A new type of sulfite reductase - a novel coenzyme F$_{420}$-dependent enzyme from the methanarchaeon *Methanocaldococcus jannaschii*

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Running title: Coenzyme F$_{420}$-dependent sulfite reductase from *M. jannaschii*

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*Methanocaldococcus jannaschii* is a hyperthermophilic, strictly hydrogenotrophic, methanogenic archaeon of ancient lineage isolated from a deep-sea hydrothermal vent. It requires sulfide for growth. Sulfite is inhibitory to the methanogens. Yet, we observed that *M. jannaschii* grows and produces methane with sulfite as the sole sulfur source. We found that in this organism sulfite induces a novel, highly active, coenzyme F$_{420}$-dependent sulfite reductase (Fsr) with a cell extract specific activity of 0.57 µmol sulfite reduced min$^{-1}$ mg$^{-1}$ protein. The cellular level of Fsr protein is comparable to that of methyl-coenzyme M reductase, an enzyme essential for methanogenesis and a possible target for sulfite. Purified Fsr reduces sulfite to sulfide using reduced F$_{420}$ (H$_2$F$_{420}$) as the electron source (K$_m$: sulfite, 12 µM; H$_2$F$_{420}$, 21 µM). Therefore, Fsr provides *M. jannaschii* an anabolic ability and protection from sulfite toxicity. The N-terminal half of the 70 kDa Fsr polypeptide represents a H$_2$F$_{420}$ dehydrogenase and the C-terminal half a dissimilatory-type siroheme sulfite reductase, and Fsr catalyzes the corresponding partial reactions. Previously described sulfite reductases use nicotinamides and cytochromes as electron carriers. Therefore, this is the first report of a coenzyme F$_{420}$-dependent sulfite reductase. Fsr homologs were found only in *Methanopyrus kandleri* and *Methanothermobacter thermotrophicus*, two strictly hydrogenotrophic thermophilic methanogens.

fsr is the likely ancestor of H$_2$F$_{420}$ dehydrogenases, that serve as electron input units for membrane based energy transduction systems of certain late evolving archaea, and dissimilatory sulfite reductases of bacteria and archaea. fsr could also have arisen from lateral gene transfer and gene fusion events.

Methanogenesis by the methanogenic archaea is inhibited by sulfite (1). This oxyanion is a strong nucleophile and is known to be toxic to cells of all types due to its reactivity towards proteins and sulfhydryl groups (2). Methanogens perhaps have an additional reason for sulfite sensitivity. In vitro sulfite reacts with and inactivates purified methyl-coenzyme M reductase (3,4), an essential enzyme for methanogenesis (5). Yet two methanogens, *Methanothermococcus thermolithotrophicus* and *Methanothermobacter thermotrophicus*, have been reported to tolerate and even use sulfite as a sole sulfur source (6,7). Also, as shown in this report, *Methanocaldococcus jannaschii*, a deeply rooted hyperthermophilic methanogenic archaeon isolated from a deep-sea hydrothermal vent (8), grows with sulfite. However, the genome of *M. thermolithotrophicus* and *M. jannaschii* do not carry a clear homolog of a sulfite reductase (9,10); the genome sequence of *M. thermolithotrophicus* is yet to be determined. With the goal of identifying the sulfite detoxification and assimilation mechanisms of these organisms, we have studied sulfite metabolism of *M. jannaschii*. As shown below, this work has led to the discovery of a new type of sulfite reductase.
MATERIALS AND METHODS

Growth of M. jannaschii - The organism was grown on H₂ + CO₂ (80:20, v/v; 3 x 10⁵ Pa) in a mineral salts medium in sealed 500 ml serum bottles, as described previously (8,11), with either sodium sulfite (1 mM) or sulfide (1 mM) as the sole sulfur source and medium reductant. The cells were harvested by centrifugation at 9600 x g and 4 °C anaerobically under an N₂-CO₂ atmosphere (80:20 v/v).

Methane measurement - Methane was assayed by use of a Hewlett Packard model HP5890 gas chromatograph (Agilent Technologies, Inc., Palo Alto, CA) fitted with a flame ionization detector and a 0.5 mm x 30 m HP-PLOT (aluminum oxide, 15 µm) column. The column, detector and injector were maintained at 100, 150 and 150°C, respectively. The carrier gas (N₂) flow rate was 1 ml per min. A methane standard (Matheson Tri-Gas, Montgomeryville, PA) was used for calibration.

