Molecular biology and biochemical characterization of the CO dehydrogenase-linked ferredoxin from *Methanosarcina thermophila* strain TM-1

by

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## ABSTRACT

The CO dehydrogenase-linked ferredoxin from acetate-grown *Methanosarcina thermophila* was characterized to determine the structure and biochemical properties of the iron-sulfur clusters. Chemical and spectroscopic analyses indicated that the ferredoxin contained two [4Fe-4S] clusters per monomer of 6,790 Da, although a [3Fe-4S] species was also detected in the oxidized protein. The midpoint potentials of the [4Fe-4S] and [3Fe-4S] clusters at pH 7 were -407 mV and +103 mV, respectively. Evidence from biochemical and spectroscopic studies indicated that the [3Fe-4S] species may have been formed from [4Fe-4S] clusters when ferredoxin was oxidized.

The gene encoding the CO dehydrogenase-linked ferredoxin (*fdxA*) in *Ms. thermophila* had the coding capacity for a 6,230-Da protein which contained eight cysteines with spacings typical of 2[4Fe-4S] ferredoxins. A second open reading frame (ORF1) was also identified which had the potential to encode a 2[4Fe-4S] bacterial-like ferredoxin (5,850 Da). The deduced proteins from *fdxA* and ORF1 were 62% identical. *fdxA* and ORF1 were present as single copies in the genome and each was transcribed on a monocistronic mRNA. Both *fdxA* and ORF1 were transcribed in cells grown on methanol and trimethylamine, but only the *fdxA*-specific transcript was detected in acetate-grown cells. The apparent transcriptional start sites of *fdxA* and ORF1 were downstream of

sequences which had high identity with the consensus methanogen promoter.

The heterodisulfide of two cofactors unique to the methanogenic microorganisms, HS-HTP and HS-CoM, was enzymatically reduced in cell extracts of *Ms. thermophila* using electrons from the oxidation of either $H_2$ or CO. The homodisulfides of either HS-HTP or HS-CoM were not reduced under the same conditions. The results indicated that methane is formed by reductive demethylation of $CH_3$-S-CoM using HS-HTP as a reductant in *Ms. thermophila*. Coupling of CO oxidation with reduction of the heterodisulfide suggested that the CO dehydrogenase-linked ferredoxin may be involved, although the details of electron flow are not known.

## FORWARD

This dissertation focusses on ferredoxin-dependent electron transport reactions in *Methanosarcina thermophila*. Sections I and II are intended to serve as an introduction to biological methanogenesis and, more specifically, acetate conversion to $CH_4$ and $CO_2$. Sections III-V describe the research pertaining to the CO-dehydrogenase-linked ferredoxin from *Ms. thermophila* and a summary is presented in section VI. The literature cited in sections I, II, and VI is included as section VII. The studies described in sections III and V have been published (or submitted for publication) as follows:

> Clements, A. P. and J. G. Ferry. 1992. Cloning, nucleotide sequence, and transcriptional analyses of the gene encoding a ferredoxin from *Methanosarcina thermophila*. J. Bacteriol. 174:5244-5250.
>
> Clements, A. P., R. H. White, and J. G. Ferry. Structural characterization and physiological function of component B from *Methanosarcina thermophila*. Submitted.

The research presented in section IV is being prepared for publication as:

> Clements, A. P., L. Kilpatrick, T. G. Spiro, W-P. Lu, S. W. Ragsdale, and J. G. Ferry. Characterization of the iron-sulfur clusters in a ferredoxin from *Methanosarcina thermophila*.

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# SECTION I. INTRODUCTION

Anaerobic digestion of complex organic matter requires the concerted efforts of three distinct groups of microorganisms (59). The fermentative bacteria hydrolyze complex substrates such as proteins, lipids, and polysaccharides and then ferment the products to fatty acids, $H_2$, and $CO_2$. The $H_2$-producing, acetogenic bacteria, then convert some of the higher fatty acids to formate, acetate, $H_2$, and $CO_2$. Finally, the methanogenic microorganisms produce methane from $H_2$, $CO_2$, formate, acetate, methanol, and methylated amines. When properly harnessed, this type of consortium can be used to convert organic waste to methane, a clean-burning fuel. Projects have been successfully set up to recover methane produced in landfills for use in home heating and lighting (51).

The methane-producing organisms convert the end products of the other organisms in the consortium to gases ($CH_4$ and $CO_2$) which are subsequently released from the digestor environment. As a result, end products do not accumulate and inhibit anaerobic carbon cycling. Low partial pressures of $H_2$, which are maintained in anaerobic digestors by specific methanogenic microorganisms and bacteria, are important in (i) favoring the production of specific compounds by the fermentative bacteria, (ii) making degradation of

these compounds by the acetogenic bacteria energetically favorable (59), and (iii) preventing inhibition of methanogenesis from acetate (4, 26). Fatty acids, including acetate, are not effectively consumed in the absence of both bacteria and methanogenic microorganisms which consume $H_2$. Accumulation of fatty acids can result in a pH decrease and a slowing of the digestion process. Therefore, turnover of organic matter in anaerobic ecosystems is dependent on the ability of the methanogenic organisms to successfully compete in specific environments.

Since the majority of the methane produced in anaerobic environments is derived from the reduction of either $CO_2$ or the methyl group of acetate, studies have focussed on methanogenic microorganisms that catalyze these reactions. *Methanosarcina thermophila* strain TM-1 is an acetotrophic organism which can also utilize methanol or trimethylamine as a carbon and energy source. Many of the details of acetate catabolism in *Ms. thermophila* are known (39), but until recently, the path(s) of electron flow and method(s) of energy conservation had not been studied. The CO dehydrogenase enzyme complex of *Ms. thermophila* is a key protein in acetate metabolism and previous studies revealed that a ferredoxin accepted an electron pair from the CO dehydrogenase following oxidation of CO to $CO_2$ (2, 79). The ferredoxin was partially characterized and its involvement in several important redox reactions has been reported (39, 78). As a result, the properties of the protein and the gene encoding ferredoxin were

examined in an effort to characterize and study the synthesis of one of the electron-transport components involved in methanogenesis from acetate.

## SECTION II. LITERATURE REVIEW

### BIOLOGICAL METHANE FORMATION

Recently, it was proposed that all forms of life can be divided into three distinct domains based on the comparison of 16S rRNA (88). Plants, animals, and fungi are included in the *Eucarya* domain while procaryotes such as *Escherichia*, *Clostridium*, *Anabaena*, *Streptomyces*, and *Rhodospirillum* are members of the *Bacteria*. Methane-producing microorganisms, extreme thermophiles, and extreme halophiles make up the *Archaea* domain, an eclectic collection of phylogenetically-diverse organisms. Members of the *Archaea* share many features with the *Bacteria* such as circular chromosomes, phages, plasmids, bacterial-like ribosome structures, polycistronic mRNAs, ribosome-binding sequences on mRNAs, and transcription termination signals (44). However, promoter sequences and the single DNA-dependent RNA polymerase of the *Archaea* are more similar to those found in the *Eucarya* (44). Despite the similarities with organisms in the other domains, the members of the *Archaea* are distinct enough to warrant their placement in a separate domain. For example, all organisms in the *Archaea* contain membrane-associated polar lipids which consist of polyisoprenoids ether linked to glycerol; corresponding lipids in the *Bacteria* and *Eucarya* contain ester-linked fatty acids (44). Although the

*Archaea* are only distantly related to *Bacteria* and *Eucarya*, metabolic reactions and enzyme mechanisms appear to be similar for organisms in all three domains (44). Variations in metabolic pathways and enzymes used by the methane-producing *Archaea* may be an adaptation for the specialized process of coupling methane synthesis with growth.

The methanogenic microorganisms are ubiquitous in anaerobic environments where nitrate and sulfate levels are low, such as some freshwater and marine sediments, landfills and sewage digestors, and digestive tracts of animals (44). Although the process of methanogenesis requires strictly-anaerobic conditions, there is evidence that some methane-producing microorganisms can survive in dried soils stored in the presence of oxygen (58). The methanogenic *Archaea* were first recognized as a distinct group based on their unique physiological characteristics (7), and the subsequent discovery of several unusual cofactors required for methanogenesis (17) and comparison of 16s rRNA structures (6) supported this taxonomic grouping. However, despite the apparent similarities, the methane-producing microorganisms are very diverse and comprise three different orders (*Methanobacteriales*, *Methanococcales*, and *Methanomicrobiales*) and 14 distinct genera (42). Given the deep phylogenetic divisions and the range of environments inhabited by the methanogenic *Archaea*, it is not surprising that amongst these organisms there is considerable variation in: (i) morphology (rod, coccus, sarcina, spirillum, plane);

(ii) growth substrates ($H_2$ and $CO_2$, secondary alcohols and $CO_2$, formate, acetate, methanol, methylated amines); (iii) optimum growth temperature (20 to 85°C) and pH (6.0 to 9.0); (iv) cell envelope composition (pseudomurein, protein, glycoprotein, heteropolysaccharide); (v) %G+C (25.8 to 61.2); and (vi) nutritional requirements (ranging from complete autotrophy to a requirement for pre-formed coenzyme M) (44). Organisms in the *Methanomicrobiales* are the most diverse based on morphology and substrates utilized.

## CATABOLIC REACTIONS IN METHANOGENIC MICROORGANISMS

The methanogenic *Archaea* are the only known organisms which couple methane synthesis with energy generation to support growth; inhibition of growth, however, does not stop methane production (44). The methane-producing microorganisms are limited to relatively simple carbon and energy sources (Table 1). Reduction of $CO_2$ with $H_2$ and catabolism of acetate account for almost all of the biologically-formed methane (74). Metabolism of either $H_2$ and $CO_2$ or formate involves a pathway in which $CO_2$ is serially reduced to methane; reduction of $CO_2$ to $CH_4$ with electrons derived from the oxidation of ethanol, 1-propanol, 2-propanol, or 2-butanol (87) probably involves a similar mechanism. Conversion of acetate, methanol, and trimethylamine to methane is accomplished via three different pathways. *Methanobacterium*

Table 1. Substrates for methanogenesis (32, 87).

| Net reaction | $\Delta G^{\circ\prime}$ (kJ/mol $CH_4$) |
|---|---|
| $4H_2 + CO_2 \text{ -----> } CH_4 + 2H_2O$ | -136 |
| $4HCOOH \text{ -----> } CH_4 + 3CO_2 + 2H_2O$ | -144 |
| $4CO + 2H_2O \text{ -----> } CH_4 + 3CO_2$ | -211 |
| $CH_3COOH \text{ -----> } CH_4 + CO_2$ | -37 |
| $4CH_3OH \text{ -----> } 3CH_4 + CO_2 + 2H_2O$ | -107 |
| $4(CH_3)_3N + 6H_2O \text{ -----> } 9CH_4 + 3CO_2 + 4NH_3$ | -76 |
| $4CH_3CHOHCH_3 + CO_2 \text{ -----> } 4CH_3COCH_3 + CH_4 + 2H_2O$ | -36 |
| $2CH_3CH_2OH + CO_2 \text{ -----> } 2CH_3COOH + CH_4$ | -116 |

*thermoautotrophicum* is reported to grow slowly on CO as the sole carbon and energy source, possibly using a reversal of the acetate catabolism pathway (15). Regardless of the growth substrate, the final steps of methane formation are the same in all of the methane-producing microorganisms studied to date.

## Methanogenesis from $H_2$ and $CO_2$

Most of the methane-producing *Archaea* reduce $CO_2$ to $CH_4$ and studies of this pathway in *Methanobacterium thermoautotrophicum* (order *Methanobacteriales*) led to the identification of six unusual cofactors which are necessary for methanogenesis (17). These cofactors and their functions are listed in Table 2. The presence of the fluorescent cofactor, $F_{420}$, in all methanogenic microorganisms can be utilized to rapidly identify these organisms

Table 2. Unusual cofactors involved in methanogenesis.

| Cofactor | Function |
|---|---|
| Methanofuran | formyl carrier |
| Methanopterin | $C_1$ carrier |
| Coenzyme M (2-mercaptoethanesulfonate) | $-CH_3$ carrier |
| Coenzyme $F_{420}$ | electron carrier |
| $F_{430}$ | prosthetic group of methylreductase |
| HS-HTP (7-mercaptoheptanoylthreonine $PO_4$) | electron donor to methylreductase |

with fluorescent microscopy (19). The existence of many isofunctional derivatives of methanofuran, methanopterin, and $F_{420}$ (17) confirm that the methanogenic Archaea are phylogenetically diverse. Other cofactors present in methane-producing microorganisms which are commonly found in other organisms include thiamine, riboflavin, pyridoxine, $B_{12}$-like cobamides, biotin, niacin, pantothenate, p-aminobenzoic acid, and the molybdopterin cofactor of formate dehydrogenase (44).

The pathway for conversion of $H_2$ and $CO_2$ to methane is shown in Figure 1. $CO_2$ is initially reduced to the formyl level and then bound to methanofuran (44). The formyl group is transferred from methanofuran to

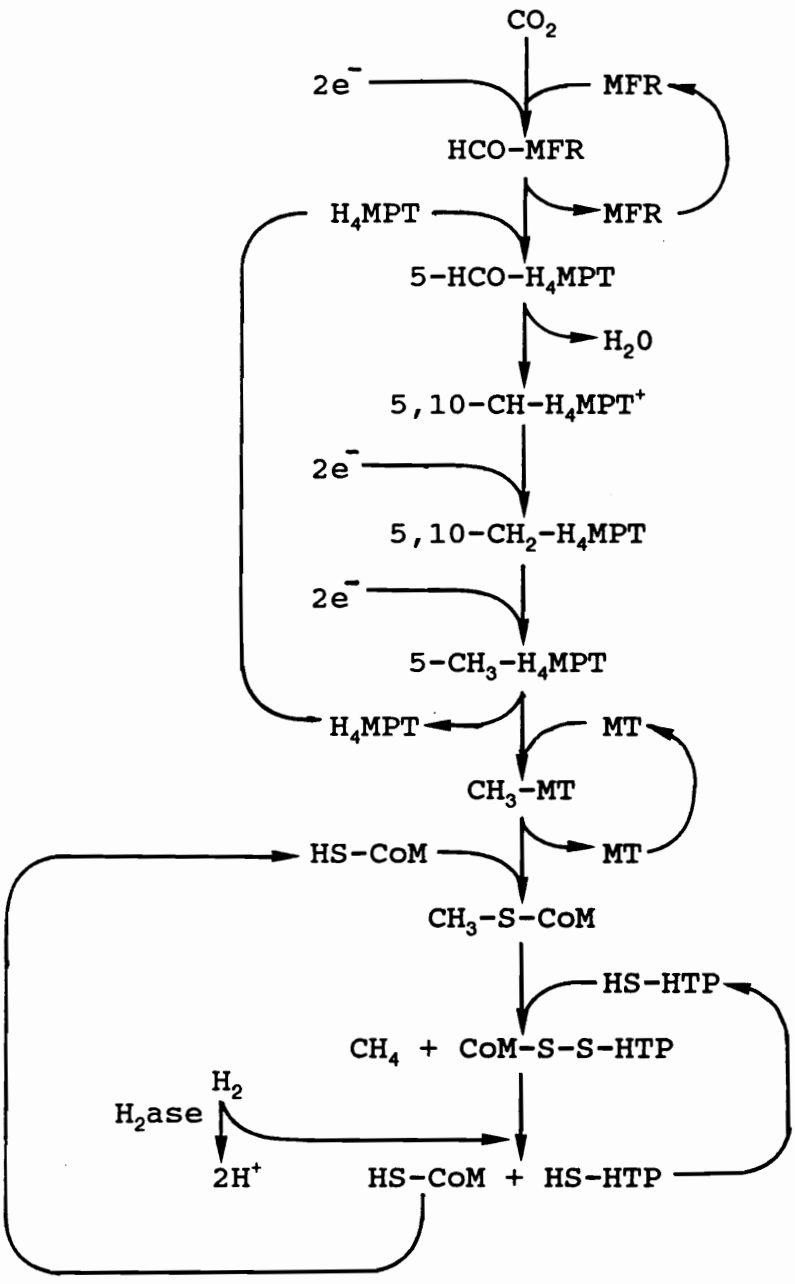



Figure 1. $CO_2$ reduction to $CH_4$ in *Mb. thermoautotrophicum*. MFR, Methanofuran; $H_4MPT$, tetrahydromethanopterin; MT, methyltransfer protein; HS-CoM, 2-mercaptoethanesulfonate; HS-HTP, 7-mercaptoheptanoylthreonine phosphate; $H_2$ase, hydrogenase.

tetrahydromethanopterin ($H_4$MPT), a folate analog. The formyl moiety is sequentially reduced to the methyl level with reducing equivalents generated by a hydrogenase which uses coenzyme $F_{420}$, an 8-hydroxy 5-deazaflavin, as an electron carrier. The methyl group of $CH_3$-$H_4$MPT is transferred to coenzyme M (HS-CoM), possibly involving a corrinoid-containing methyltransferase (46). Methyl coenzyme M methylreductase, which binds the nickel-containing porphinoid cofactor $F_{430}$, catalyzes the reductive demethylation of $CH_3$-S-CoM using HS-HTP as a reductant (65). The products of the final step of methanogenesis are $CH_4$ and a heterodisulfide of HS-CoM and HS-HTP (CoM-S-S-HTP). Regeneration of the free forms of HS-CoM and HS-HTP is catalyzed by a specific reductase (10, 24) which uses electrons supplied by $H_2$ through an unknown series of electron carriers.

