isomer shift, δ, of 0.45 mm/s and an average quadrupole splitting, ΔEQ, of 1.22 mm/s at 4.2 K, with the quadrupole splitting decreasing slightly (ΔEQ = 1.12 mm/s) at 100 K. These parameters are typical for [4Fe-4S] clusters in the 2+ oxidation state. The line shape of the quadrupole doublet indicates either that the iron within the cluster are not identical (one possible deconvolution: ΔEQ(1) = 1.6 mm/s, ΔEQ(2) = 1.3 mm/s, ΔEQ(3) = 1.16 mm/s, ΔEQ(4) = 0.85 mm/s) or that there is a microheterogeneous population of iron-sulfur clusters in the Isf molecules, each having slightly different quadrupole splittings.

In the Mössbauer spectrum of the reduced Isf protein recorded at 100 K (Fig. 6B), a single, very broad, nonsymmetrical quadrupole doublet is observed with an average isomer shift, δ = 0.55 mm/s, and an average quadrupole splitting, ΔEQ = 1.30
These parameters are typical of [4Fe4S] clusters in the 1+ oxidation state. The nonsymmetrical shape of the doublet, particularly noticeable in the right component, indicates that the irons within the cluster have distinct quadrupole splittings. The spectrum was fit using four distinct $D_{EQ}$ values (see Fig. 6B), which gives a reasonable but not unique deconvolution, since the quadrupole doublets of the four iron sites are not resolved. The line shape of the doublet is Voigt (gaussian distribution of a Lorentzian line shape) rather than Lorentzian, indicative of a microheterogenous environment for the iron sites.

Above 40 K, the electronic spin of the iron-sulfur cluster relaxes fast and only quadrupole interactions are observed in Mössbauer spectra; however, below 40 K paramagnetic hyperfine interactions also are observed. Mössbauer spectra of the reduced Isf protein recorded at 4.2 K (Fig. 7) exhibit paramagnetic hyperfine structure, as expected for a reduced [4Fe-4S] cluster with $S = \frac{1}{2}$. The shape of the spectra and the derived hyperfine interactions in weak applied fields are similar to those observed for the [4Fe-4S] clusters of *Bacillus subtilis* (29), *E. coli* sulfite reductase (24), and *Azobacter vinelandii* ferredoxin (25). The average of the derived hyperfine interactions for the reduced Isf protein ($A_{ave(4Fe-4S)} = 25 \pm 2$ MHz, $A_{ave(ferrous pair)} = 17 \pm 2$ MHz) are comparable with those observed for the [4Fe-4S]$^{1+}$ clusters of the ferredoxins from *B. subtilis* (29), *E. coli* sulfite reductase (24), and *A. vinelandii* ferredoxin (25). A detailed data analysis of the paramagnetic hyperfine interactions will require a high field...
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Mössbauer study.

Potentiometric Titrations of FMN and the [4Fe-4S] Cluster—The visible spectrum of Isf was monitored between 300 and 700 nm during potentiometric titration (Fig. 8). The FMN absorption dominates the spectrum of the oxidized protein. At potentials of −0.305 and −0.342 V, the flavin is almost completely reduced and the spectrum of the oxidized [4Fe-4S] cluster is dominant. The ratio of the oxidized:reduced flavin was determined by monitoring the FMN absorbance peak at 480 nm, instead of 452 nm, to minimize possible spectral interference from the [4Fe-4S] cluster. Since [4Fe-4S] clusters have their maximum absorbance between 390 and 420 nm, the cluster exhibits a negligible contribution to the 480 absorbance value. The difference extinction coefficient is calculated to be 48 mM−1 cm−1 for the dimeric protein. An $E_m^0$ value of −0.277 ± 0.003 V was determined for the FMN/FMNH$_2$ couple of Isf (Fig. 8A, inset). A Nernst plot gave a 34-mV slope for the potentiometric measurement, which is near the theoretical value of 58 mV for a one-electron transfer. The redox reaction was fully reversible, since the reduction was transiently stable semiquinone can exist under physiological conditions during the electron transfer from CODH/ACS to Isf.

The midpoint potential determined for the 2+/1+ couple of the [4Fe-4S] cluster was −0.394 V at pH 7.0 (Figs. 4 and 10). The slope of the log-linear plot was 53 mV, which is close to the theoretical value of 58 mV for a one-electron transfer. The redox reaction was fully reversible, since the reduction was titrated in both the oxidative and reductive directions. Thus, the midpoint potential of the [4Fe-4S] cluster is more than 100 mV lower than that of the FMN/FMNH$_2$ couple (Figs. 8 and 10) and is similar to the value reported for other low potential [4Fe-4S] clusters (26, 27).