Protein analysis - SDS-PAGE was performed according to Laemmli (12), and the same method but employing buffers without SDS and omitting the sample denaturation and reduction step was used for non-denaturing gel electrophoresis. The identity of a polypeptide in a gel band was determined by in-gel trypsin digestion, MALDI-TOF mass spectrometry and database searches as described previously (13,14). Protein was assayed according to Bradford (15). Gel filtration chromatography was also conducted as described previously (16) but with the following modifications. The mobile phase was made anaerobic by purging it with helium and was maintained such under a helium blanket. To the anaerobic column with a constant flow of mobile phase, an anaerobic Fsr sample was applied via auto-injection from a sealed via. These precautions ensured a separation under anaerobic condition.

Enzyme assays - F₄₂₀-dependent sulfite reductase (Fsr) activity was assayed spectrophotometrically under strictly anaerobic conditions. It involved monitoring the oxidation of reduced F₄₂₀ (H₂F₄₂₀) with sodium sulfite at 400 nm. The anaerobic assay method has been described previously (17). The reaction rate was calculated from an extinction coefficient of 25 mM⁻¹ cm⁻¹ for F₄₂₀ at 400 nm (18). For each standard assay a 1 ml reaction mixture containing the following components was used: 50 mM potassium phosphate (KPi) buffer, pH 7, 40 µM reduced F₄₂₀ (H₂F₄₂₀), and 1.5 mM sodium sulfite. The assay was initiated by the addition of enzyme. For pH studies, the KPi buffer was replaced with constant ionic strength buffers (14). H₂F₄₂₀ was generated by chemical reduction of F₄₂₀, that was purified from Methanothermobacter thermautotrophicus (17,19), in water with NaBH₄ (20). Unreacted NaBH₄ was titrated with HCl. When methylviologen (MV⁺) replaced sulfite as an electron acceptor, the assay mixture also contained 1.44 mM metronidazole (20). In this assay the oxidation of H₂F₄₂₀ could be followed at 400 nm without an interference from reduced methylviologen (MV⁰), because the latter was continuously re-oxidized by metronidazole (20). For determining the rate of oxidation of MV⁰ with sulfite, H₂F₄₂₀ in the standard assay was replaced with MV⁰ and the reaction was followed at 560 nm (18); MV⁰ was generated by reducing MV⁺ with titanium citrate (21).

Assay for sulfide produced in Fsr reaction - A 160 µl standard Fsr reaction mixture containing H₂F₄₂₀, sodium sulfite, and 0.2 µg purified enzyme was used. To avoid interference from reactants and side products, the sulfide produced in the reaction was recovered from the reaction mixture by use of a micro gas diffusion cell prior to assay (22). The resulting Na₂S was quantified by the methylene blue method (23). To determine the amount of F₄₂₀ generated by the reaction, an aliquot of the assay mixture was diluted in KPi buffer, pH 7, and the absorbance of this solution at 400 nm was determined. From this absorbance data and by use of the extinction coefficient for F₄₂₀, as indicated in the preceding section, the amount of the oxidized coenzyme formed was calculated.