## Methanogenesis from acetate

To date, acetotrophic growth has been demonstrated only by the *Methanosarcina* and *Methanothrix* (order *Methanomicrobiales*). Comparison of a thermophilic *Methanosarcina* strain and a thermophilic *Methanothrix* strain revealed that the former organism had a higher growth rate and a higher $K_s$ for acetate (61); the *Methanothrix* organism, with a higher affinity for acetate, was only able to compete at low concentrations of acetate. As a result, the ratio of

*Methanosarcina* to *Methanothrix* in environments where acetate is available may be partially dependent on the concentration of acetate. Many of the *Methanosarcina* can also produce methane from methyl-containing substrates such as methanol and methylated amines. Studies with anaerobic consortia demonstrated that the methyl group of acetate was reduced to methane while the carbonyl group was oxidized to $CO_2$ (14, 67, 75). In the *Methanosarcina* (39, 81), acetate kinase and phosphotransacetylase catalyze the activation of acetate to acetyl coenzyme A, which is the substrate for an enzyme complex with CO dehydrogenase activity (Figure 2). Acetyl coenzyme A is formed in *Methanothrix* by acetyl coenzyme A synthetase (acetate thiokinase) at the expense of two high-energy phosphate bonds (47). After both the C-C and C-S bonds of acetyl coenzyme A are cleaved by the CO dehydrogenase complex of the *Methanosarcina* and the carbonyl group is oxidized to $CO_2$, the methyl group is transferred to $H_4$MPT (27). Following a series of methyl-transfer steps which involve unidentified carriers, and possibly a corrinoid-containing enzyme (21), the methyl group is ultimately transferred to HS-CoM (54). The reactions involving reductive demethylation of $CH_3$-S-CoM and $H_2$-dependent CoM-S-S-HTP reduction appear to be identical to those observed during $CO_2$ reduction to $CH_4$ in *Mb. thermoautotrophicum*. However, heterodisulfide reduction in *Ms. barkeri* is also coupled to CO oxidation (28). Low levels of $F_{420}$ in *Ms. barkeri* (22) and the apparent ability of cell extracts to convert acetyl coenzyme A to

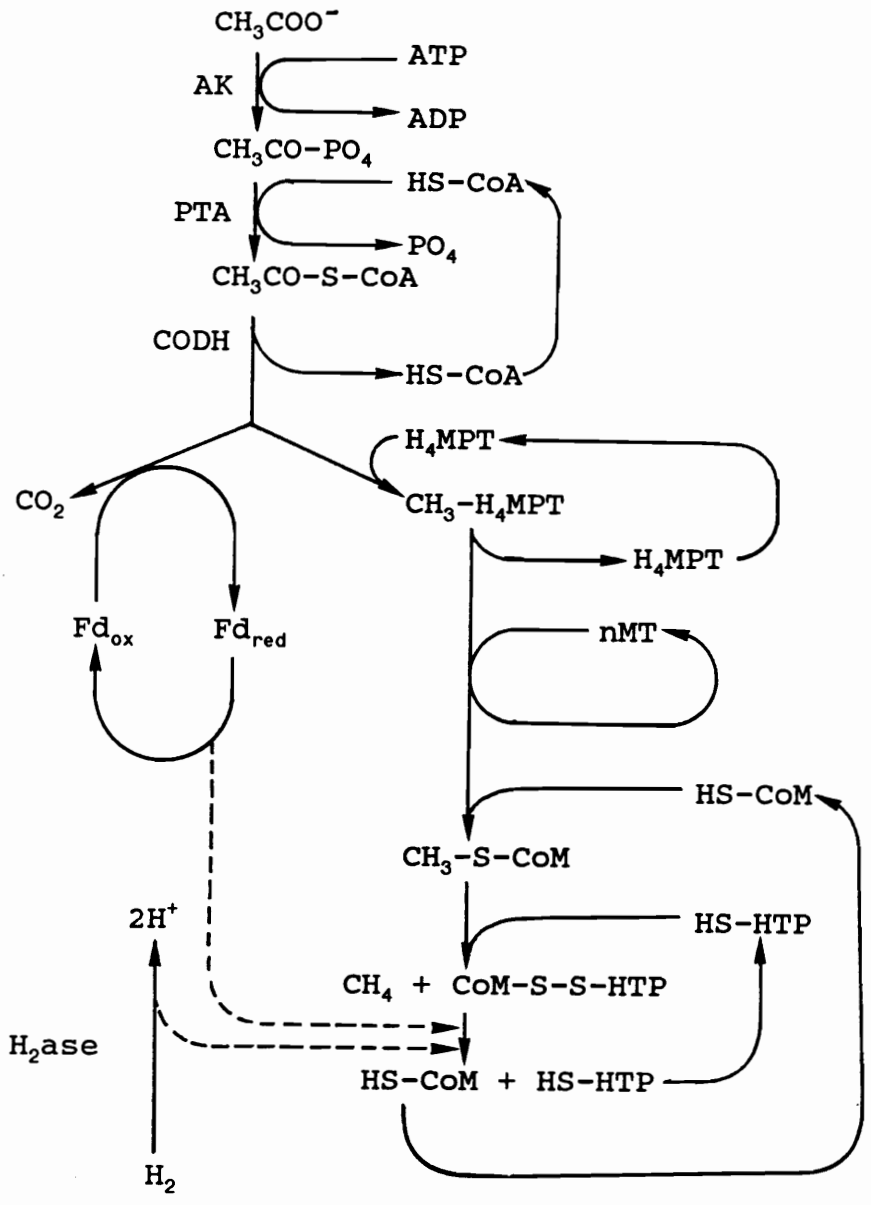



Figure 2. Conversion of acetate to $CH_4$ and $CO_2$ by the *Methanosarcina*. AK, Acetate kinase; PTA, phosphotransacetylase; CODH, CO dehydrogenase enzyme complex; Fd, ferredoxin (oxidized or reduced); HS-CoA, coenzyme A; nMT, unknown number of methyltransferase proteins. For other abbreviations, see legend to Figure 1. Dashed lines indicate unknown steps in the pathway.

$CH_4$ and $CO_2$ in the absence of $F_{420}$ (27) suggest that this cofactor is not a major component in acetate catabolism in this organism. $H_2$ production and consumption during methanogenesis from acetate has been reported; although a mechanism for generating a proton gradient via $H_2$ cycling has been proposed (29, 53), the physiological function of hydrogenase during acetate catabolism has not been determined.

## Methanogenesis from methanol and trimethylamine

Conversion of methanol and trimethylamine to methane involves transfer of an intact methyl group from each substrate (84) to HS-CoM by specific methyltransferase proteins (Figure 3). Two separate methyltransferases, $MT_{M1}$ and $MT_{M2}$, are present in methanol-grown *Ms. barkeri* (82). $MT_{M1}$, which contains an oxygen-sensitive cobamide, catalyzes the transfer of the methyl group of methanol to the enzyme-bound cobamide. $MT_{M2}$ mediates the transfer of the methyl group from $MT_{M1}$ (or cobamides) to HS-CoM. A different methyltransferase is present in trimethylamine-grown *Ms. barkeri* and catalyzes the transfer of methyl groups from trimethylamine to HS-CoM (Figure 3) (63). Reducing equivalents for the reduction of the methyl groups derived from methanol or trimethylamine are most likely generated by oxidizing the methyl groups of either substrate to $CO_2$, possibly using a reverse $CO_2$-reduction

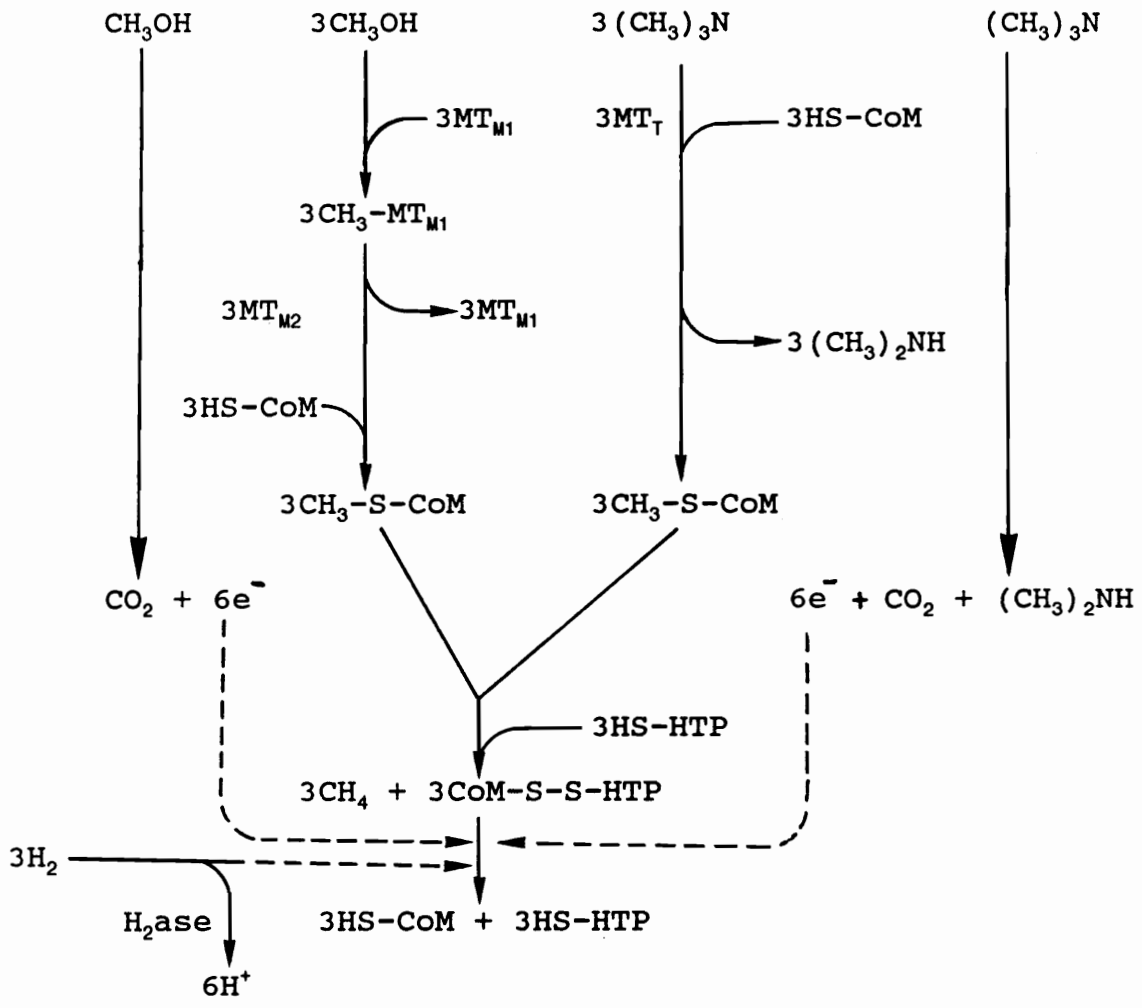



Figure 3. Conversion of methanol and trimethylamine to $CH_4$ by the *Methanosarcina*. $MT_{M1}$, methanol-specific methyltransferase 1; $MT_{M2}$, methanol-specific methyltransferase 2; $MT_T$, trimethylamine-specific methyltransferase. For other abbreviations, see legends to Figures 1 and 2.

pathway (73, 92). Alternately, electrons derived from the oxidation of $H_2$ or acetate can support reduction of the methyl groups derived from methanol (26, 73, 85, 91, 92). $H_2$ metabolism has been observed during methanogenesis from methanol or trimethylamine (53).

## BIOSYNTHETIC REACTIONS IN METHANOGENIC MICROORGANISMS

The pathways for acetate catabolism in the *Methanosarcina* and synthesis of acetate by *Clostridium thermoaceticum* (52) are shown in Figure 4. The similarities between the two pathways suggest that acetate biosynthesis in the methanogenic *Archaea* may simply be a reversal of acetate catabolism. *Methanococcus maripaludis* (order *Methanococcales*) is normally able to grow autotrophically on $H_2$ and $CO_2$, but some mutants with low levels of CO dehydrogenase activity were shown to require acetate for growth (50). Studies with *Mb. thermoautotrophicum* suggest that the carbonyl group of acetyl coenzyme A is derived from $CO_2$ which is reduced to CO and then condensed with a bound methyl group (76). Acetyl coenzyme A synthesis from $CH_3I$, CO, and coenzyme A has been demonstrated using the purified CO dehydrogenase complex of *Ms. thermophila* (1) and in some experiments a CO dehydrogenase-linked ferredoxin stimulated the reaction. It has been proposed that a ferredoxin also functions in acetate biosynthesis in *C. thermoaceticum* (68).

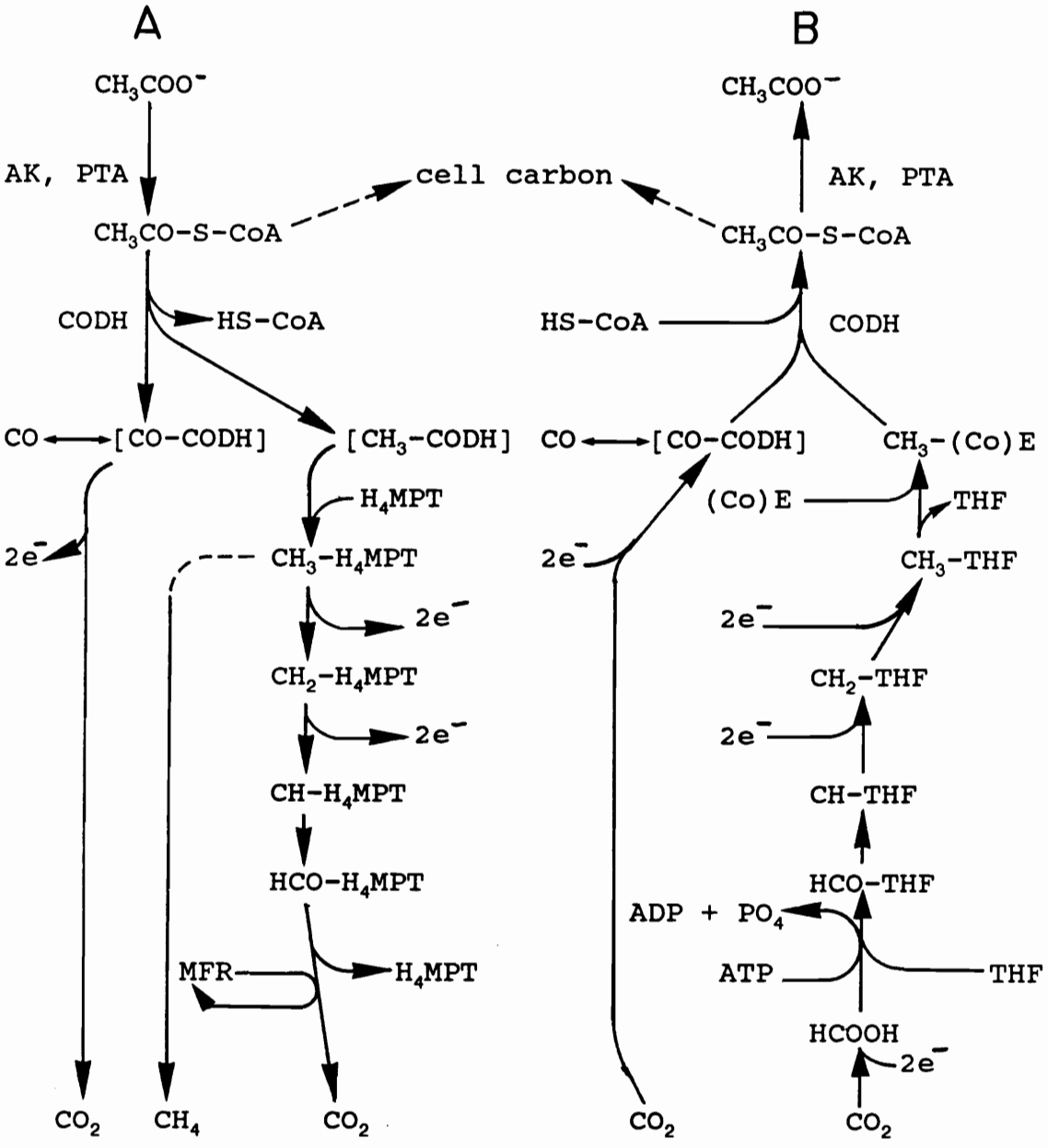



Figure 4. Anabolism and catabolism of acetate in procaryotic organisms. A, pathway for acetate catabolism in the methanogenic *Archaea*. B, pathway for acetate synthesis in *C. thermoaceticum*. THF, Tetrahydrofolate; (Co)E, corrinoid-containing enzyme. For other abbreviations, see legends to Figures 1-3. Enzyme-bound intermediates are indicated with brackets.

Thus, in addition to catalyzing the cleavage of acetyl coenzyme A in *Methanosarcina* and *Methanothrix*, the CO dehydrogenase enzyme complex appears to be essential for synthesis of acetyl coenzyme A for cell carbon in all three orders of the methanogenic *Archaea*. Inhibition by cyanide of both methanogenesis from acetate and synthesis of cell carbon from $H_2$ and $CO_2$ are consistent with the proposed dual function of the CO dehydrogenase complex (20, 72). There is also evidence that tetrahydromethanopterin and cobamides are involved in acetate synthesis in *Mb. thermoautotrophicum* (36).

Studies with *Methanococcus* (50) and *Methanobacterium* (30, 31, 90) suggest that pyruvate is synthesized from acetyl coenzyme A, and then ultimately used to form oxaloacetate. The precursors for many biosynthetic reactions are synthesized via modified tricarboxylic acid (TCA) cycles (Fig 4). *Methanobacterium*, *Methanospirillum*, and *Methanococcus* use an incomplete reductive TCA cycle to synthesize $\alpha$-ketoglutarate from succinyl-CoA (44) while an incomplete oxidative cycle is used by the *Methanosarcina* to form $\alpha$-ketoglutarate from citrate (86). Labelling studies with *Methanospirillum hungatei* (order *Methanomicrobiales*) (23) indicate that carbohydrates, amino acids, lipids, and nucleosides are synthesized from pyruvate, acetyl coenzyme A, $CO_2$, and intermediates of the TCA cycle.

## ELECTRON TRANSPORT AND ENERGY CONSERVATION DURING METHANOGENESIS

The low oxidation-reduction potential of many of the metabolic reactions involved in methanogenesis requires the use of low-potential electron carriers, such as ferredoxin, $F_{420}$, and cytochromes. Ferredoxins are small redox proteins and, unlike other iron-sulfur proteins, do not have any known enzymatic activity. The iron-sulfur clusters in ferredoxins are redox active and the types of clusters ([2Fe-2S], [3Fe-4S], and [4Fe-4S]) and their midpoint potentials vary considerably (-450 mV to +400 mV (60); thus, it is possible for ferredoxins to function as electron carriers for a wide variety of enzymes (13, 89). Ferredoxins are present in the methanogenic *Archaea* and several functions have been described. A ferredoxin isolated from *Methanococcus thermolithotrophicus* is proposed to function as an electron donor to CO dehydrogenase (34). Since this organism in unable to grow with acetate as the sole carbon and energy source, CO dehydrogenase and ferredoxin are probably involved in synthesis of acetyl coenzyme A for biosynthetic reactions. In *Ms. barkeri*, ferredoxin-dependent reactions include (i) reductive activation of methyltransferase $MT_{M1}$ (83), (ii) acetyl coenzyme A conversion to methane, (iii) CO-dependent reduction of CoM-S-S-HTP (28), and (iv) $H_2$ production during pyruvate conversion to acetyl coenzyme A and $CO_2$ (33). A ferredoxin isolated from *Ms. thermophila* accepts

electrons from the nickel/iron-sulfur component of the CO dehydrogenase complex (2) and may function in reductive activation of the methyl coenzyme M methylreductase (39).