DISCUSSION

The methanogenic fermentation of acetate by the methanosarcina involves cleavage of acetyl-CoA into carbonyl and methyl groups, the latter of which is reduced to CH$_4$ with an electron pair derived from oxidation of the former to CO$_2$ (1). Cleavage of acetyl-CoA and oxidation of the carbonyl group is catalyzed by a five-subunit CODH/ACS complex. The methyl group is transferred to tetrahydrodipicolinate and then to coenzyme M. Reductive demethylation of the methyl group of CH$_3$-CoM to CH$_4$ requires the electron donor coenzyme B (IIS-CoB), which pro-
dues the heterodisulfide CoB-S-S-CoM as a second reaction product. The heterodisulfide is reduced to the corresponding active sulfhydryl forms of the cofactors by HDR. The electron pair for this reduction originates from oxidation of the carbonyl group of acetyl-CoA by the CODH/ACS complex. Oxidation of either exogenous CO or the carbonyl group of acetyl-CoA is proposed to take place at the nickel/Fe-S center (center C) in the CdhA subunit of the CODH/ACS complex (28, 29), which also contains a low potential [4Fe-4S] center (28) that is proposed to shuttle electrons from center C to a low potential 8Fe ferredoxin (12). Electron transfer from ferredoxin to HDR apparently involves membrane-bound electron carriers consistent with the formation of a transmembrane proton gradient (9, 30). Possible membrane-bound electron carriers include methanophenazine (31) and/or b-type cytochromes. Cytochromes of the b-type are reduced by CO and oxidized by CoB-S-S-CoM, which suggests that they could play a role in electron transport during the fermentation of acetyl-CoA by the CODH/ACS complex. Oxidation of either exogenous CO or the carbonyl group of acetyl-CoA is proposed to take place at the nickel/Fe-S center (center C) in the CdhA subunit of the CODH/ACS complex (28, 29), which also contains a low potential [4Fe-4S] center (28) that is proposed to shuttle electrons from center C to a low potential 8Fe ferredoxin (12). Electron transfer from ferredoxin to HDR apparently involves membrane-bound electron carriers consistent with the formation of a transmembrane proton gradient (9, 30). Possible membrane-bound electron carriers include methanophenazine (31) and/or b-type cytochromes. Cytochromes of the b-type are reduced by CO and oxidized by CoB-S-S-CoM, which suggests that they could play a role in electron transport during the fermentation of acetyl-CoA by the CODH/ACS complex.
ace/acetyl-CoA synthase; FdxA S-S-CoM.

ferredoxin and the CODH/ACS complex (11). The flavin of Isf are reduced by CO in the presence of both redox centers of Isf are involved in electron transfer reaction (12), reduced ferredoxin would be expected to efficiently donate electrons to one-electron accepting b-type cytochromes.

from the [4Fe-4S] center to FMN. The [4Fe-4S] cluster exhibits a broad absorption feature in the physiological electron acceptor for Isf (which is unknown) could be a two-electron carrier; Isf would then function as a one-electron/two-electron switch. Involvement of the obligate two-electron carrier coenzyme F_{420} in the electron transport chain has been excluded (30); however, other two-electron carriers, like methanophenazine, cannot be ruled out. Another possibility is that the semiquinone radical, which is indeed transiently formed, does transfer electrons to one-electron accepting b-type cytochromes. It is plausible that the semiquinone state in Isf may become even more stabilized when Isf is complexed with other physiological electron donors/acceptors. For example, in nitrate reductase, the semiquinone is highly stabilized upon reduction by its physiological electron donor NADH although the amount of semiquinone species formed during potentiometric titrations is just 1% of the FAD present (40). This allows one electron transfer from the flavin to the b-type cytochrome in nitrate reductase. Thus, despite the instability of the flavin semiquinone in Isf during the potentiometric titrations, it is still feasible that the flavin mediates one-electron transfers from the Fe-S cluster in vivo.

The H2- or CO-dependent reduction of Isf by extracts of M. thermoaurotrophicum and the occurrence of Isf homologs in the genomes of M. thermoaurotrophicum and M. jannaschii suggest that Isf functions in CO2-reducing methanoarchaea. Cells of M. thermoaurotrophicum grow and produce CH4 with CO as the sole energy source (41), indicating a physiological role for CODH in the energy metabolism. Furthermore, methanoarchaea contain CODH in the synthesis of CO for incorporation into acetyl-CoA for cell carbon (42). The CODHs from either M. thermoaurotrophicum or M. jannaschii have not been purified and, therefore, the electron acceptor is unknown. The results presented here are consistent with ferredoxin as the electron acceptor; however, purification of the CODH is necessary to prove this hypothesis. The gene organization in M. jannaschii (20) provides additional support for diverse functions of Isf homologs in the methanoarchaea. In this organism, ORF MJ731 has a deduced sequence 40% identical to Isf from M. thermophila and is located in a gene cluster containing an ORF (MJ728) identified as CODH (20). The sequence of this putative enzyme has greatest identity to the CODH from Rhodospirillum rubrum, which catalyzes the oxidation of CO but not acetyl-CoA synthesis (43). MJ731 and MJ728 are also located near an ORF (MJ722) identified as an 8Fe ferredoxin (20), consistent with a role for this electron carrier as an electron donor to Isf. However, none of the deduced sequences of ORFs with identity to Isf in the genome of M. thermoaurotrophicum (MTH1350, MTH1474, and MTH1585) are organized near CODH or any other redox proteins (21).

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