Purification of F₄₂₀-dependent sulfite reductase - All steps, except the centrifugation of ammonium sulfate treated extract (see below), were performed inside an anaerobic chamber containing a gas.
atmosphere of N\textsubscript{2} and H\textsubscript{2} (95:5, v/v) and maintained at 25 °C. \textit{M. jannaschii} cells (1.8 g wet weight) grown with sulfite were lysed in 20 ml of 25 mM potassium phosphate (KP\textsubscript{i}) buffer pH 7.0 (buffer A) via osmotic shock and subsequent three passages through a 25 g needle. The lysate was centrifuged at 1230 x g and 25 °C for 20 min to collect a supernatant. The pellet was resuspended in 15 ml buffer A and the suspension was centrifuged to obtain additional supernatant. The two supernatants were combined and the pool was fractionated by precipitation with ammonium sulfate at 30% and 60% saturation on ice. At each step a tube containing the treated extract was sealed inside the chamber and then it was centrifuged outside the chamber at 10,000 x g. After centrifugation, the tube was transferred inside the chamber where the supernatant was collected. Further fractionation steps involved chromatography; the (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} and NaCl solutions used at these steps contained 25 mM KP\textsubscript{i} buffer, pH 7.0. The column fractions with Fsr activity were examined via SDS-PAGE for purity. The volume, protein content, and activity of the enzyme preparation recovered at each of these steps are given in Table 2. First, the 60% supernatant was loaded onto a 1.5 cm x 6 cm Phenyl-Sepharose column (Amersham Biosciences, Piscataway, NJ) (bed volume, 10.6 ml) that was pre-equilibrated with 1.5 M (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}. The bed was then washed with 20 ml equilibration solution and eluted with a 75 ml reversed gradient of 1.5 to 0 M (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}. The column fractions with 379-205 mM (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} contained Fsr activity, of which those with a minimum number of non-Fsr polypeptides were pooled. This pool was diluted with buffer A to lower the (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} concentration to 25 mM. The diluted enzyme was loaded onto a 0.7 cm x 5.5 cm QAE-Sephadex column (Amersham Biosciences, Piscataway, NJ) (bed volume, 2.1 ml) that was equilibrated with buffer A. The column was washed with 10 ml buffer A and the enzyme was eluted at a NaCl concentration of about 325 mM under a 80 ml gradient of 0.0-1.0 M NaCl. The active fractions were pooled. This preparation was homogeneous.

**Sequence and phylogenetic analysis** - The ClustalW program was used for the comparative analysis of amino acid sequences of proteins (25). For drawing phylogenetic inferences, aligned protein sequences were analyzed by using the neighbor-joining bootstrap analysis (26) in the CLUSTALX program (27). The phylogenetic tree was developed using NJ plot (28).

**RESULTS**

Growth of \textit{M. jannaschii} with sulfite as the sulfur source and expression of a novel sulfite reductase - \textit{M. jannaschii} used sulfite as the sole sulfur source for growth; the growth and methane formation patterns were similar to that seen with a culture grown with the same level of sulfide (Fig. 1). A medium without an added sulfur source did not support growth. When grown with sulfite, the organism expressed a 70 kDa polypeptide (Fig. 2A). As judged from the SDS-PAGE data for cell lysates, this polypeptide was absent in cells that received sulfide. Mass spectrometry with an unseparated tryptic digest and database searches matched several peptide fragments of the 70 kDa band with ORF MJ0870 (9) (Fig. 3) (Table 1, to be published as supplemental data in JBC On-line); the matches covered 25% of the identified ORF. MJ0870 has been annotated as the β subunit of a coenzyme F\textsubscript{420}-reducing hydrogenase (FrhB) (9). However, from a comparative sequence analysis (25), it was apparent that this 620-residue polypeptide had two distinct domains. As shown below, the N-terminal half comprised of 311 residues represented a reduced coenzyme F\textsubscript{420} dehydrogenase and the C-terminal half (296 residues) was homologous to siroheme-dependent dissimilatory sulfite reductases. Thus, the 70 kDa polypeptide had the potential of using F\textsubscript{420} as electron carrier for reducing sulfite into sulfide. Accordingly, we called this enzyme a coenzyme F\textsubscript{420}-dependent sulfite reductase or Fsr. We examined \textit{M. jannaschii} cell extracts for Fsr activity, which was measured in terms of the
oxidation of H$_2$F$_{420}$ with sulfite as the electron acceptor. Extracts of cells grown with sulfite catalyzed this reaction at a specific rate of 1.3-1.7 $\mu$mol H$_2$F$_{420}$ oxidized min$^{-1}$ mg$^{-1}$ protein. This activity was absent in cells grown with sulfide.