Many enzymes which are NAD-, NADP-, or ferredoxin-dependent in the *Bacteria*, *Eucarya*, and some *Archaea* are $F_{420}$-dependent in the methanogenic microorganisms, including hydrogenase, formate dehydrogenase, pyruvate synthase, and $\alpha$-ketoglutarate synthase (17, 44); additionally, secondary alcohol dehydrogenases, $H_4$MPT-dependent enzymes, and NADP/$F_{420}$ oxidoreductase also use $F_{420}$ as an electron carrier. Levels of $F_{420}$ vary greatly in methanogenic organisms (22), possibly reflecting different requirements for the electron carrier in specific metabolic reactions. The midpoint potential ($E_m$) for $F_{420}$ is between -340 and -350 mV (41).

Two distinct hydrogenase activities have been described in the methanogenic *Archaea*. A $F_{420}$-dependent hydrogenase appears to be present in all of the methane-producing microorganisms and there is evidence that the enzyme is associated with the cytoplasmic membrane (8, 56, 62). A second isozyme is present in *Mb. thermoautotrophicum*, *Mb. formicicum*, and *Ms. barkeri* (25, 40, 43) and, although the electron carrier for this hydrogenase is not known, ferredoxin is a candidate.

Cytochromes have been detected in membranes of methylotrophic methane-producing microorganisms, but are not present in organisms that

reduce $CO_2$ to methane; thus it has been proposed that cytochromes are required for oxidation of the methyl groups of acetate, methanol, or methylamines (48). Methanol- or trimethylamine-grown *Methanosarcina* contain two b-type cytochromes ($E_m$ = -325 and -183 mV) and a c-type cytochrome; cells grown on acetate have an additional b-type cytochrome ($E_m$ = -250 mV) (49). Membranes of *Ms. barkeri* contain a b-type cytochrome which can be reduced by a membrane-associated hydrogenase and reoxidized in the presence of $CH_3$-S-CoM (45). $H_2$-dependent reduction of a b-type cytochrome has also been reported for *Ms. thermophila* (78).

Iron-sulfur clusters ([3Fe-4S] and [4Fe-4S]) have been detected in membranes of many of the methanogenic *Archaea* using electron paramagnetic resonance spectroscopy (70); the redox states of the [3Fe-4S] clusters in *Mb. thermoautotrophicum* are affected by the addition of either $F_{420}$ or $CH_3$-S-CoM. Corrinoids have been detected in high levels in methane-producing organisms and are proposed to function in methyl-transfer and electron-transport reactions (17).

The requirement for ATP in the activation of acetate prior to cleavage of the C-C bond and the observation that more than one mole of acetate is required to synthesize one mole of ATP (73) suggest that substrate-level phosphorylation is not the mechanism used by the *Methanosarcina* for energy conservation. ATP synthesis involving an ATPase and a proton motive force

have been described in *Mb. thermoautotrophicum* and *Ms. barkeri* (18, 37). In whole cells of *Ms. barkeri*, ATP synthesis via an electrochemical gradient is coupled to CO-dependent $H_2$ evolution (12). The oxidation of either CO and $H_2$ is coupled to both the reductive demethylation of $CH_3$-S-CoM in *Ms. thermophila* (64) and heterodisulfide reduction in *Ms. barkeri* (28). Furthermore, proton extrusion and ATP synthesis are linked to the reduction of CoM-S-S-HTP in everted membrane vesicles of the methylotrophic methanogenic organism Gö1 (16). The involvement of heterodisulfide reductase in energy conservation suggests that this redox protein is the terminal electron acceptor in all methanogenic *Archaea*. The presence of iron-sulfur clusters, corrinoids, and redox proteins in membranes and the generation of electrochemical gradients which can be used to synthesize ATP strongly indicate that a membrane-associated chemiosmotic mechanism is used for energy conservation by the methane-producing *Archaea*.

## METABOLIC REGULATION IN THE *Methanosarcina*

The free energy available for acetotrophic methane-producing organisms is about the same as that required to phosphorylate one molecule of ADP. Since the activation of acetate requires the investment of ATP, it would be energetically advantageous for the *Methanosarcina* to metabolize substrates

which are more energy rich than acetate whenever possible. There is evidence that a hierarchy for substrate usage does exist for the acetotrophic *Methanosarcina*. *Ms. barkeri* and *Ms. thermophila* both exhibit biphasic growth when cultured on a mixture of both methanol and acetate with preferential conversion of methanol to methane while acetate is either oxidized to generate reducing equivalents or incorporated into cell carbon (73, 91, 92). The same phenomenon is observed when *Ms. barkeri* is grown on either trimethylamine and acetate or $H_2$ and $CO_2$ and acetate (9, 26, 73). The addition of acetate to *Mb. barkeri* Fusaro growing on methanol stimulates not only methanol reduction to methane, but also cell growth and corrinoid production (71). There is evidence that high partial pressures of $H_2$ have an inhibitory effect on acetate catabolism (26, 92). The addition of $H_2$ and $CO_2$ to *Ms. barkeri* growing on methanol, however, does not affect methanogenesis, but the source of reducing equivalents does shift from methanol to $H_2$ (85). Thus, methanol oxidation to $CO_2$ is regulated in the presence of $H_2$ while methanol reduction to methane is unaffected. Studies with *Ms. thermophila* grown separately on acetate, methanol, or acetate plus methanol indicate that proteins involved in methanol conversion to methane are inducible and the presence of methanol represses the expression of proteins necessary for acetate catabolism (91). The levels of acetate kinase and phosphotransacetylase and the activities of CO dehydrogenase and carbonic anhydrase are higher in cells of *Ms. thermophila* grown on acetate than in those

grown on methanol (3, 5, 55, 80). Analyses of total cellular proteins from methanol- and acetate-grown cells clearly demonstrated that substrate-specific regulation affects the levels of at least 150 different proteins (38). The inability to detect trimethylamine:HS-CoM methyltransferase activity in methanol-grown cells of *Ms. barkeri* suggests that this enzyme may also be regulated (63). The presence of a hierarchical system for substrate usage offers the opportunity to study metabolic regulation in the methanogenic *Archaea*. Essential steps in determining the mechanism(s) of regulation include (i) identification of proteins whose synthesis is regulated, and (ii) cloning and characterizing the genes encoding these proteins. Once identified, the regulatory systems of the *Archaea* can be compared to those of the *Bacteria* and *Eucarya* in order to determine if any of the mechanisms are similar or if the archaeal regulatory systems are unique.

## ELECTRON TRANSPORT STUDIES IN *Methanosarcina thermophila*

*Ms. thermophila* strain TM-1 was originally isolated from a thermophilic sewage sludge digestor (92) and has been shown to compete well with other thermophiles under varying growth conditions (57). The organism has a growth optimum of 50°C and uses acetate, methanol, or trimethylamine as a carbon and energy source. The doubling times for cells grown separately on acetate,

methanol, or a mixture of acetate and methanol are 12, 7-10, and 5 hours, respectively (92). Previously, *Ms. barkeri* has been used to study acetate conversion to methane, but since *Ms. thermophila* is unable to grow well on $H_2$ and $CO_2$ (92) some of the complexity of overlapping systems can be avoided. Additionally, *Ms. thermophila* has a doubling time on acetate of about 12 hours at 50°C compared to about 24 hours for *Ms. barkeri* at 37°C (74). The faster growth rate is advantageous in obtaining more cell mass for the large-scale isolation of cellular components. Since the enzymology of acetate activation (3, 55) and acetyl coenzyme A cleavage (2, 77) have been well studied in *Ms. thermophila*, this organism is ideal for studying electron transport reactions. Additionally, the N-terminal sequences of several key proteins in the acetate pathway, including ferredoxin, have been determined making it possible to construct DNA probes for cloning the corresponding genes.

The identification of ferredoxin as the first electron carrier during acetate conversion to $CH_4$ and $CO_2$ in *Ms. thermophila* makes it possible to begin a comprehensive study of electron transport components in the *Methanosarcina*. Determination of the midpoint potential of the iron-sulfur clusters and the complete primary protein sequence of the *Ms. thermophila* ferredoxin will be useful in comparing this protein with ferredoxins isolated from organisms in the *Archaea*, *Bacteria*, and *Eucarya*. The small size of ferredoxins and their lack of enzymatic activities makes this type of protein ideal for spectroscopic studies

since there are no other prosthetic groups which can interfere with the iron-sulfur clusters. Cloning and sequencing of the gene encoding ferredoxin from *Ms. thermophila* will be useful for several reasons. First, a gene in the methyl viologen-reducing hydrogenase operon of *Mb. thermoautotrophicum* encodes a polyferredoxin (35, 69) which contains tandem repeats of bacterial-like ferredoxins. Thus, it must be established whether the ferredoxin isolated from acetate-grown *Ms. thermophila* is encoded by a single gene or is a product of a processed polyferredoxin. Second, the native molecular weight of the *Ms. thermophila* ferredoxin as determined by gel-filtration chromatography was 16,400 Da (79), suggesting that the protein may be either a large monomer or a complex of smaller monomers. Lastly, the apparent regulation of the CO dehydrogenase complex (80), which is the electron donor to ferredoxin, raises the possibility that ferredoxin may be also be regulated according to the growth substrate. Previous studies show that synthesis of ferredoxins in *Anabaena* is dependent on the availability of iron (66) or heterocyst differentiation (11). Regardless of whether the gene encoding ferredoxin in *Ms. thermophila* is regulated, identification of promoter structures and transcriptional start sites will be valuable in studying genes and their expression in the methanogenic *Archaea*.

## SECTION III. CLONING, NUCLEOTIDE SEQUENCE, AND TRANSCRIPTIONAL ANALYSES OF THE GENE ENCODING A FERREDOXIN FROM *Methanosarcina thermophila*


### SUMMARY

A mixed 17-mer oligonucleotide deduced from the N terminus of a ferredoxin isolated from *Methanosarcina thermophila* was used to probe a lambda gt11 library prepared from *Ms. thermophila* genomic DNA; positive clones contained either a 5.7-kbp or 2.1-kbp *Eco*RI insert. An open reading frame (*fdxA*) located within the 5.7-kbp insert had a deduced amino acid sequence that was identical to the first 26 N-terminal residues reported for the ferredoxin isolated from *Ms. thermophila*, with the exception of the initiator methionine. *fdxA* had the coding capacity for a 6,230-Da protein which contained eight cysteines with spacings typical of 2[4Fe-4S] ferredoxins. An open reading frame (ORF1) located within the 2.1-kbp *Eco*RI fragment also had the potential to encode a 2[4Fe-4S] bacterial-type ferredoxin (5,850 Da). *fdxA* and ORF1 were present as single copies in the genome and each was transcribed on a monocistronic mRNA. While the *fdxA*- and ORF1-specific mRNAs were detected in cells grown on methanol and trimethylamine, only the *fdxA*-specific transcript was present in acetate-grown cells. The apparent transcriptional start

sites of *fdxA* and ORF1, as determined by primer extension analyses, lay 21-28 bases downstream of sequences with high identity to the consensus methanogen promoter.

## INTRODUCTION

Ferredoxins are small, acidic proteins containing nonheme iron and acid-labile sulfur in clusters coordinated by cysteines. These ubiquitous electron-transport proteins participate in a wide variety of redox reactions and many organisms, including *Rhodospirillum rubrum* (28), *Streptomyces griseolus* (15), and *Clostridium thermoaceticum* (7), contain multiple ferredoxins. Ferredoxins from three different methane-producing microorganisms have been purified and characterized (9-11, 25); however, the genes encoding these proteins have not been cloned. Each ferredoxin has a subunit molecular mass of about 6,000 Da and accepts electrons from either pyruvate:ferredoxin oxidoreductase or carbon monoxide dehydrogenase.

*Methanosarcina thermophila* is a moderately-thermophilic organism that produces methane from acetate, methanol, and methylated amines. A ferredoxin, isolated from acetate-grown cells, and partially sequenced (25) accepts electrons from the purified *Ms. thermophila* CO dehydrogenase enzyme complex which catalyzes cleavage of the C-C and C-S bonds of acetyl coenzyme

A and oxidation of the carbonyl group to $CO_2$ (1). This ferredoxin is also required for CO-dependent $H_2$ production (24) and recent studies suggest that reduced ferredoxin may be involved in activation of the methyl-coenzyme M methylreductase (13). A ferredoxin is required for the conversion of acetyl coenzyme A to methane in cell extracts of *Methanosarcina barkeri* MS (8); thus, ferredoxin appears to be essential for acetate conversion to methane in the *Methanosarcina*. Here we report the cloning and nucleotide sequence of the gene encoding the ferredoxin isolated from *Ms. thermophila*, as well as an open reading frame (ORF) with the potential to encode a second ferredoxin in this organism.

## MATERIALS AND METHODS

**Materials.** T4 polynucleotide kinase, T4 DNA ligase, *Escherichia coli* DH5$\alpha$ MAX Efficiency competent cells, Nick Translation System, DNA restriction endonucleases, pUC18, and pUC19 were obtained from Bethesda Research Laboratories, Gaithersburg, MD. RNase A, ampicillin, 5-bromo-4-chloro-3-indolyl-$\beta$-D-galactoside (X-gal) were purchased from Sigma Chemical Co., St. Louis, MO. GeneScreen Plus and Colony/PlaqueScreen hybridization membranes, NENSORB 20 purification cartridges, and radiolabeled nucleotides were from DuPont-New England Nuclear Corp., Boston, MA. Lambda gt11

phage arms, the Packagene lambda DNA packaging system, RQ1 DNase, rRNasin, and avian myeloblastosis virus reverse transcriptase were supplied by Promega Corp., Madison, WI. Sequenase enzyme kits and sequencing primers were obtained from United States Biochemicals Corp., Cleveland, OH. All other materials used were reagent grade.

**Preparation and labeling of DNA probes.** A mixed oligonucleotide (probe I; 5'-TGYGTNGAYGARTGYCC-3', where Y is either pyrimidine, R is either purine, and N is any nucleotide) was synthesized based on amino acids 15-20 of the N-terminal protein sequence of ferredoxin from *Ms. thermophila* (25). The *fdxA*-specific probe II (5'-AACTGCGATTCCTTTCTCTTC-3') was complementary to nucleotides +85 to +105 (see Figure 2). The ORF1-specific probe III (5'-TTACTCAATTGTGATTGCATT-3') was complementary to nucleotides +157 to +177 (see Figure 3). Oligonucleotides were 5' end labeled with [$\gamma$-$^{32}$P]ATP as described before (19). The 762-bp *Eco*RI/*Pst*I fragment of plasmid pMT500 (probe IV) and the 727-bp *Sst*I/*Eco*RI fragment of plasmid pMT200 (probe V) were isolated from agarose gels with an Elutrap apparatus (Schleicher and Schuell, Inc., Keene, NH) and labeled with [$\alpha$-$^{32}$P]dATP using the Nick Translation System as described by the supplier. After excess label was removed using a NENSORB 20 cartridge, probes were added to the hybridization solution to yield >1 X $10^6$ cpm/ml (oligonucleotide probes) or >1 X $10^5$ cpm/ml (nick-translated probes).

**Growth of cells and isolation of DNA.** *Methanosarcina thermophila* strain TM-1 (29) was grown in fermentors as described before (22) with sodium acetate (100 mM), methanol (100 mM), or trimethylamine (50 mM) as the carbon and energy source, and cells were stored in liquid nitrogen. Frozen cells (3 g) were added to 5 ml of STES buffer (50 mM NaCl, 50 mM Tris-HCl [pH 7.5], 10 mM EDTA, 1% [wt/vol] sodium dodecyl sulfate [SDS]) and thawed at 23°C for 15 min. The cell suspension was mixed gently with 5 ml phenol saturated with Tris-HCl (pH 7.5) for 15 min. The mixture was centrifuged at 6,800 x *g* and the aqueous phase was collected. The phenol phase was mixed with 2.5 ml STES, incubated for 5 min at 23°C, and centrifuged, and the aqueous phase was collected. The aqueous phases were combined, extracted once with phenol and once with chloroform, and stored at -20°C following the addition of 0.5 volume 7.5 M ammonium acetate and 2 volumes 95% (vol/vol) ethanol. DNA was recovered by centrifugation, resuspended in TE buffer (10 mM Tris-HCl [pH 7.3], 1 mM EDTA) and treated with DNase-free RNase A. DNA was then extracted separately with phenol and chloroform and ethanol precipitated as described above. A total of 750 μg of DNA was recovered, and agarose gel electrophoresis indicated that the genomic DNA was >23 kbp long. Isolation of genomic DNA from *Escherichia coli* strain Y1090 was performed by the same procedure.

DNA from phage lambda gt11 was purified as described before (21) and

plasmid DNA was isolated using an alkaline lysis procedure (19).

**Construction and screening of genomic library.** Genomic DNA from *Ms. thermophila* was digested to completion with *Eco*RI and ligated with dephosphorylated lambda gt11 phage arms and packaged according to the supplier's recommendations. *E. coli* strain Y1090 was transfected with the packaged phage as described before (19) and plated to yield about 300 plaques per plate. Plaque lifts were performed in duplicate, and the nylon membranes were prehybridized (4 h at 37 or 42°C) in 50 mM Tris-HCl (pH 7.5)-1% (wt/vol) SDS-10% (wt/vol) dextran sulfate-1 M NaCl-10-25% (vol/vol) deionized formamide. End-labeled oligonucleotide probe I was added to the prehybridization solution and hybridized 12 to 18 h at 37 or 42°C. Membranes were washed for a minimum of 40 min at 42°C with 2X SSC (0.3 M sodium chloride, 0.03 M sodium citrate) containing 0.1% (wt/vol) SDS; washes at higher temperatures were performed as needed to reduce background. Following autoradiography, positive plaques were picked and stored at 4°C as described before (19). Secondary and tertiary phage purifications were performed in a similar manner.

**Construction of plasmids and transformation of cells.** DNA restriction fragments from recombinant phage or plasmids were recovered from agarose gels using a GeneClean kit (Bio 101, Inc., La Jolla, CA.) and ligated with vector DNA (plasmids pUC18 or pUC19) as described before (19). *E. coli* DH5$\alpha$

competent cells were transformed with the resulting recombinant plasmids as suggested by the supplier. Recombinant clones were selected by plating cells on LB medium supplemented with ampicillin and X-gal.

**Southern hybridizations.** DNA digested to completion with restriction endonucleases was separated by agarose gel electrophoresis in 45 mM Tris-borate [pH 8.3]-1 mM EDTA buffer and transferred to GeneScreen Plus nylon membranes as recommended by the manufacturer, with an electroblotting apparatus. Prehybridization, hybridization, and washing of the membranes were performed as described for plaque hybridizations.

**DNA sequencing.** Recombinant plasmids containing overlapping *Ms. thermophila* DNA fragments served as templates for double-stranded sequencing by the dideoxynucleotide termination method (20). Sequencing primer A and M13 reverse sequencing primer were used to determine the nucleotide sequence of both strands of DNA with Sequenase (version 2.0) as recommended by the manufacturer.

**RNA isolation.** *Ms. thermophila* cells (1 g) were thawed in 10 ml of a solution which contained 50 mM Tris-HCl (pH 8), 20 mM EDTA, and 1% (wt/vol) SDS and passed through a French pressure cell (Aminco, Urbana, IL) at 18,000 lb/in$^2$. The resulting lysate was transferred directly into a mixture of 10 ml phenol (equilibrated with 1 M sodium acetate, pH 4) and 2 ml chloroform. Following the addition of 1 ml of 2 M sodium acetate (pH 4), total RNA was

isolated essentially as described before (17) and stored at -20°C in 50% (vol/vol) isopropanol. For all subsequent manipulations, RNA was collected by centrifugation and washed twice with 70% (vol/vol) ethanol. Treatment of RNA preparations with RNase-free DNase in the presence of rRNasin (an RNase inhibitor) was performed as suggested by the supplier. RNA samples were then extracted once with phenol and once with chloroform and precipitated at -20°C with isopropanol.

**Northern (RNA blot) hybridizations.** Total RNA (20 μg) and molecular size standards (4 to 8 μg) were glyoxalated and separated in a 1.4% agarose gel (19). The transfer of RNA to GeneScreen Plus membranes, hybridization, and wash procedures were done as described above for Southern hybridizations. Probes used to detect the *fdxA*- and ORF1-specific mRNAs were derivatives of plasmid pUC19 which contained DNA restriction fragments cloned from *Ms. thermophila*.

**Primer extension assays.** The 5' ends of the *fdxA*- and ORF1-specific mRNAs were mapped as described before (3) except that [$\alpha$-$^{35}$S]dATP (1000-1500 Ci/mmol) was substituted for [$\alpha$-$^{32}$P]dATP. The 21-mer oligonucleotide probes II (complementary to nucleotides +85 to +105 of *fdxA*; see Figure 2) and III (complementary to nucleotides +157 to +177 of ORF1; see Figure 3) were used as primers. The *fdxA*- or ORF1-specific primers (15 ng) were annealed to 10 μg or 25 μg, respectively, of total *Ms. thermophila* RNA which

had been treated with DNase. Sequencing ladders were generated from plasmid clones using the same oligonucleotides.

**Nucleotide sequence accession numbers.** The *fdxA* and ORF1 nucleotide sequences have been submitted to the GenBank database and assigned accession numbers M83187 and M83188, respectively.

## RESULTS

**Cloning of the ferredoxin gene.** A synthetic 17-mer mixed oligonucleotide (probe I), corresponding to a sequence in the N terminus of a *Ms. thermophila* ferredoxin (25), was used to probe Southern blots of completely digested *Ms. thermophila* DNA (Figure 1). The results indicated that probe I hybridized with two fragments when genomic DNA was digested separately with each restriction enzyme. A genomic library was constructed by cloning *Eco*RI-restricted DNA fragments of *Ms. thermophila* into phage lambda gt11. A primary screening of the library (approximately 6,000 plaques) detected 10 phage isolates that hybridized with probe I. After two additional phage purifications, 4 of the 10 isolates continued to hybridize to probe I; 3 contained an *Eco*RI insert of approximately 5.7-kbp and 1 contained a 2.1-kbp *Eco*RI insert. These inserts were approximately the same sizes as the DNA fragments identified with probe I in Southern hybridizations of *Ms. thermophila* genomic DNA digested to

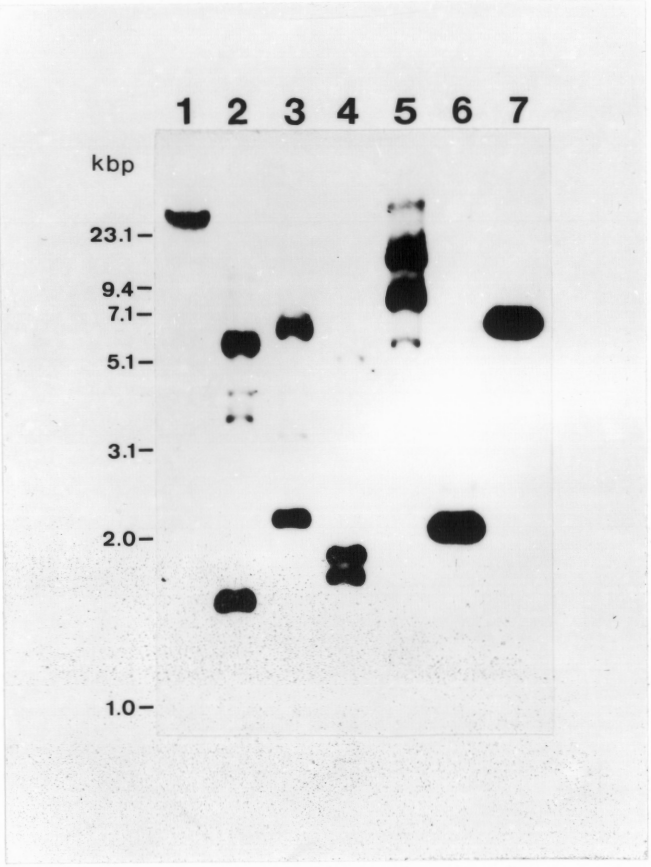
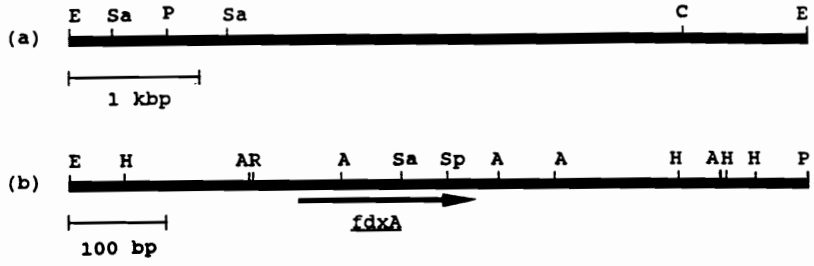



Figure 1. Southern blot analyses. DNA from *Ms. thermophila* or *E. coli* Y1090 was digested to completion with restriction endonucleases, separated by electrophoresis through a 0.7% agarose gel, and transferred to a nylon membrane for hybridization with oligonucleotide probe I. Lane 1, *E. coli* Y1090 DNA (20 μg) digested with *Eco*RI. Lanes 2, 3, 4, and 5, *Ms. thermophila* genomic DNA (20 μg) digested with *Cla*I, *Eco*RI, *Pst*I, and *Sal*I, respectively. Lane 6, plasmid pMT200 (75 ng) digested with *Eco*RI. Lane 7, plasmid pMT500 (75 ng) digested with *Eco*RI. Molecular size standards (Bethesda Research Laboratories) were the combination of a 1-kbp DNA ladder and lambda DNA digested with *Hin*dIII.

completion with *Eco*RI (Figure 1, lanes 3, 6, and 7). The 2.1- and 5.7-kbp fragments were not detected by probe I in *E. coli* genomic DNA digested to completion with EcoRI, which confirmed that the two inserts in the isolated phage were not derived from *E. coli*. However, a large DNA fragment from *E. coli* did hybridize to probe I (Figure 1, lane 1), which suggested that a sequence similar to the two inserts from *Ms. thermophila* was also present in *E. coli*. The existence of two hybridizing fragments when *Ms. thermophila* genomic DNA was restricted separately with various enzymes and the isolation of phage species with one of two different inserts suggested either duplicate copies of the gene encoding ferredoxin or two similar DNA sequences which hybridized with probe I.

Plasmids pMT200 and pMT500 were constructed by subcloning the 2.1-kbp and 5.7-kbp *Eco*RI inserts from the purified phage into plasmid pUC18. Further subcloning and Southern blot analyses identified a 762-bp *Eco*RI/*Pst*I fragment of plasmid pMT500 and a 727-bp *Sst*I/*Eco*RI fragment of plasmid pMT200 which hybridized to probe I; the nucleotide sequence revealed an ORF within each fragment (Figures 2 and 3). The ORF contained within the *Eco*RI/*Pst*I fragment included a DNA sequence which corresponded to an amino acid sequence (Figure 2) identical to the first 26 N-terminal residues reported for a ferredoxin from *Ms. thermophila* (25). The results indicated that the ORF (designated *fdxA*) encoded this ferredoxin, assuming that the initiator

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-235  GAATTCGATTTTTTACTTCTTCTTTTATTTATAAATTAAATGATATA

-188  TTTTAATCCCCGGCTAGTTGACTCCATCCTGATTTTGAAGGCTGCAT

-141  TTTGTTAAAAAGCGATAAGTTTTTATGTTTATCCTGTCAATGGCGTT

 -94  ATGACCAAACTTTTCGATAACCTATGGAAGTTTTAAAAACGAGCTGT

 -47  ACAATTTCCAGGTATATATCGAGTAACAAAAAGGAGTGTGAAAAAAT

  +1  ATG CCA GCA TTA GTT AAT GCA GAT GAA TGT TCT GGA
      M   P   A   L   V   N   A   D   E   C*  S   G

 +37  TGC GGA AGC TGT GTC GAT GAA TGC CCC TCT GAA GCA
      C*  G   S   C*  V   D   E   C*  P   S   E   A

 +73  ATC ACC CTT GAT GAA GAG AAA GGA ATC GCA GTT GTC
      I   T   L   D   E   E   K   G   I   A   V   V

+109  GAC CAG GAC GAA TGC GTA GAG TGC GGC GCA TGT GAA
      D   Q   D   E   C*  V   E   C*  G   A   C*  E

+145  GAA GCA TGC CCG AAC CAG GCA ATT AAA GTA GAA GAG
      E   A   C*  P   N   Q   A   I   K   V   E   E

+181  TAAAACGGCAATTAGTTGGAATAAGCTCTTTATTCCACTTTTCTTTT

+228  TCCTTTTTATCAATCATTTTTTCTTTTTCGGAGGTAGCT
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Figure 2. Partial restriction map of the 5.7-kbp *Eco*RI lambda gt11 insert and nucleotide sequence of *fdxA* and flanking DNA. (a) 5.7-kbp *Eco*RI insert. (b) 762-bp *Eco*RI/*Pst*I fragment derived from the 5.7-kbp *Eco*RI insert. The size and orientation of *fdxA* are shown with an arrow. The nucleotide sequence of the coding strand of the 501-bp *Eco*RI/*Alu*I fragment containing *fdxA* is shown, with the deduced amino acid sequence below the nucleotide sequence in single-letter code. Numbering of nucleotides is relative to the first nucleotide of the coding region. The proposed transcriptional start site is indicated by a solid triangle. Potential promoter and ribosome-binding sequences are overlined and double underlined, respectively. Inverted repeats are indicated with converging arrows. Cysteines postulated to bind iron-sulfur clusters are indicated with asterisks. Abbreviations: A, *Alu*I; C, *Cla*I; E, *Eco*RI; H, *Hpa*II; P, *Pst*I; R, *Rsa*I; Sa, *Sal*I; Sp, *Sph*I.

methionine was removed. The amino acid sequence of the mature protein (59 amino acids; 6,080 Da) deduced from *fdxA* was 92% identical to the protein sequence determined for the 3Fe ferredoxin isolated from *Methanosarcina barkeri* MS (11). In addition, the deduced amino acid sequence of *fdxA* was 34, 42, and 37% identical to the protein sequences determined for the 2[4Fe-4S] ferredoxins isolated from *Methanococcus thermolithotrophicus* (5), *Clostridium pasteurianum* (23), and *Peptococcus aerogenes* (2, 5), respectively (Figure 4). The spacing of the eight cysteines in the ferredoxin encoded by *fdxA* (Figure 2) could enable the protein to coordinate two clusters of the [3Fe-4S] or [4Fe-4S] type.

An apparent ribosome-binding site, complementary to the sequence at the 3' terminus of the 16S rRNA in *Ms. barkeri* (3'-UCCUCCACUA) (4) was separated by 11 bases from the initiator codon of *fdxA* (Figure 2). An inverted repeat, which started 11 bases downstream of the stop codon (Figure 2), has the potential to form a stem-loop structure. Such a structure, if present, may be a signal for termination of transcription or function as a stabilizer of mRNA, but this has yet to be determined. The G+C content of *fdxA* was 49%.

**Cloning of an ORF with the potential to encode a second ferredoxin.** The nucleotide sequence of the ORF (ORF1) and flanking DNA located within the 727-bp *Sst*I/*Eco*RI fragment of plasmid pMT200 is shown in Figure 3. An in-frame stop codon started 96 bases upstream of the initiator codon which indicated that ORF1 was not part of a larger ORF. An apparent ribosome-

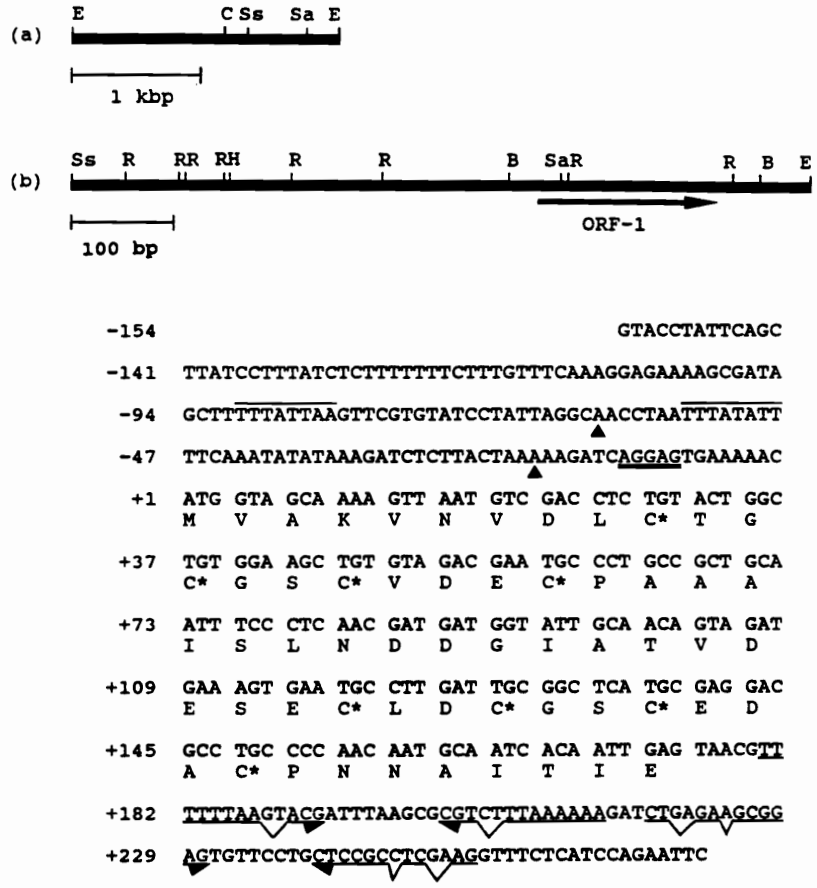


Figure 3. Partial restriction map of the 2.1-kbp *Eco*RI lambda gt11 insert and nucleotide sequence of ORF1 and flanking DNA. (a) 2.1-kbp *Eco*RI insert. (b) 727-bp *Sst*I/*Eco*RI fragment derived from the 2.1-kbp *Eco*RI insert. The size and orientation of ORF1 are shown with an arrow. The nucleotide sequence of the coding strand of the 423-bp *Rsa*I/*Eco*RI fragment containing ORF1 is shown, with the deduced amino acid sequence below the nucleotide sequence in single-letter code. Other details are explained in the legend to Figure 2.
Abbreviations: B, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hpa*II; R, *Rsa*I; Sa, *Sal*I; Ss, *Sst*I.

binding sequence was separated by eight bases from the initiator codon, and regions with dyad symmetry were located downstream of the stop codon (Figure 3). The G+C content of ORF1 was 49%.

The deduced amino acid sequence of ORF1 (58 amino acids; 5,850 Da) had 62% identity with the deduced sequence of *fdxA* and contained eight cysteines spaced identically to those in the predicted *fdxA* gene product and other ferredoxins containing [3Fe-4S] or [4Fe-4S] centers (Figure 4). The deduced ORF1 gene product lacked arginine, histidine, aromatic amino acids and contained no methionines (except for the initiator); these properties are characteristic of ferredoxins isolated from *Methanosarcina* species. The predicted ORF1 protein, minusthe initiator methionine, had 32, 42, and 40% identity with the 2[4Fe-4S] ferredoxins of *Mc. thermolithotrophicus*, *C. pasteurianum*, and *P. aerogenes*, respectively (Figure 4). The results suggested that ORF1 encodes a second ferredoxin in *Ms. thermophila*.