**Purification and molecular properties of F$_{420}$-dependent sulfite reductase (Fsr)** - The Fsr activity in cell extracts was highly oxygen sensitive. About 92% activity was lost if the cell extract was exposed to air for 60 min. Dithiothreitol and 2-mercaptoethanol neither protected the enzyme from inactivation nor restored activity in an air-inactivated enzyme preparation. Therefore, all purification steps were conducted under strictly anaerobic conditions. Table 2 provides a summary of the results from a purification experiment. At the ammonium sulfate precipitation step, 67.5% of cell extract Fsr activity was recovered in the 60% saturated supernatant. From this supernatant the enzyme was purified to homogeneity via phenyl-Sepharose, coenzyme F$_{420}$-Sepharose, and QAE-Sephadex chromatography steps. In a non-denaturation polyacrylamide gel electrophoresis experiment the final enzyme preparation showed a single band (Fig. 2B). In a SDS-PAGE gel, the same preparation exhibited three bands (Fig. 2C). Mass spectrometric analyses (13,14) showed that each of these bands corresponded to MJ0870 (data not shown). Therefore, we concluded that the 70 kDa band in Fig. 2C was due to intact MJ0870 and the 42 and 140 kDa bands were, respectively, for a degradation product and a covalently linked dimer that were probably created during sample preparation. Accordingly, the enzyme obtained from the QAE-Sephadex chromatography was homogeneous. The fractionation scheme used in this work provided a final yield of 2.6% (Table 2). This apparently low value was the result of an aggressive approach that was necessary for generating a homogeneous Fsr preparation. Our early efforts for obtaining a homogeneous Fsr preparation were not successful. Therefore, in further attempts at each step of purification the column fractions with Fsr activity were examined for purity via SDS-PAGE. Only those fractions with a minimum number of non-Fsr polypeptides were pooled. This strategy provided a homogeneous preparation of the enzyme, but resulted in a substantial loss of Fsr protein and consequently a poor overall yield. The fold of purification was 14 (Table 2). This value is justified by the fact that the cell extract contained a very high level of Fsr (Fig. 2A). Therefore, only a moderate fold of purification provided a homogeneous preparation of the enzyme.

From the gel filtration data the hydrodynamic radius and the apparent native molecular mass of the enzyme were estimated to be 69.5 Å and 350 kDa, respectively. The UV-visible spectrum of Fsr in as isolated form exhibited peaks at 280, 395 and 590 nm (Fig. 4).

**Catalytic properties of Fsr** - The purified enzyme oxidized H$_2$F$_{420}$ with sodium sulfite. The optimum pH and temperature for this activity were 7.0 and >95 °C, respectively. Activities at temperatures above 95 °C were not determined due to technical limitations. From the straight line section of the Arrhenius plot (25-75 °C), a value of 45.9 kJ/mol was obtained for the activation energy. Within a sulfite concentration range of 1.4-300 $\mu$M and with a fixed H$_2$F$_{420}$ concentration of 40 $\mu$M, the apparent K$_m$ value for SO$_3^{2-}$ was 12.2 ± 1 $\mu$M and the corresponding apparent V$_m$ value was 16 $\mu$mol H$_2$F$_{420}$ oxidized or 32 $\mu$mol electron transferred /min/mg. Similarly, at a fixed SO$_3^{2-}$ concentration of 290 $\mu$M and within a H$_2$F$_{420}$ concentration range of 4-60 $\mu$M, the apparent K$_m$ value for H$_2$F$_{420}$ was 21.2 ± 3.8 $\mu$M and the value for V$_m$ was 11.6 $\mu$mol H$_2$F$_{420}$ oxidized or 23.2 $\mu$mol electron transferred /min/mg. Fsr also oxidized H$_2$F$_{420}$ with methylviologen (MV$^+$), and at H$_2$F$_{420}$ and MV$^+$ concentrations of 40 $\mu$M and 2.3 mM, respectively, the specific activity was 110 $\mu$mol electrons transferred min$^{-1}$ mg$^{-1}$ protein. Similarly, Fsr oxidized reduced methylviologen (MV$^0$) with SO$_3^{2-}$, and with 0.3 mM MV$^0$ and 5.8 mM SO$_3^{2-}$ the specific activity was 90 $\mu$mol electrons transferred min$^{-1}$ mg$^{-1}$ protein.