**Gene copy number and relative proximity of *fdxA* and ORF1.** Genomic DNA digested separately with either *Cla*I, *Eco*RI, *Pst*I, or *Sal*I, was probed with the synthetic 21-mer oligonucleotides specific for either *fdxA* or ORF1 (probes II and III, respectively). The results indicated that a single copy of each gene was present in the *Ms. thermophila* genome (data not shown). Similar results were obtained when genomic DNA restricted separately with either *Cla*I, *Eco*RI, or *Pst*I, was probed with DNA restriction fragments (probes IV and V) which

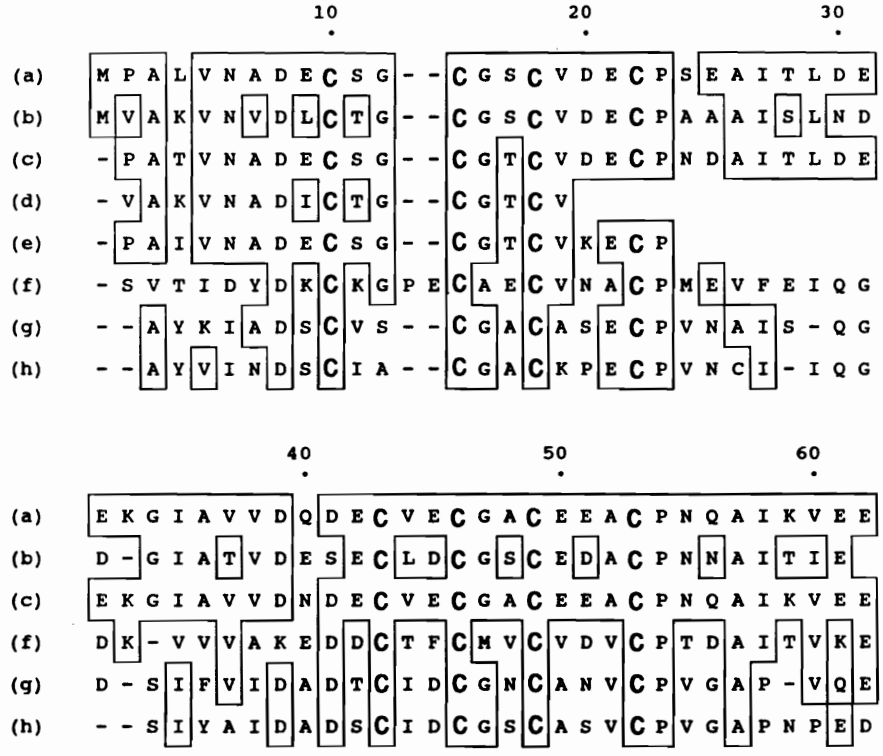


Figure 4. Comparison of amino acid sequences. (a) Deduced from *Ms. thermophila fdxA* (Figure 2); (b) deduced from *Ms. thermophila* ORF1 (Figure 3); (c) ferredoxin isolated from *Ms. barkeri* strain MS (11); (d) peptide B isolated from *Ms. barkeri* strain MS (11); (e) N terminus of the ferredoxin isolated from *Ms. barkeri* strain Fusaro (9); (f) ferredoxin isolated from *Mc. thermolithotrophicus* (5); (g) ferredoxin isolated from *C. pasteurianum* (23); (h) ferredoxin isolated from *P. aerogenes* (2, 5). Boxed regions indicate identity with the *Ms. thermophila* ferredoxin sequence deduced from *fdxA*. Dashes were used as packing characters to obtain maximum alignment. Conserved cysteines are shown in large boldface letters.

contained either the fdxA- or ORF1-specific sequences; however, the presence of a *Sal*I restriction site in probes IV and V (Figures 2 and 3) yielded an additional *Sal*I fragment not detected when the synthetic 21-mer oligonucleotide probes were used. Based on the fragment sizes observed when genomic DNA was hybridized with probes II through V, the minimum distance between *fdxA* and ORF1 in the genome was approximately 7.0 kbp.

**Northern blot analyses.** Total RNA from *Ms. thermophila* was probed with two different plasmid pUC19 derivatives, the inserts of which contained either the *fdxA*- or ORF1-specific sequences. Experiments with plasmid pUC19 as a control probe showed no hybridization with RNA from acetate-, methanol-, or trimethylamine-grown cells (data not shown). An mRNA species (approximately 330 bases) was detected with the *fdxA*-specific probe in Northern blots with RNA isolated from acetate-, methanol-, or trimethylamine-grown cells (Panel I, Figure 5). The results indicated that *fdxA* was not part of a polycistronic operon and that the gene was not transcriptionally regulated according to the growth substrate. The ORF1-specific probe hybridized to an approximately 310-base mRNA species (Panel II, Figure 5) present in cells grown on methanol or trimethylamine but not in cells grown on acetate. This ORF1-specific mRNA appeared to be less abundant in trimethylamine-grown cells. The absence of the monocistronic ORF1-specific mRNA in acetate-grown cells suggested that ORF1 was transcriptionally regulated.

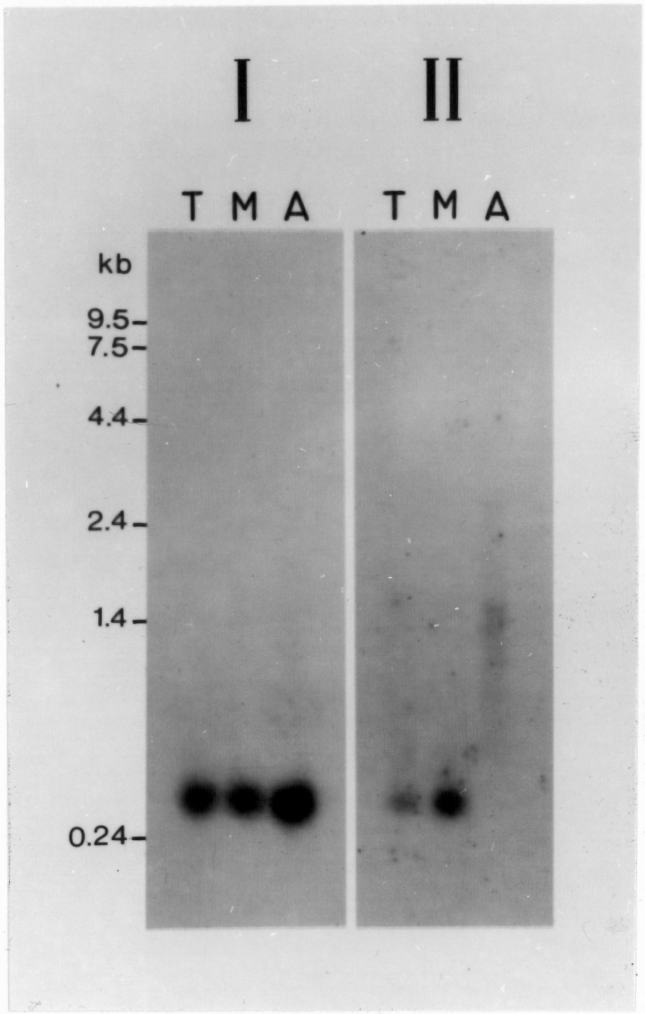


Figure 5. Northern blot analyses. Total RNA (20 μg) from *Ms. thermophila* grown on acetate (A), methanol (M), or trimethylamine (T) was separated by gel electrophoresis, transferred to a nylon membrane, and hybridized with either a *fdxA*- or an ORF1-specific probe. (Panel I) The probe contained the 161-bp *Alu*I fragment (+45 to +205 in Figure 2), which included most of *fdxA*. (Panel II) The probe contained the 159-bp *Rsa*I fragment (+31 to +189 in Figure 3), which included most of ORF1. Molecular size standards (Bethesda Research Laboratories) were a 0.24 to 9.5-kb RNA ladder.

**Primer extension analyses.** Primer extension of total RNA using the primer specific for *fdxA* (oligonucleotide II) resulted in the formation of one major extension product (Figure 6); minor extension products were also present, which may have resulted from premature termination. The 5' end of the major *fdxA*-specific mRNA (Panel I, Figure 6) mapped to the G nucleotide located 92 bases upstream of the first base of *fdxA* (Figure 2). The major primer extension product was the same length regardless of whether the RNA was isolated from acetate-, methanol-, or trimethylamine-grown cells (data not shown). A sequence (TTTATGTT) separated by 20 bases from the proposed transcriptional start site (Figure 2) had high identity with the consensus promoter sequence for methanogens (TTTATATA) (26).

Two faint extension products were observed when primer extension analyses were performed using the primer specific for ORF1 (oligonucleotide III) (Panel II, Figure 6). The 5' ends of the two ORF1-specific mRNA species mapped to the A nucleotides located 63 bases and 20 bases upstream of the first base of ORF1, and were 21 bases and 28 bases downstream of potential promoter sequences (TTTATTAA and TTTATATT, respectively) (Figure 3). While both extension products were detected regardless of whether RNA was isolated from cells grown on methanol or trimethylamine, the larger of the two extension products appeared to be slightly more abundant. No primer extension products were detected when RNA from acetate-grown cells was used (data not

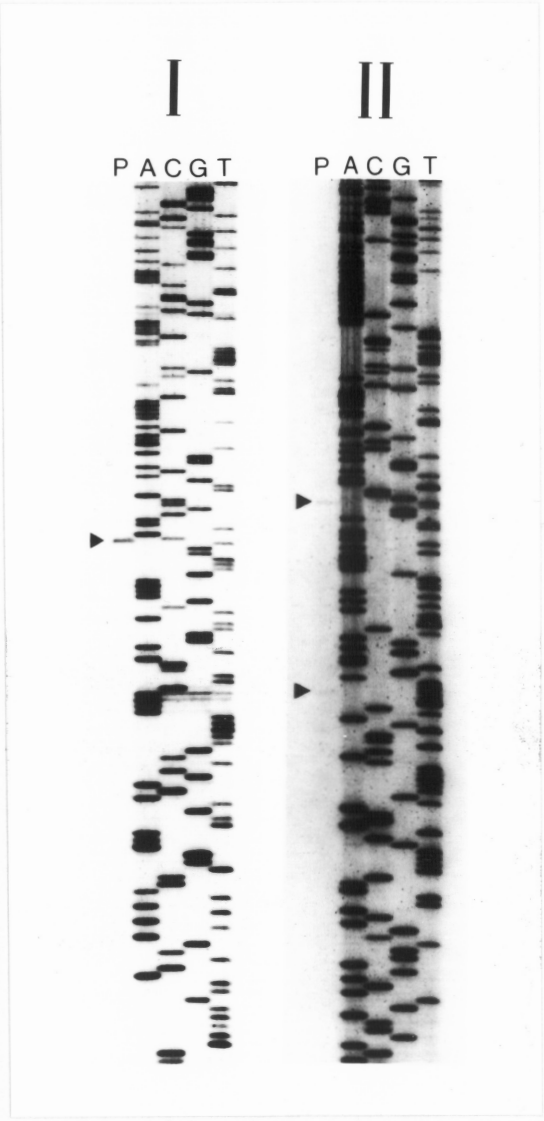


Figure 6. Primer extension analyses of *fdxA*- and ORF1-specific mRNAs. Primers were annealed with total RNA from *Ms. thermophila* grown on acetate (panel I) or methanol (panel II), extended with reverse transcriptase, and coelectrophoresed with DNA sequencing reactions (lanes A, C, G, T) initiated with the same oligonucleotide. Lane P, primer extension reaction. (Panel I) Primer was complementary to the +85 to +105 region of *fdxA* (Figure 2). (Panel II) Primer was complementary to the +157 to +177 region of ORF1 (Figure 3). Solid triangles indicate the locations of the major primer extension products.

shown), confirming the Northern blot results which indicated that ORF1 was transcribed at very low levels, or not at all, in acetate-grown cells. The finding of two extension products with the ORF1-specific primer may have resulted from either (i) recognition of the two potential promoter sequences by RNA polymerase or (ii) premature termination during the primer extension reactions at the A located 20 bases upstream of ORF1.

Comparison of DNA upstream of the coding regions of *fdxA* and ORF1 (Figure 7) showed regions of identity surrounding the potential promoters and the proposed transcriptional start sites. These regions may be important for recognition of the promoters by RNA polymerase.

## DISCUSSION

The gene (*fdxA*) encoding the ferredoxin previously isolated from *Ms. thermophila* has been cloned and sequenced. Based on the N-terminal amino acid sequence of the purified protein (25), the initiator methionine is processed to produce a mature protein of 59 amino acids. The difference between the predicted subunit molecular mass (6,080 Da) and that reported for the isolated ferredoxin ($M_r$ 16,400) (25) may result from atypical migration of ferredoxin during gel filtration chromatography or formation of an oligomeric complex. The spacing of the eight cysteines in the predicted *fdxA* gene product suggested


Figure 7. Comparison of DNA upstream of *fdxA* and ORF1. Numbering is relative to the first base of each coding region (see Figures 2 and 3). Bases that are identical between the *fdxA* and ORF1 sequences are indicated with asterisks. Potential promoter sequences are underlined and the proposed transcriptional start sites are indicated with large boldface letters.

that the protein could potentially coordinate two iron-sulfur clusters of the [3Fe-4S] or [4Fe-4S] type. Iron and sulfur analyses and electron paramagnetic resonance spectroscopy (6) indicate that the purified ferredoxin contains two [4Fe-4S] centers per 6,080-Da monomer. The predicted gene product of *fdxA* has very high identity (92%) with the ferredoxin from *Ms. barkeri* MS (peptide A), although the *Ms. barkeri* ferredoxin was reported to contain a [3Fe-3S] center (14); the basis for the difference in number and types of clusters in these two ferredoxins is unknown. The ferredoxin from *Mc. thermolithotrophicus* is also linked to CO dehydrogenase, but the similarity of this protein with the ferredoxins from *Methanosarcina* species is surprisingly low. There is a deep phylogenetic separation between *Methanococcus* and *Methanosarcina,* which belong to different taxonomic orders (4); thus, it is unknown if the differences in ferredoxin sequences reflect only evolutionary distances or also result in different biochemical properties. It is interesting that the deduced *fdxA* and ORF1 gene products of *Ms. thermophila* have higher identity with the *C. pasteurianum* ferredoxin than with the *Mc. thermolithotrophicus* ferredoxin.

Although it remains to be established that the gene product of ORF1 is a ferredoxin, the presence of multiple ferredoxins in one organism is common (7, 15, 28). ORF1 is postulated to encode a second ferredoxin based on the following characteristics of the predicted gene product: (i) size; (ii) the spacing of cysteines in clusters; (iii) similarity of the deduced amino acid sequence with

the *fdxA*-encoded and bacterial 2[4Fe-4S] ferredoxins; and (iv) absence of specific amino acids as noted in ferredoxins of *Methanosarcina* spp. The predicted ORF1 gene product has 81% identity with the 16 N-terminal amino acids of peptide B (Figure 4) from *Ms. barkeri* MS, which supports the proposal that peptide B is a fragment derived from a second ferredoxin in this organism (11). Thus, *Ms. barkeri* MS apparently has two ferredoxins with sequences similar to those of the deduced *fdxA* and ORF1 gene products of *Ms. thermophila*, suggesting that both organisms may require specific ferredoxins for different reactions. Less than half of the differences in the amino acid sequence between the deduced ORF1 and *fdxA* gene products are conservative; thus, *Ms. thermophila* may have two ferredoxins, possibly containing iron-sulfur clusters with different oxidation-reduction potentials, which may interact with different redox proteins. As with the deduced gene product of *fdxA*, the predicted ORF1 protein contained two conserved regions of amino acids, supporting the hypothesis (16) that a gene duplication event occurred prior to divergence of the recently proposed (27) *Bacteria* and *Archaea* domains.

Southern blots indicated that the *fdxA* and ORF1 genes are not clustered in the genome, and Northern blot analyses of RNA from *Ms. thermophila* confirmed that the two coding regions are not cotranscribed with other genes. *Methanobacterium thermoautotrophicum* contains a hydrogenase-linked ORF which is predicted to encode a "polyferredoxin" with six tandemly repeated

bacterial-ferredoxin-like domains, each with the potential to coordinate 2[4Fe-4S] clusters (18). The results presented here, and the recent purification of the 44-kDa polyferredoxin protein (12), clearly establish that the previously isolated *Ms. thermophila* ferredoxin is not derived from a processed polyferredoxin.

Although there is considerable similarity between the sequences surrounding the transcriptional start sites of *fdxA* and ORF1, additional promoter sequences for constitutive and regulated genes from *Ms. thermophila* must be identified before any predictions can be made concerning gene regulation.

## ACKNOWLEDGMENTS

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## SECTION IV. CHARACTERIZATION OF THE IRON-SULFUR CLUSTERS OF A FERREDOXIN FROM *Methanosarcina thermophila*


### SUMMARY

Chemical and spectroscopic analyses of a ferredoxin from *Methanosarcina thermophila* indicated that the as-isolated protein contained two [4Fe-4S] clusters per monomer of 6,790 Da, although a [3Fe-4S] species was also detected in the oxidized protein. The clusters in as-isolated ferredoxin were unstable in the presence of air and cluster loss accelerated with increasing temperature. The midpoint potentials of the [4Fe-4S] and [3Fe-4S] clusters at pH 7 were -407 mV and +103 mV, respectively. Reconstitution of ferredoxin with iron and sulfide resulted in only a slight decrease in the total amount of [3Fe-4S] clusters. As-isolated and reconstituted ferredoxin showed no significant differences in ability to accept and donate electrons *in vitro*. Evidence from spectroscopic studies indicated that the [3Fe-4S] species may be an artifact formed from [4Fe-4S] clusters when ferredoxin was oxidized.


### INTRODUCTION

Ferredoxins are ubiquitous electron-transport proteins which contain

active sites of nonheme iron and acid-labile sulfur. Although all iron-sulfur clusters are coordinated by cysteines and can accept a single electron, the clusters present in ferredoxins can be [2Fe-2S], [3Fe-4S], or [4Fe-4S]. While some ferredoxins contain a single cluster, others contain either two [4Fe-4S] clusters or one [3Fe-4S] and one [4Fe-4S]. The structure, stability, and redox potential of a particular iron-sulfur cluster depends on the environment in which it is located and is therefore influenced by protein structure. However, the amino acid sequence of a ferredoxin is not sufficient to accurately predict the number and type of iron-sulfur clusters present, although there appears to be some correlation between the sequence and clusters. For example, 2[4Fe-4S] ferredoxins are typically composed of about 60 amino acids, eight of which are cysteines, and have low levels of specific amino acids (8).

The methane-producing microorganisms are the most phylogenetically-diverse group in the recently proposed *Archaea* domain (46). The most metabolically diverse of the methanogenic *Archaea* are the *Methanosarcina* which can use $H_2$ and $CO_2$, acetate, methanol, or methylated amines as growth substrates. Although the biochemistry of methanogenesis from these substrates has been well studied, the mechanisms for electron transport and energy conservation have not been determined. A ferredoxin was previously isolated from *Methanosarcina thermophila* (44) and partially characterized, but the composition and biochemical properties of the iron-sulfur clusters were not

determined. The ferredoxin functions in accepting electrons from a CO dehydrogenase (CODH) enzyme complex following oxidation of CO to $CO_2$ and is required in a CO-dependent $H_2$-evolving system (43); synthesis of ATP is coupled to CO oxidation and proton reduction in whole cells of *Methanosarcina barkeri* (6). It has been proposed that CODH catalyzes both the cleavage of the C-C and C-S bonds of acetyl coenzyme A and oxidation of the carbonyl group to $CO_2$ during acetate conversion to $CH_4$ and $CO_2$ in *Ms. thermophila* (1); ferredoxin is then reduced by the electron pair. Acetyl coenzyme A conversion to $CH_4$ and $CO_2$ as well as CO-dependent $H_2$ production in cell extracts of acetate-grown *Ms. barkeri* MS are reported to be ferredoxin dependent (15). Thus, ferredoxin appears to be essential for energy conservation during methanogenesis from acetate in the *Methanosarcina*.

The amino acid sequence deduced from the gene (10) which encodes the *Ms. thermophila* ferredoxin has high identity with the available sequences of two ferredoxins isolated from different strains of *Ms. barkeri*. The ferredoxin purified from *Ms. barkeri* MS is reported to contain a single [3Fe-3S] cluster (35), while there is evidence for two [4Fe-4S] clusters in the ferredoxin isolated from *Ms. barkeri* Fusaro (20). A CODH-linked ferredoxin purified from *Methanococcus thermolithotrophicus* also contains two [4Fe-4S] clusters (21), but the protein sequence (7) has surprisingly low identity (~33%) with those of the three ferredoxins from the *Methanosarcina*. Ferredoxin from *Ms. thermophila*

was further characterized to study the properties of the iron-sulfur clusters, including their redox potentials. This data will be necessary for future studies designed to identify the route(s) of electron flow during acetate conversion to $CH_4$ and $CO_2$ in *Ms. thermophila*. Additionally, information about the biochemical properties of the *Ms. thermophila* ferredoxin will be valuable in assessing how closely related the methane-producing *Archaea* are to each other and to organisms in the *Bacteria* and *Eucarya* domains.