Assuming that Fsr utilized a flavin as an intermediate electron carrier, the effects of added FAD and FMN on the purified enzyme’s activity were tested. Two types of experiments were performed. First, Fsr was assayed in a standard
reaction mixture containing FAD or FMN or both flavins. These assays provided the following relative activity values (% of the value recorded in the absence of an added flavin; averages from three assays): 1. FAD concentration, activity: 2.5 µM, 93±3%; 5 µM, 87±4%. 2. FMN concentration, activity: 2.5 µM, 97±5%; 5 µM, 88±6.5%. 3. FAD+FMN concentration, activity: 1.25 µM + 1.25 µM, 90±8%; 2.5 µM + 2.5 µM, 79±8%. Second, a solution of Fsr (protein concentration, 0.2 µg/ml) in 50 mM KPi buffer, pH 7, was incubated at 4 °C for 14 h in the presence of FAD (0.5 mM) or FMN (0.5 mM) or FAD and FMN (0.25 mM of each); an enzyme solution incubated without an added flavin was the control. Assays for Fsr activities in these mixtures after incubation yielded the following relative activity values (% of the activity in the control at the start of the experiment; averages from three assays): control, 106±5%; FAD, 93±2%; FMN, 75±3%; FAD + FMN, 85±5%.

Due to carry over with the enzyme, the assays for the flavin-treated Fsr were conducted in the presence of flavins at following concentrations: FAD, 0.5 µM; FMN, 0.5 µM; FAD+FMN; 0.25 µM + 0.25 µM.

The results of a sulfide production assay showed that from 0.366 µmol H2F420 and 3.66 µmol sulfite, Fsr produced 0.362 ± 0.003 µmol H2F420 and 0.088 ± 0.004 µmol sulfide; these values represent averages of data from three independent assays. Sodium salts of thiosulfate and sulfate did not serve as the electron acceptor for H2F420 oxidation by Fsr. Also, Fsr could not use NADH and NADPH for the reduction of sulfite.

**Homologs of MJ0870** - The ORF MTH280 of Methanothrophicthermautotrophicus (10,29) and ORF MK0799 of Methanopyrus kandleri (10,29) were found to be homologous to MJ0870 or Fsr (Fig. 5). None of these MJ0870 homologs have been studied experimentally. MTH280 has been annotated as FrhB (10) and MK0799 as a protein with FrhB and nitrite reductase characteristics (29), respectively. Similar to MJ0870 or Fsr, each of these homologs possesses an N-terminal H2F420 dehydrogenase domain and a C-terminal dissipilatory sulfite reductase domain (Fig. 5). Interestingly, Methanococcus maripaludis, a close relative of *M. jannaschii* and a mesophile, lacked an Fsr homolog (30).

**Sequence features of the N-terminal half of Fsr** - The N-terminal half of MJ0870 (residues 1-311 of MJ0870; henceforth called Fsr-N) showed substantial sequence similarities to FqoF and FpoF which are the H2F420 dehydrogenase subunits of H2F420:quinone oxidoreductase (Fqo) complex of A. fulgidus and H2F420:phenazine oxidoreductase (Fpo) complex of Methanosarcina mazei, respectively (Fig. 5A and 6A) (31,32). *M. mazei* is a hyperthermophilic methylotrophic methanogenic archaeon and *A. fulgidus* is a hyperthermophilic sulfite reducing non-methanogenic archaeon (32,33). Two CXXCXXCXCP or ferredoxin-type [Fe4-S4] motifs of A. fulgidus FqoF (C65C68C71C75P76 and C96C99C102C106P107) and *M. mazei* FpoF (C14C17C20C24P25 and C55C58C61C65P66) aligned with the C15C18C21C25P26 and C42H47C50C54P55 (3-Cys+His) of MJ0870, respectively (marked with black bullets in Fig. 5A). These centers are absent in the F420-interacting subunits of the F420-reducing hydrogenases of the methanogens (FrhB, FruB; Fig. 5A) and are located at the C-terminus of the homologous subunit (β or FdhB) of F420-dependent formate dehydrogenase (the segment not shown). Four fully conserved Cys residues of MJ0780 (C160, C201, C256, and C259; marked with black bullets in Fig. 5A) that do not lie close to each other were also found conserved in all F420-interacting polypeptides shown in Fig. 5A. A great number of non-Cys residues of MJ0870 were conserved in FrhB, FruB, and FdhB (Fig. 5A). As judged from an analysis by use of 3D-PSSM, web-based program for protein fold recognition (http://www.sbg.bio.ic.ac.uk/~3dpssm) (34), none of these residues were the part of a known motif. Fsr did not possess a recognizable coenzyme F420-binding sequence feature (35,36). All of the above-described features of MJ0870 were present in its homologs, MTH280 and MK0799 (Fig. 5A).

**Sequence features of the C-terminal half of Fsr** - We use Fsr-C as an abbreviation for the C-terminal
half of Fsr (residues 325-620 in MJ0870). Fsr-C and the corresponding regions of MTH280 and MK0799 were compared with the hemoprotein subunit (SirHP) of *E. coli* assimilatory sulfite reductases or ASR, the A and B subunits (DsRA and DsRB) of archaeal or bacterial dissimilatory sulfite reductases or DSR, and the siroheme containing subunit (AsrC) of a small sulfite reductase that is expressed in *Salmonella enterica* under anaerobic growth conditions (Fig. 5B) (33,37-39). As explained in the following section, *S. enterica* AsrC is a dissimilatory sulfite reductase (DSR). In an ASR or DSR, three sequence elements, designated H1, H2 and H3, are thought to house a siroheme-[Fe4-S4]-cluster and bind sulfite (37). These elements were conserved in the C-termini of the MJ0870 and its homologs (Fig. 5B). The Arg355 and Arg423 of MJ0870 (marked ♦ in Fig. 5B) corresponded to the sulfite binding Arg residues of sulfite reductases (37). The siroheme-[Fe4-S4]-binding CXXCXXCXX/C sequence is fully conserved in SirHP, DsRA and AsrC and only partially conserved in a DsRB (Fig. 5B). This element was found fully conserved in MJ0870, MTH280 and MK0799 (C428C434C468C472 in MJ0870, Fig. 5B). However, in overall sequence features, the C-terminal regions of the archael proteins were more similar to DSRs (Fig. 5B) and not to the *E. coli* Sir-HP (data not shown). The P488C495C498C501C505 and C524C527C530C534P535 of MJ0870 have the potential of forming two [Fe4-S4] clusters and both of these were found conserved in AsrC, MTH280 and MK0799. The first of these clusters was absent in both DsRA and DsRBs and the second was fully conserved in the DsRBs but partially in DsRA (Fig. 5B).

**Phylogenetic analyses** - Fsr-N and the N-terminal halves of MTH280 and MK0799 were closely related to FqoF (Fig. 7A). The closest relative of the Fsr-C and its homologs was *S. enterica* AsrC (Fig. 7B); a close relationship was also seen with the subunits of dissimilatory sulfite reductases, DsRA and DsRB. AsrC is a dissimilatory enzyme (39,40); the abbreviation Asr means anaerobic sulfite reduction and not assimilatory sulfite reductase (39). Therefore, Fsr-C belonged to the dissimilatory sulfite reductase group (AsrC, DsRA and DsRB).

**DISCUSSION**

Sulfide is an essential nutrient for *M. jannaschii* (8) and sulfite is inhibitory to the methanogens (1). Yet we found that this methanogenic archaeon was able to use sulfite as a sole sulfur source and did not show a significant inhibition of methanogenesis by this oxyanion (Fig. 1). As discussed below, this ability was due to a novel enzyme that was expressed on demand. We also discuss the physiological, ecological and evolutionary implications of our findings.

**A novel sulfite reductase** - Sulfite induced the expression of a sulfite reductase in *M. jannaschii* (Fig. 2A) that was encoded by orf MJ0870. The subunit size of this enzyme was 70 kDa (Fig. 2C). It reduced sulfite into sulfide and thereby allowed *M. jannaschii* to grow with sulfite as sole sulfur source. Interestingly, this sulfite reductase used reduced coenzyme F420 (H2F420) as the electron source. Previously described sulfite reductases use nicotinamides and cytochromes as electron carriers (Fig. 8A-C) (37). F420 is a naturally occurring 5-deazaflavin that was originally discovered in the methanogenic archaea (41). It is an obligatory 2-electron or hydride transferring coenzyme (41,42). The midpoint potential values presented below (42,43) show that under standard conditions H2F420 is a potent reductant for sulfite (SO32-) or bisulfite (HSO3-). At pH 7, half of the added SO32- exists as HSO3- (pKa for SO32-/HSO3-, 6.91; (44)). The overall reaction leading to the reduction of sulfite with H2F420 is exergonic.