## MATERIALS AND METHODS

**Materials.** All chemicals used were reagent grade.

**Growth of organism and protein purification.** *Methanosarcina thermophila* strain TM-1 (48) was grown on sodium acetate as the carbon and energy source as described before (40) and stored in liquid $N_2$. All purification steps and manipulations of protein fractions were performed at 25-30°C in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) filled with $N_2$:$H_2$ mixture (19:1). Cell lysate was prepared as previously described (44), except that 2-mercaptoethanol was omitted from the cell breakage buffer. Ferredoxin was isolated as described before (44), without the pressure dialysis step, and collected in buffer A (50 mM $K^+$Tes [N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] pH 6.8, 1 mM $MgCl_2$, 10% [vol/vol] ethylene glycol)

which contained 0.6-1 M KCl. Purified ferredoxin (oxidized) had a 388 nm/300 nm absorption ratio of 0.77-0.82 and migrated as a single band during denaturing polyacrylamide gel electrophoresis.

The CO dehydrogenase enzyme complex (CODH) was partially purified as described previously (45). Ferredoxin and CODH were stored under $N_2$ at -20°C.

**Reconstitution of ferredoxin.** Dithiothreitol, $Fe(NH_4)_2(SO_4)_2$, and $Na_2S$ were dissolved individually in anaerobic buffer B (50 mM $K^+$Tes, pH 6.8) and added to as-isolated ferredoxin to yield 10, 8, 8, 1 moles of each, respectively. The mixture was incubated at 27°C for 6 h and then applied to a Mono-Q HR10/10 column (Pharmacia, Piscataway, NJ) equilibrated with buffer A. The column was washed with 20 ml of buffer A prior to elution of the reconstituted protein with 0.7 M KCl in buffer A. Reconstituted ferredoxin had a 388 nm/300 nm absorption ratio of 0.71-0.85.

**Analytical methods.** Ferredoxin protein concentrations were determined by the micro method with the bicinchoninic acid protein assay reagent (Pierce Chemical Co., Rockford, IL). Dialyzed and lyophilized ferredoxin from *Ms. thermophila* was the standard. Denaturing polyacrylamide gel electrophoresis (12% acrylamide) was performed using the Laemmli buffer system (31) and protein was visualized with silver stain (34).

Acid-labile sulfur and iron were quantitated as previously described (4,

16). Ferredoxin dialyzed against 5 mM sodium phosphate buffer (pH 7.5) was analyzed using a Jarrell-Ash 965 inductively-coupled plasma emission spectrophotometer and compared to standards of 40 elements, including selenium and transition metals known to be biologically important in proteins.

**Absorption spectroscopy and activity assays.** CODH activity was determined as previously described (45); one unit of CODH activity was defined as 1 μmol of methyl viologen reduced per min. The standard assay for metronidazole reduction (9) contained 103.6 μg CODH (3.5 U activity), 0.1 μmole metronidazole, 5-20 μg ferredoxin, and CO-saturated buffer A in a final volume of 1 ml; the final KCl concentration was 0.06 M. The standard assay for reduction of ferredoxin by CODH contained 65.5 μg ferredoxin, 4.6 μg CODH (0.2 U), and CO-saturated buffer A in a final volume of 0.5 ml; the final KCl concentration was 0.34 M. Reduction of ferredoxin was followed by monitoring the decrease in absorbance at 388 nm. All rates and spectra were collected on a Perkin-Elmer Lambda 4B UV/Vis spectrophotometer using the kinetics or spectral processing software.

**Stability of the iron-sulfur clusters in ferredoxin.** As-isolated ferredoxin (0.13 mg/ml) in buffer A containing 0.34 M KCl was incubated at the indicated temperatures either in the presence of air or in the anaerobic chamber. Integrity of the iron-sulfur clusters was determined by periodically collecting spectra (200-700 nm) and comparing the remaining absorbance at 388 nm to the

initial absorbance.

**Resonance Raman (RR) spectroscopy.** As-isolated ferredoxin in buffer A containing 1 M KCl was concentrated approximately 3 fold by incubating the sample at 35°C under $N_2$ 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 needed. RR spectra of the frozen protein solution held *in vacuo* (12) were obtained by collecting scattering 135° from the incident beam 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 using the spectral analysis program, Lab Calc.

**Electron paramagnetic resonance (epr) spectroscopy and potentiometric titrations.** As-isolated and reconstituted ferredoxin samples were frozen in liquid $N_2$ prior to epr analyses. The iron-sulfur clusters in as-isolated ferredoxin (100 μM protein in 700 μl of 50 mM Tes [pH 7], 1 mM MgCl, 8.5% [vol/vol] ethylene glycol, 0.6 M KCl) were titrated using an electrochemical epr cell as described previously (19). The intensity of the g = 1.94 signal at potentials between -300 mV and -500 mV vs. the Normal Hydrogen Electrode was used to monitor the extent of oxidation/reduction of the [4Fe-4S] clusters in the presence of benzyl viologen and methyl viologen (approximately 100 μM each).

Disodium anthraquinone-1,5-disulfonic acid, potassium indigotetrasulfonate, methylene blue, and phenasine methosulfate (100 μM each) were used in titrating the g = 2.02 signal of the [3Fe-4S] cluster at potentials higher than -300 mV. The midpoint potentials ($E_m$) of each iron-sulfur species at pH 7.0 and the number of electrons (n) involved in the reaction were calculated using the Nernst equation ($E_h = E_m + 59/n \cdot \log([ox]/[red])$ where $E_h$ is the poised potential measured at equilibrium. Spectra were recorded on a Bruker ESP300 (ECS106) epr spectrophotometer equipped with an Oxford ITC4 temperature controller. Double integration of epr signals was performed using 1 mM copper perchlorate as the standard.

## RESULTS

**Properties of as-isolated ferredoxin.** Ferredoxin purified from *Ms. thermophila* contained 6.7-8.5 atoms of iron and 5.6-6.9 atoms of acid-labile sulfur per monomer of 6,790 Da; dialyzed samples contained 6.2-7.5 atoms of iron. Based on elemental analyses, iron was the only metal present in as-isolated ferredoxin; selenium, a component of some iron-sulfur proteins, was not detected. The calculated extinction coefficients for the absorption maxima at 388 nm and 300 nm were 24.8-30.3 and 31.1-38.6 $mM^{-1} \cdot cm^{-1}$, respectively. Despite the variation in iron levels between different batches of as-isolated

ferredoxin, the ratio of the molar extinction coefficient at 388 nm to moles of iron was consistently about 3,600, which is typical for ferredoxins containing [4Fe-4S] clusters (47). The results of the chemical analyses suggested that as-isolated ferredoxin contained two chromophores (either [3Fe-4S] or [4Fe-4S]) per monomer. The variation in the amounts of iron and acid-labile sulfur as well as the difference in extinction coefficients indicated that some of the iron-sulfur clusters may have been damaged during purification.

As-isolated ferredoxin incubated aerobically exhibited a substantial decrease in absorbance at 388 nm when compared to ferredoxin samples incubated under anaerobic conditions (Figure 1). The stability of the clusters in the presence of air appeared to decrease as the incubation temperature was increased. The rate of absorbance loss during aerobic incubation of ferredoxin at 4°C was not affected by the concentration of KCl (0.07, 0.34, or 0.70 M) (data not shown). The spectral changes which occurred during air exposure indicated that the iron-sulfur clusters of as-isolated ferredoxin were sensitive to either oxygen or high redox potentials.

The previously-reported absorption spectrum of ferredoxin from *Ms. thermophila* (44) was typical of ferredoxins which contained either [3Fe-4S] or [4Fe-4S] centers. As a result, other spectroscopic studies were needed to differentiate between the two cluster types. Resonance Raman (RR) analyses of as-isolated ferredoxin produced bands at both 337 $cm^{-1}$ and 348 $cm^{-1}$ (Figure

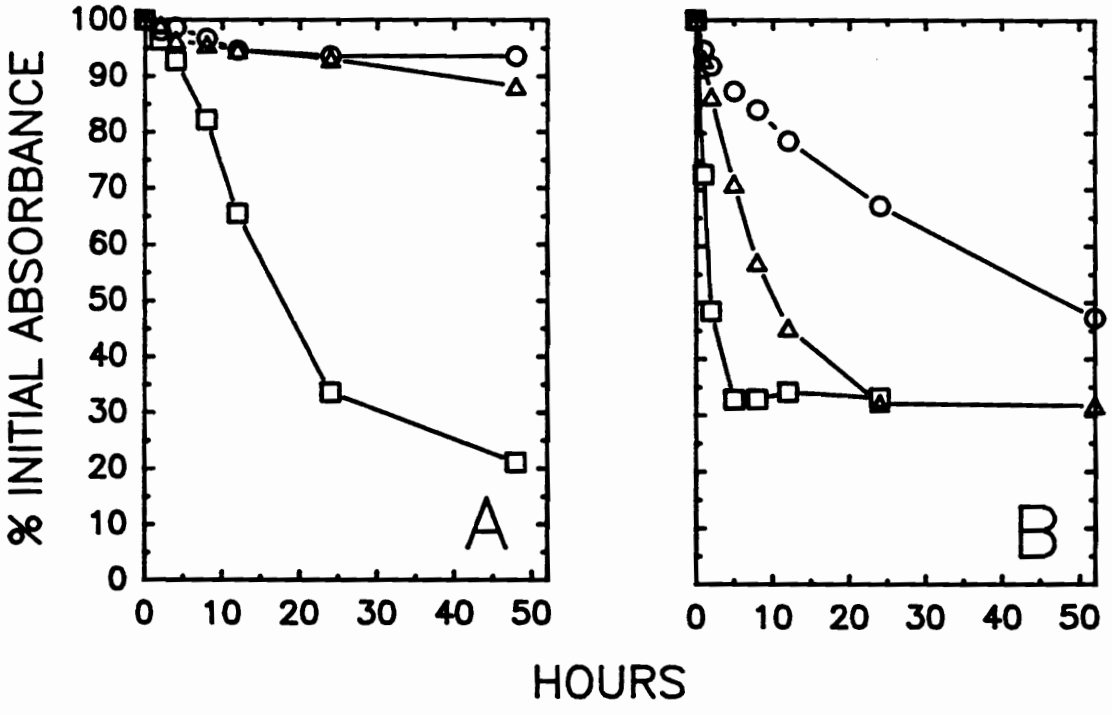


Figure 1. Stability of the iron-sulfur clusters in as-isolated ferredoxin. Ferredoxin (0.13 mg/ml) was incubated anaerobically (panel A) or aerobically (panel B) in buffer A containing 0.34 M KCl at 4°C (circle), 23°C (triangle), or 50°C (square). The decrease in absorbance at 388 nm was monitored and compared to the initial absorbance (approximately 0.480).

2A). The intensity of the 337 cm-1 signal was significantly greater than the one at 348 cm-1, even when the latter signal was maximally enhanced at 488.0 nm (Figure 2B). Vibrations at 337 cm-1 have been previously assigned to the A1 breathing modes of [4Fe-4S] clusters (13), while [3Fe-4S] centers have a characteristic signal at 348 cm-1 (25). Thus, the RR spectra indicated that as-isolated ferredoxin from *Ms. thermophila* contained both [3Fe-4S] and [4Fe-4S] species. Although RR data are not quantitative, the results suggested that [4Fe-4S] clusters were the prominent species.

As-isolated ferredoxin was analyzed by electron paramagnetic resonance (epr) spectroscopy to quantitate the two cluster types detected by RR and to determine the redox potential of each iron-sulfur cluster. A paramagnetic iron species with an apparent g value of 2.02, which is characteristic of [3Fe-4S] clusters, was detected in ferredoxin at potentials greater than 0 mV (Figure 3). This [3Fe-4S] signal corresponded to 0.4 spins per monomer of ferredoxin when the clusters were completely oxidized ($E_h$ = +142 mV). A signal at g = 4.3, characteristic of a $Fe^{3+}$ species, was observed at +80 mV (data not shown). An additional feature at g = 2.08 was detected at +50 mV (Figure 3), but the significance of this signal was not known. As the ambient redox potential was decreased to below -335 mV, the g = 2.02 signal was replaced by a complex epr signal (~g = 2) which reached its maximum intensity at a potential of -480 mV (Figure 4). Complex epr signals such as these have been previously attributed to

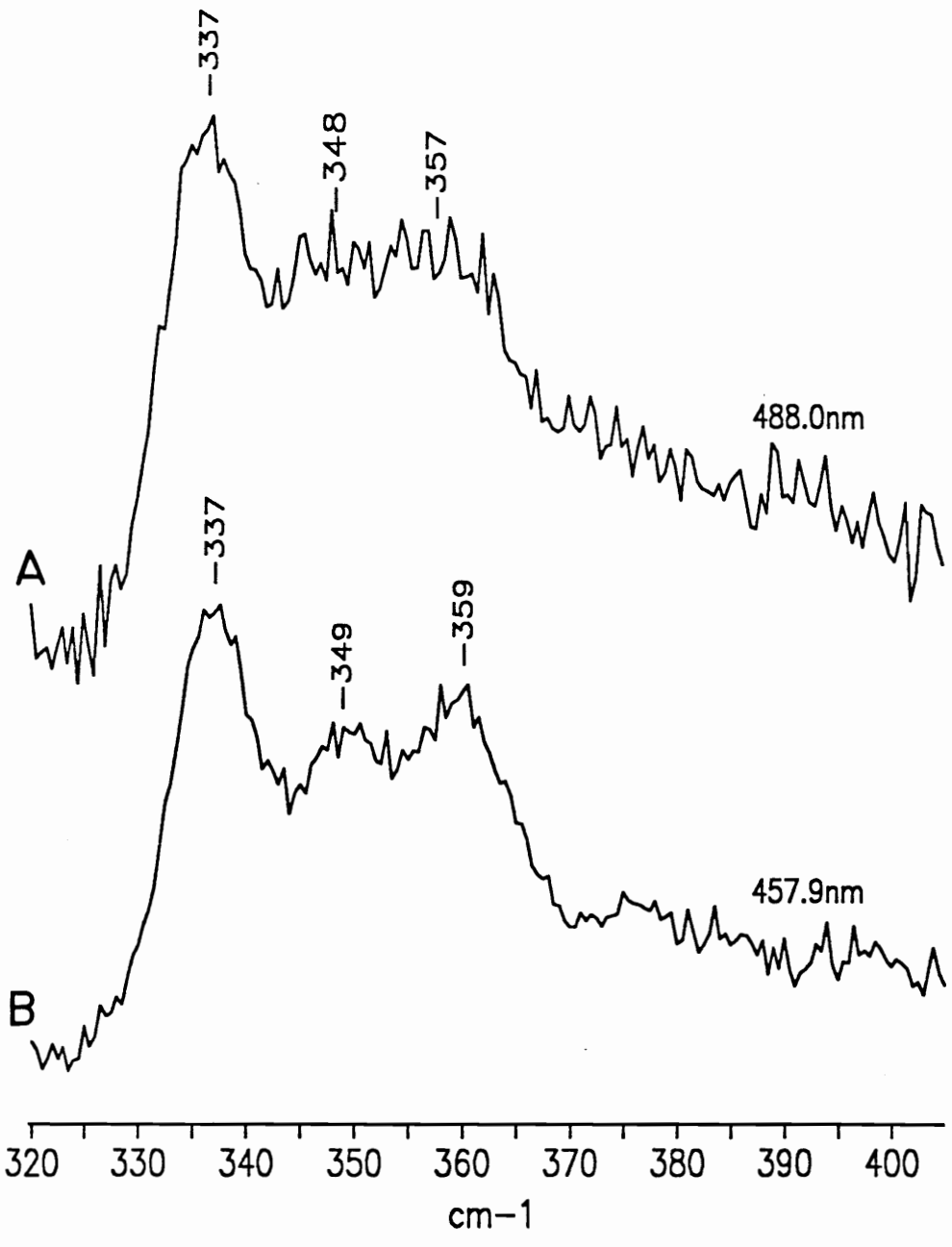


Figure 2. Resonance Raman spectra of as-isolated ferredoxin. As-isolated ferredoxin was thawed and loaded immediately into the sample cell of the spectrophotometer (5 min air exposure). Experimental conditions: laser power, 200 mW; slit width, 6 $cm^{-1}$; wavelength settings, 457.9 (spectrum A) or 488.0 nm (spectrum B).

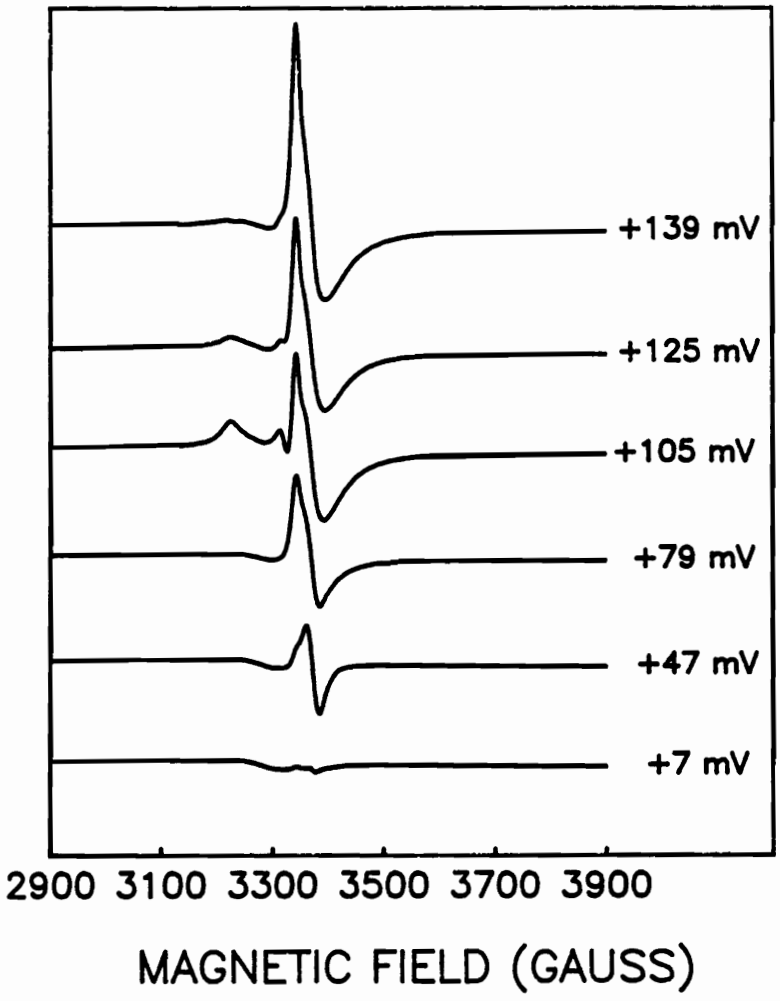


Figure 3. High potential epr spectra of as-isolated ferredoxin. Experimental conditions: ferredoxin, 100 μM; temperature, 9 K; microwave power, 2 mW; microwave frequency, 9.400 GHz; receiver gain, 2 x e4; modulation amplitude, 10 G; modulation frequency, 100 KHz.

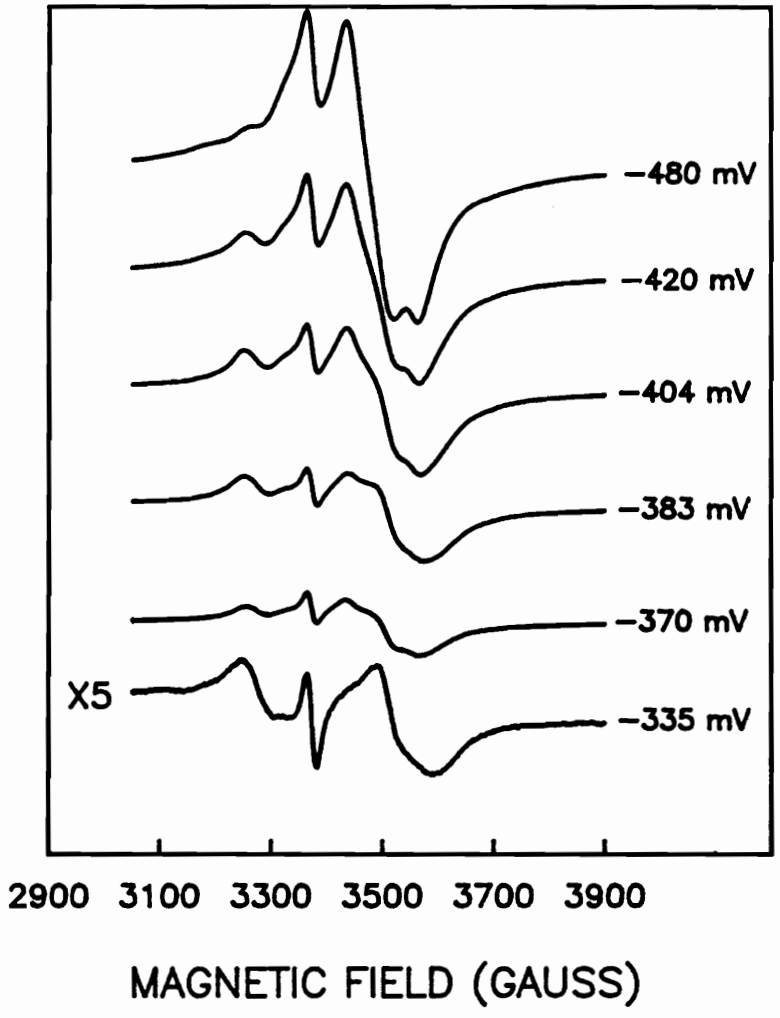


Figure 4. Low potential epr spectra of as-isolated ferredoxin.
Experimental conditions were the same as described in the legend for Figure 3.

the spin-spin coupling which results from dipolar interactions between two paramagnetic $[4Fe\text{-}4S]^{1+}$ clusters (32). Double integration of the fully-reduced spectrum ($E_h$ = -480 mV), after subtraction of the radical signal which resulted from the mediator dyes, corresponded to about 2.1 spins per monomer of ferredoxin. The presence of two [4Fe-4S] clusters which accounted for approximately 2 spins/monomer indicated that there were no other types of clusters (i.e. [3Fe-4S]) in fully-reduced ferredoxin. The epr spectrum of ferredoxin recorded at -335 mV (apparent g values of 2.007, 1.922, and 1.880) was typical of a single (S=1/2) $[4Fe\text{-}4S]^{1+}$ cluster (32, 38). Spectra recorded at potentials between -335 and -480 mV showed a mixture of the singly- and fully-reduced forms of as-isolated ferredoxin (Figure 4). These spectra were almost identical to those recorded for the *Clostridium pasteurianum* ferredoxin (3, 32, 38), although a feature at g = 2.12 in the fully-reduced *C. pasteurianum* ferredoxin (38) was not observed in ferredoxin from *Ms. thermophila*. A feature at g = 2.073 in the *Ms. thermophila* ferredoxin increased in intensity as the potential was decreased from -335 to -410 mV, but the intensity did not change further at potentials lower than -410 mV. A similar feature at g = 2.06 was previously observed in the partially-reduced *C. pasteurianum* ferredoxin (38), but this feature decreased in intensity as the potential was decreased from about -410 mV. There was no apparent signal resulting from $Fe^{3+}$ (g = 4.3) at potentials lower than -300 mV (data not shown).

The [3Fe-4S] and [4Fe-4S] clusters in as-isolated ferredoxin from *Ms. thermophila* (7.0 Fe/monomer) were titrated in the presence of redox mediator dyes using epr spectroscopy. The midpoint potential of the [4Fe-4S] chromophore ($E_m$, pH 7 = -407 ± 7.5 mV; slope = 62 ± 3 mV) was determined by plotting the intensity of the [4Fe-4S] signal versus redox potential (Figure 5A). Alternately, an $E_m$ value of -414 ± 4 mV (slope = 59.5 ± 2 mV) was calculated when signal amplitude at g = 1.94 was plotted versus redox potential. The single slope of ~60 mV when either method was used indicated that both [4Fe-4S] clusters had essentially the same $E_m$. This phenomenon has been reported for the two [4Fe-4S] clusters in ferredoxin from *C. pasteurianum* (38). The midpoint potential (pH 7) of the [3Fe-4S] cluster in the *Ms. thermophila* ferredoxin was +103 ± 10 mV (slope = 60 ± 2.5 mV) (Figure 5B). Signal amplitude of the g = 2.02 signal, instead of signal intensity, was used to determine the $E_m$ value of the [3Fe-4S] cluster since the shape of the epr signal varied somewhat with changing redox potential. The midpoint potential of the [3Fe-4S] cluster in the *Ms. thermophila* ferredoxin was outside the usual range of about -30 to -425 mV for [3Fe-4S] clusters in other ferredoxins (2, 18, 21, 24).

**Properties of reconstituted ferredoxin.** As-isolated ferredoxin (8.5 moles Fe/monomer) was incubated with $Fe^{2+}$ and $S^{2-}$ in the presence of dithiothreitol and repurified by anion-exchange chromatography. Reconstituted ferredoxin contained 8.2 moles of iron and, although the absorption spectra were similar to

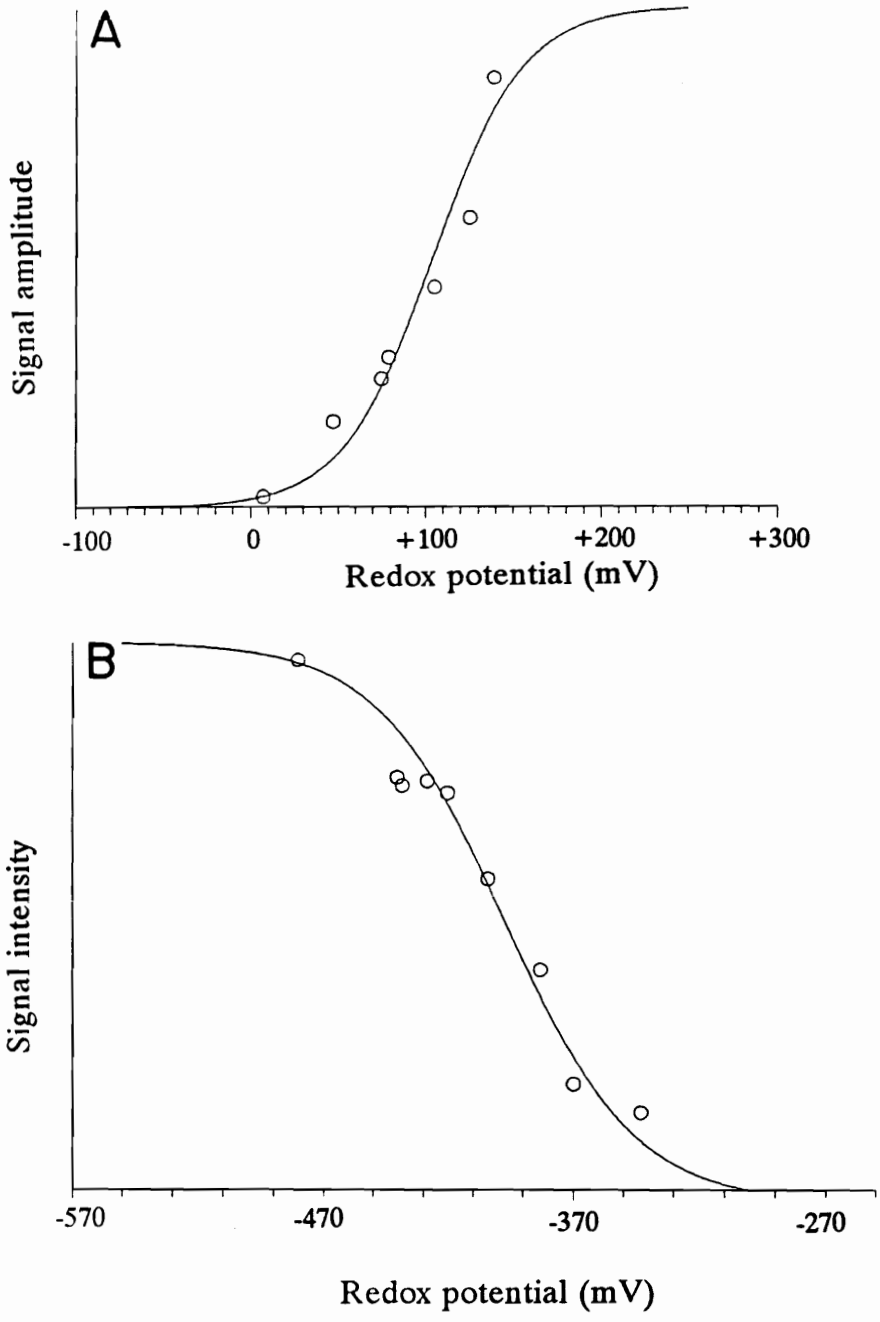


Figure 5. Potentiometric titrations of the [3Fe-4S] and [4Fe-4S] clusters. Points in all redox titrations were readily reversible. (Panel A) Titration of the [3Fe-4S] clusters. (Panel B) Titration of the [4Fe-4S] clusters. Epr conditions were the same as described in the legend for Figure 3.

those of the as-isolated protein (data not shown), the extinction coefficients of the reconstituted protein at 388 nm (25.4-39.5 $mM^{-1} \cdot cm^{-1}$) and 300 nm (32.1-46.5 $mM^{-1} \cdot cm^{-1}$) were higher than those of as-isolated ferredoxin. The g = 2.02 and g = 1.94 epr signals detected in as-isolated ferredoxin corresponded to 0.24 and 1.02 spins/monomer, respectively. Although the epr spectra of reconstituted ferredoxin were identical in shape to those of the as-isolated protein (data not shown), there was a slight decrease in the intensity of the [3Fe-4S] signal in reconstituted ferredoxin (0.16 spins/monomer) and a significant increase in the [4Fe-4S] signal (2.06 spins/monomer). The results suggested that some cluster conversion took place; however, the slight decrease in the number of [3Fe-4S] clusters following reconstitution was not enough to account for the substantial increase in the [4Fe-4S] signal from reconstituted ferredoxin. These observations indicated that under certain conditions some of the [3Fe-4S] centers in as-isolated ferredoxin could be converted to the [4Fe-4S] type *in vitro* although a complete conversion to [4Fe-4S] clusters was not achieved. Both the higher extinction coefficients and the significant increase in spins associated with reduced [4Fe-4S] clusters in reconstituted ferredoxin suggested that entire clusters were incorporated when as-isolated ferredoxin was reconstituted.

**Activities of as-isolated and reconstituted ferredoxin.** The ability of as-isolated and reconstituted ferredoxins to transfer electrons was compared using CODH as an electron donor and metronidazole as an electron acceptor. The

rates of reduction of ferredoxin by CODH and the rates of electron transfer from CO (via CODH and ferredoxin) to metronidazole were not significantly different with either as-isolated or reconstituted ferredoxin (data not shown). The results indicated that both the as-isolated and reconstituted ferredoxins were competent in transferring electrons, but it was not possible to make any conclusions concerning the physiological significance of the [3Fe-4S] clusters.

## DISCUSSION

Ferredoxin from *Ms. thermophila* contains two [4Fe-4S] clusters per monomer of approximately 6,790 Da, based on chemical and spectroscopic analyses. The ferredoxin from *Ms. thermophila* is similar to the 2[4Fe-4S] ferredoxin isolated from *C. pasteurianum*, based on the type of iron-sulfur clusters and comparison of the available primary protein sequences (32, 38, 42). The similarities are important since the *C. pasteurianum* protein is proposed to closely resemble the ancestral ferredoxin (5, 33). It is interesting that the ferredoxins purified from *Ms. barkeri* MS (22) and *Ms. thermophila* are 92% identical, yet the two proteins apparently coordinate different types and numbers of clusters. Despite the low identity (33%) between the protein sequences of the ferredoxins from *Ms. thermophila* and *Methanococcus thermolithotrophicus* (7), it appears that both proteins coordinate two [4Fe-4S] clusters. This

apparent inconsistency is not surprising in view of the fact that there is considerable diversity in both the methanogenic *Archaea* and the family of ferredoxins.

The low midpoint potential of the [4Fe-4S] clusters in the *Ms. thermophila* ferredoxin is consistent with the need for low-potential redox centers to accept electrons following oxidation of CO to $CO_2$ by CODH. Functional [3Fe-4S] clusters have been characterized in ferredoxins from *Azotobacter vinelandii* (24) and *Desulfovibrio africanus* (2), but the $E_m$ of these iron-sulfur centers is much lower than the +103 mV of the [3Fe-4S] species in the *Ms. thermophila* ferredoxin. The final electron acceptor in methanogenesis is proposed to be a membrane-associated heterodisulfide reductase ($E_m$ ~ -200 mV) (23, 27) which catalyzes the reduction of the disulfide of two cofactors, HS-CoM and HS-HTP, which are involved in methane formation. CoM-S-S-HTP reduction coupled to both proton pumping and ATP synthesis has been observed in one methanogen (14). Recently, CO- and $H_2$-dependent heterodisulfide reductase activities were detected in *Ms. thermophila* (11) and the former activity is known to be ferredoxin dependent in *Ms. barkeri* (15). It seems unlikely that the native ferredoxin from *Ms. thermophila* would contain a [3Fe-4S] cluster with a potential significantly higher than -200 mV since it would be especially difficult for any of the acetotrophic methane-producing *Archaea* ($\Delta G^{o'}$ for acetate = -8.9 kcal/mol $CH_4$) to grow with an inefficient system for conserving energy.

Therefore, the redox potential of the [3Fe-4S] cluster in the *Ms. thermophila* ferredoxin may be too high for physiological reactions.

It has been reported that the 2[4Fe-4S] ferredoxins isolated from *Thermodesulfobacterium commune* (18), *Mc. thermolithotrophicus* (21), and *C. pasteurianum* (26) also contained small amounts of [3Fe-4S] centers; it was proposed that the [3Fe-4S] species in the *T. commune* ferredoxin resulted from oxidative damage during purification. Although the *Ms. thermophila* ferredoxin was purified under anaerobic conditions, thus making it impossible to rule out the existence of [3Fe-4S] clusters in the native protein, the data presented here suggest that the [3Fe-4S] species is an artifact. The destruction of the iron-sulfur clusters in aerobically-incubated ferredoxin may explain the presence of [3Fe-4S] clusters in the as-isolated protein as well as the variation in iron and acid-labile sulfur levels in different ferredoxin preparations. The absorbance change at 388 nm observed when ferredoxin was exposed to air may be the result of conversion of [4Fe-4S] clusters to the [3Fe-4S] type followed by destruction of one or both cluster types. A similar reaction which resulted in the gradual destruction of the [4Fe-4S] and [3Fe-4S] centers in the ferricyanide-oxidized *A. vinelandii* ferredoxin I was previously reported (41). A conversion of the *C. pasteurianum* ferredoxin from a 2[4Fe-4S] protein to a 1[3Fe-4S] form has also been documented (26). Increased unfolding of the *Ms. thermophila* ferredoxin at high temperatures may increase the strain on the clusters, thus

contributing to the instability of the centers in the presence of air. It is also possible that the [3Fe-4S] clusters form in ferredoxin when the redox potential is raised to greater than 0 mV. Studies of model compounds suggest that [4Fe-4S] clusters can be converted to [3Fe-4S] clusters via a process of two electron oxidation (39) and that during this conversion some of the iron in the cluster is converted to $Fe^{3+}$. A $Fe^{3+}$ species was observed in as-isolated ferredoxin from *Ms. thermophila* at high potentials (+80 mV), but not when spectra were recorded below -300 mV. The spin quantitation values for as-isolated ferredoxin indicated that [3Fe-4S] clusters are generated from the [4Fe-4S] clusters during oxidation of the protein and converted back to the [4Fe-4S] form upon reduction.

Reconstitution of as-isolated *Ms. thermophila* ferredoxin with excess iron resulted in a slight decrease in the intensity of the [3Fe-4S] signal. Thus, some of the iron-sulfur clusters of ferredoxin can be interconverted *in vitro* under the conditions used in this report. However, the iron content of ferredoxin did not change significantly after reconstitution which suggests that adventitiously-bound iron was incorporated into the clusters upon reconstitution or during reduction of the reconstituted protein. Conversion of [3Fe-4S] clusters to the [4Fe-4S] type occurs in other ferredoxins under strongly-reducing conditions without the addition of excess iron (18, 26). If a conversion of [3Fe-4S] clusters to the [4Fe-4S] type occurred under reducing conditions, the reduced as-isolated and

reduced reconstituted ferredoxin from *Ms. thermophila* may have had almost identical cluster compositions and would be expected to transfer electrons at the same rate. Although cluster interconversion may occur upon reduction of the as-isolated *Ms. thermophila* protein, reconstitution with excess iron (in the presence of sulfide and dithiothreitol) appears to have been responsible for the formation of additional [4Fe-4S] clusters in the reconstituted ferredoxin (1.02 spins vs. 2.06 spins). There is evidence that under certain conditions the cluster in *Desulfovibrio gigas* ferredoxin I can be interconverted *in vitro* (29, 37) and studies with cell extracts indicated that this interconversion may be physiological (36). Thus, specific regulation of electron-transport components, which may result in a change in redox potential and reactivity with other electron carriers, could be valuable to the cell for limiting oxidative damage to other cellular components. $F_{420}$, an electron carrier involved in reduction of $CO_2$ to $CH_4$, is reversibly modified by the enzymatic addition of either AMP or GMP to form $F_{390}$ when cells of *Methanobacterium thermoautotrophicum* are exposed to oxygen (30). $F_{390}$ has a higher redox potential than $F_{420}$, (-320 vs. -350 mV) (17), and a lower affinity for the $F_{420}$-reducing hydrogenase (28). If a non-functional [3Fe-4S] cluster is formed in the *Ms. thermophila* ferredoxin under non-physiological oxidative conditions, the activity and redox potential of the protein could potentially be altered, thus affecting electron flow.

## ACKNOWLEDGMENTS

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## SECTION V: HETERODISULFIDE REDUCTASE ACTIVITIES IN CELL EXTRACTS OF ACETATE-GROWN *Methanosarcina thermophila*


### SUMMARY

The heterodisulfide of HS-HTP (7-mercaptoheptanoylthreonine phosphate) and HS-CoM (coenzyme M, 2-mercaptoethanesulfonate) was enzymatically reduced in cell extracts of *Ms. thermophila* using electrons supplied by either $H_2$ or CO. The homodisulfides of either HS-HTP or HS-CoM were not reduced under the same conditions which indicated that the heterodisulfide reductase recognized both structures on the disulfide substrate. The results suggested that during the final steps of methanogenesis from acetate in *Ms. thermophila* $CH_3$-S-CoM is reductively demethylated by the methyl coenzyme M methylreductase, using HS-HTP as an electron donor, yielding methane and the heterodisulfide as products.


### INTRODUCTION

Methane-producing microorganisms are the largest and most phylogenetically-diverse group in the *Archaea* (16). The final step in methanogenesis from all known substrates is the reductive demethylation of

$CH_3$-S-CoM by the methyl coenzyme M methylreductase system which utilizes component B as an electron donor. The structure of component B from the $CO_2$-reducing organism *Methanobacterium thermoautotrophicum* strain $_{\Delta}$H (12) and the methylotrophic organism *Methanosarcina thermophila* (3) has been identified as 7-mercaptoheptanoylthreonine phosphate. The heterodisulfide (CoM-S-S-HTP) which is formed during reduction of $CH_3$-S-CoM to $CH_4$ by the methylreductase of *Mb. thermoautotrophicum* is subsequently reduced by a heterodisulfide reductase to yield the free sulfhydryl forms of each cofactor (2, 8). In addition to regenerating HS-CoM and HS-HTP, heterodisulfide reductase may also be involved in energy flow in the methanogenic *Archaea*. Both proton translocation and ATP synthesis are coupled to the reduction of CoM-S-S-HTP in everted membrane vesicles of the methylotrophic methanogen Gö1 (4).

*Ms. thermophila* reduces the methyl group of acetate, methanol, or methylamines to methane. The pathway for acetate catabolism (10) involves activation of acetate to acetyl coenzyme A which is then cleaved by a CO dehydrogenase enzyme complex. The enzyme complex catalyzes both the cleavage of the C-C and C-S bonds and oxidation of the carbonyl group of acetyl coenzyme A to $CO_2$ (1). The methyl group is ultimately transferred to HS-CoM and the resulting $CH_3$-S-CoM is reductively demethylated to $CH_4$ by methyl coenzyme M methylreductase. The purified methylreductase utilizes HS-HTP as an electron donor (10); thus it is proposed that CoM-S-S-HTP is a product of

the reaction and that the heterodisulfide is subsequently reduced by an electron pair generated from oxidation of the carbonyl group to $CO_2$.

## MATERIALS AND METHODS

**Materials.** CoM-S-S-HTP, CoM-S-S-CoM, and HTP-S-S-HTP were synthesized as previously described (2, 13) and were supplied by Thomas A. Bobik at the University of Illinois, Urbana, IL. 5,5' dithiobis-(2-nitrobenzoic acid), HS-CoM, and bovine $\gamma$-globulins were obtained from Sigma Chemical Co., St. Louis, MO.

**Growth of cells and preparation of cell-free extracts.** *Methanosarcina thermophila* strain TM-1 (17) was grown on sodium acetate as the carbon and energy source (14) and stored in liquid nitrogen. Cell extracts were prepared anaerobically under a $N_2$ atmosphere as described (11) except that the breakage buffer consisted of 50 mM Bis-Tris pH 6.5, 1 mM $MgCl_2$, 10% (vol/vol) glycerol, and 0.015 mg/ml DNase I. Cell lysate was centrifuged at 1,500 x *g* for 15 minutes at 4°C and the resulting supernatant was stored in liquid nitrogen.

**Enzyme assays.** CO dehydrogenase and hydrogenase activities were determined as previously described (15), except that the methyl viologen assay mixture contained 50 mM sodium phosphate buffer (pH 7.5) instead of Tris. One unit of enzyme activity was defined as 1 µmol of methyl viologen reduced

per min.

The heterodisulfide reductase assay mixture (0.5 ml) contained 0.51 mg of cell extract protein and 250 nmol disulfide (CoM-S-S-HTP, CoM-S-S-CoM, or HTP-S-S-HTP) in 50 mM Bis-Tris (pH 6.5). All components were added to serum-stoppered 8.5 ml amber vials which contained 1 atmosphere of $N_2$ and were placed on ice. $H_2$ or CO was added to the vials and incubation was continued for 5 min at 4°C. Reactions were initiated by transfer of the vials to a reciprocating water bath (220 rpm) at 56°C. Total thiols were quantitated (6) by adding 20 µl of the reaction mixture to 980 µl of 100 mM $K_2HPO_4$ containing 5,5' dithiobis-(2-nitrobenzoic acid) at a final concentration of 0.3 mM. The absorbance at 412 nm was used to monitor free thiols in each sample. HS-CoM was used as a standard.

**Protein determination.** Protein concentrations were determined by the micro method using bicinchoninic acid protein assay reagent (Pierce Chemical Co., Rockford, IL). Bovine $\gamma$-globulins were used as a standard.

## RESULTS

Cell extracts of *Ms. thermophila* contained hydrogenase and CO dehydrogenase activities (3.6 and 2.4 U/mg protein, respectively) and catalyzed the reduction of heterodisulfide (CoM-S-S-HTP) with either $H_2$ or CO as a

source of electrons (Figure 1); no significant reduction was observed under a 100% $N_2$ atmosphere. Formation of thiols was minimal (< 1 nmol produced after 12 min) when boiled cell extract replaced native cell extract or if CoM-S-S-HTP was omitted from the reaction mixture (data not shown). No significant amount of thiols (< 2 nmol) was produced after 12 min when CoM-S-S-HTP was replaced with an equal molar amount of CoM-S-S-CoM or HTP-S-S-HTP in the presence of either $H_2$ or CO (data not shown). Thus, $H_2$- and CO-dependent disulfide reduction in cell extracts was enzymatic and specific for CoM-S-S-HTP. The results indicated that the heterodisulfide reductase of *Ms. thermophila* recognized the HTP and CoM structures on the disulfide substrate. The apparent initial specific activity of heterodisulfide reduction was 954 nmol thiols produced/min·mg protein under a 1:9 $H_2$:$N_2$ atmosphere (Figure 1). Reduction of CoM-S-S-HTP under a 1:89 CO:$N_2$ atmosphere began after a lag of 0.5 min with a specific activity of 86 nmol/min·mg protein (Figure 1).

## DISCUSSION

Recently, it was reported that HS-HTP is an electron donor for the methyl coenzyme M methylreductase purified from *Ms. thermophila* (10). These results, and the results presented here, strongly indicate that reduction of $CH_3$-S-CoM to $CH_4$ yields CoM-S-S-HTP which is then reduced by a heterodisulfide

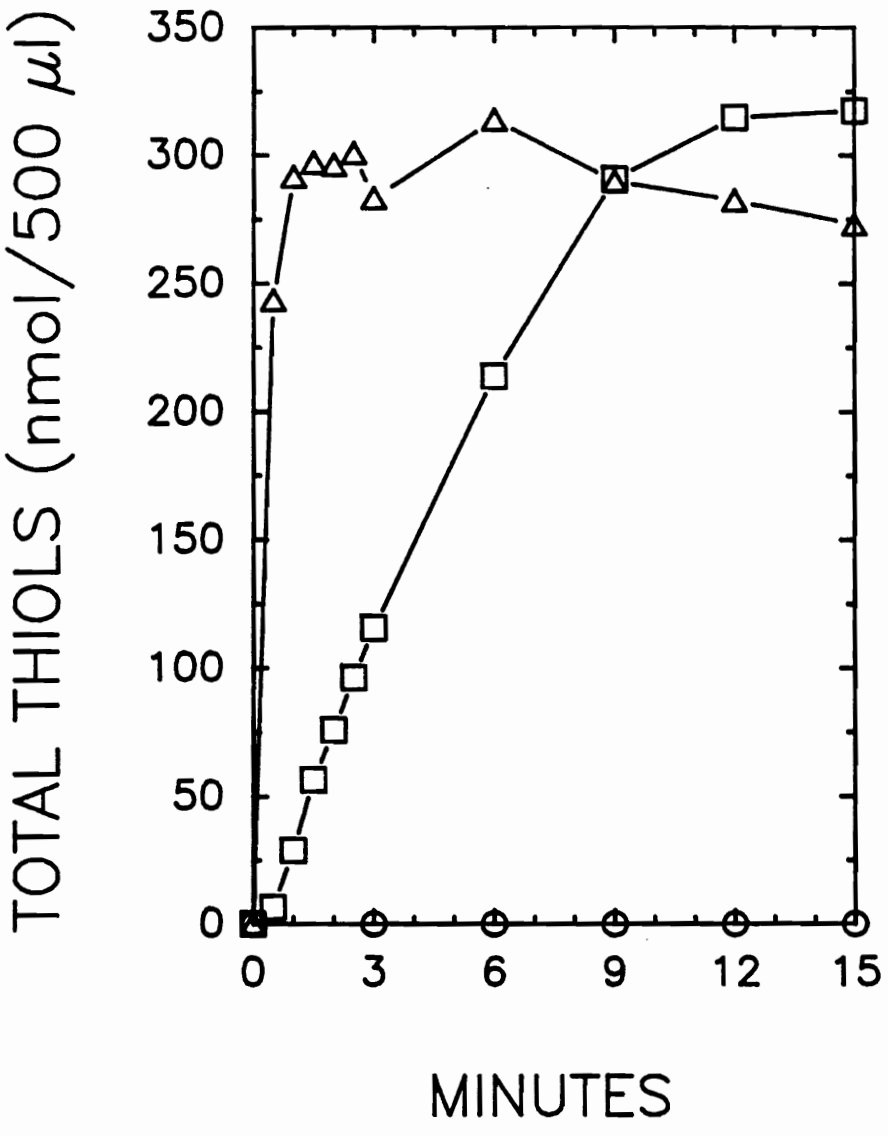


Figure 1. Reduction of CoM-S-S-HTP by cell extracts of *Ms. thermophila*. Reaction mixtures (see Materials and Methods) were under 101 kPa of 100% $N_2$ (circles), 1:8 $H_2$:$N_2$ (triangles), or 1:89 CO:$N_2$ (squares).

reductase during acetate conversion to methane and $CO_2$ in *Ms. thermophila*. Both $H_2$ and CO were sources of electrons for heterodisulfide reduction which was consistent with the previous report that either $H_2$ or CO could donate electrons for reduction of $CH_3$-S-CoM to $CH_4$ in cell extracts of *Ms. thermophila* (11). These results suggest that this organism transfers electrons to the heterodisulfide reductase using either a branched pathway or two distinct electron transport chains. Although acetate-grown cells of *Ms. thermophila* are unable to utilize $H_2$ and $CO_2$ for methanogenesis, the apparent specific activity of $H_2$-dependent heterodisulfide reductase was greater than the highest activity reported for a methane-producing microorganism which couples $CO_2$ reduction with the oxidation of $H_2$ (9); however, it is not known if this $H_2$-dependent activity is essential for acetotrophic growth of *Ms. thermophila*. It is proposed that ferredoxin, and possibly other electron carriers, transfer an electron pair to the heterodisulfide reductase following oxidation of the carbonyl group of acetyl coenzyme A to $CO_2$ by the CO dehydrogenase enzyme complex of *Ms. thermophila* (10). Consistent with this proposal, a ferredoxin is required for CO-dependent heterodisulfide reductase activity in *Methanosarcina barkeri* strain MS (7).

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## SECTION VI. SUMMARY AND DISCUSSION

An understanding of how the growth of the methanogenic *Archaea* is tied to methane formation requires characterization of both electron-transport components and elucidation of the chemiosmotic mechanism for generating a membrane potential. Studies using inhibitors of ATP synthesis and compounds that dissipate electrochemical membrane potentials have been fruitful since whole cells and membrane vesicles can be used. However, studies involving electron-transport chains have proceeded more slowly because of the need to isolate and characterize each electron carrier.

The biochemical properties of ferredoxin and its iron-sulfur clusters presented here will be valuable for future studies in determining the electron acceptor from ferredoxin. The fact that electrons derived from the oxidation of CO can be used by the heterodisulfide reductase implicates ferredoxin in this path of electron flow. Now that the first and last electron carriers are known, it should be easier to establish whether other known electron-transport components (e.g. b-type cytochrome and hydrogenase) are also involved. Additionally, the $H_2$-dependent heterodisulfide reduction also suggests the existence of multiple routes of electron flow to support the final reductive steps required for methane formation in *Ms. thermophila*. However, it is not known if the $H_2$-dependent heterodisulfide reductase activity in *Ms. thermophila* is

membrane associated.

The predicted existence of a second ferredoxin (deduced from ORF1) suggests that another enzyme besides the CO dehydrogenase complex requires a low-potential electron carrier. A ferredoxin protein was purified from *Ms. barkeri* which, along with a hydrogenase-containing fraction, was required for reductive activation the $MT_{M1}$ methyltransferase (83). This ferredoxin was approximately 12 kDa and contained 3.8-4.0 moles of iron, but the amino acid sequence was not determined. A dimer of the deduced ORF-1 protein would be approximately 12 kDa. The expression of ORF-1 in cells grown on methanol, but not acetate, is consistent with the proposal that the deduced ORF1 protein may function in activation of $MT_{M1}$. The small amount of ORF1-specific mRNA in trimethylamine-grown cells may have been a result of (i) partial repression, (ii) partial activation, or (iii) a need to use the predicted ORF1 protein in metabolizing trimethylamine.

The cloning and sequencing of *fdxA* and ORF1 which encode ferredoxin and the predicted ferredoxin-like protein, respectively, represents the beginning of an approach to studying the regulation of gene expression in *Ms. thermophila*. Although *fdxA* is not transcriptionally regulated according to the growth substrate, the proposed promoter sequence of this constitutively-transcribed gene will be useful for comparison with promoters of regulated genes. Likewise, ORF1 is interesting since it is not expressed in acetate-grown cells. Ultimately,

the identification of promoter sequences for genes that encode proteins such as CO dehydrogenase, phosphotransacetylase, acetate kinase, and carbonic anhydrase, along with the isolation and complementation of mutants of *Ms. thermophila*, will make it possible to propose models that account for the metabolic regulation previously observed in the *Methanosarcina*.

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bacteria. Ann. Rev. Microbiol. 26:139-162.

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## SECTION VIII. *CURRICULUM VITAE*

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**EDUCATION:**

1987-present:
Doctoral candidate, Department of Anaerobic Microbiology. Virginia Polytechnic Institute and State University (VPI&SU), Blacksburg, VA.

1982-1986:
B.S. Biology (Microbiology option), Chemistry minor. VPI&SU.

**PROFESSIONAL EXPERIENCE:**

May 1991-present, Sept. 1987-Sept. 1989:
Graduate Project Assistant, Department of Anaerobic Microbiology. VPI&SU.

Methane-producing organisms: anaerobic techniques, protein purification, biochemical assays, chemical and spectroscopic characterization of iron-sulfur clusters, genomic DNA isolation and cloning, DNA sequencing, RNA isolation and analyses.

Sept. 1990-May 1991:
Cunningham Dissertation Fellow, Department of Anaerobic Microbiology. VPI&SU.

(Same as Graduate Project Assistant.)

Sept. 1989-Sept 1990:
Graduate Teaching Assistant, Department of Anaerobic Microbiology. VPI&SU.

Laboratory instructor for Pathogenic Bacteriology.

Sept. 1986-Sept. 1987:
Laboratory Technician, Department of Anaerobic Microbiology, VPI&SU.

Methane-producing organisms: large-scale culturing of strictly-anaerobic organisms, isolation of plasmid DNA and transformation of *Escherichia coli*, DNA sequencing.

Sept. 1985-June 1986:
Undergraduate Research, Department of Microbiology, VPI&SU.

Isolation and biochemical analyses of groundwater bacteria.

**PERSONAL:**

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**PUBLICATIONS:**

Clements, A. P. and J. G. Ferry. 1992. Cloning, nucleotide sequence, and transcriptional analyses of the gene encoding a ferredoxin from *Methanosarcina thermophila*. J. Bacteriol. 174:5244-5250.

Clements, A. P., R. H. White, and J. G. Ferry. Structural characterization and physiological function of component B from *Methanosarcina thermophila*. Submitted.

Alber, B. E., A. P. Clements, P. E. Jablonski, M. T. Latimer, and J. G. Ferry. Enzymology of the methanogenic fermentation of acetate by *Methanosarcina thermophila*. Submitted.

Clements, A. P., L. Kilpatrick, T. G. Spiro, W-P. Lu, S. W. Ragsdale, and J. G. Ferry. Characterization of the iron-sulfur clusters in a ferredoxin from *Methanosarcina thermophila*. Manuscript in preparation.

**ABSTRACTS AND PRESENTATIONS:**

Clements, A. P. and J. G. Ferry. 1991. Cloning of the genes encoding ferredoxin and a putative ferredoxin from *Methanosarcina thermophila*. Abstracts of the Annual Meeting of the American Society for Microbiology. Abstract I116, p. 209.

Clements, A. P. and J. G. Ferry. 1991. Electron transport during acetate conversion to methane. Virginia-branch American Society for Microbiology Annual Meeting.

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