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\begin{align*}
F420 + 2e + 2H^+ & \rightarrow H2F420, \quad \Delta E^\circ = -350 \text{ mV} \\
HSO3^- + 6e + 6H^+ & \rightarrow HS^- + 3H2O, \quad \Delta E^\circ = -116 \text{ mV} \\
HSO3^- + 3H2F420 & \rightarrow HS^- + 3H2O + 3F420, \quad \Delta G^\circ = -135 \text{ kJ/mol}
\end{align*}
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The use of sulfite as a sulfur source has been observed previously with two methanogens, *M. thermolithotrophicus* and *M. thermautotrophicus* (6). However, the enzymatic basis for this observation remains unknown. A small siroheme...
sulfite reductase (size subunit, 23 kDa) has been isolated from Methanosarcina barkeri (45), but the physiological electron donor and the in vivo role for this enzyme are yet to be identified. It is not known if *M. barkeri* can use sulfite as sole sulfur source. Therefore, this is the first report of a coenzyme F₄₂₀-dependent sulfite reductase that we called Fsr.

We also determined that Fsr reduced sulfite to sulfide with H₂F₄₂₀ as the electron donor. The assay was performed in the presence of a 30-fold excess of sulfite. This calculation was based on the mol of electrons that was required for converting all of the supplied sulfite to sulfide. Under these conditions, for every 3 mol of H₂F₄₂₀ oxidized 0.73 mol sulfide was generated. If Fsr carried out only complete reduction of sulfite, producing sulfide as the sole product, this ratio would have been 3:1 (see the reaction above). The observed deviation could either be due to an experimental error or the production of partially reduced sulfur compounds in addition to sulfide. Production of partially reduced products, such as trithionate and thiosulfate, has been observed with certain sulfite reductases (46). However, the production of thiosulfate as an intermediate of sulfite reduction by Fsr is an unlikely possibility, since Fsr did not oxidize H₂F₄₂₀ with thiosulfate. We will address this issue in our future work.

Interestingly, the rates of the two Fsr partial reactions, oxidation of H₂F₄₂₀ and reduction of sulfite, as measured by use of methylviologen as the electron carrier, were about the same. However, these rates were only 20-30% of that for the overall reaction. The reason for this difference is currently unknown.

Our data did not allow us to describe the quaternary structure of Fsr. A dissimilatory sulfite reductase is composed of two DsrA and two DsrB subunits (Fig. 8C; (37)). In *E. coli* sulfite reductase, the siroheme component or Sir-HP is a tetramer and the flavoprotein component (SirFP) is octameric (Fig. 8A; (37)). Our native mass data seem to indicate that Fsr was composed of five subunits. However, a gel filtration data derived value is less reliable (47). A final conclusion on the quaternary structure of Fsr must await an accurate determination of the native molecular mass for Fsr by use of a more appropriate method such as analytical ultracentrifugation (47).

**A detoxification role for Fsr** - Fsr was induced with sulfite. The cellular level of the Fsr activity was relatively high for an anabolic enzyme. Taken together these observations indicated that Fsr was poised to protect *M. jannaschii* from the toxic effect of sulfite. Remarkably, the methane production rate in a culture with sulfite was comparable to that for a culture with the same level of sulfide (Fig. 1). Therefore, *M. jannaschii* avoided the inhibition of its methyl-coenzyme M reductase by sulfite. In a methanogen, methyl-coenzyme M reductase is expressed to a level as high as 30% of the total cell protein (48,49). *M. jannaschii* has the potential of expressing two methyl-coenzyme M reductase isoenzymes (Mcr and Mrt) and the open reading frames representing the corresponding α, β, and γ or A, B or G subunits are the following: MJ0846 (McrA, 61227 Da), MJ0842 (McrB, 47758 Da), MJ0845 (McrG, 30155 Da), MJ0083 (MrtA, 61201 Da), MJ0081 (MrtB, 47739 Da), and MJ0082 (MrtG, 30744 Da) (9). Accordingly, the 61, 48 and 30 kDa bands in Fig. 2A most likely corresponded to the methyl-coenzyme M reductase subunits. The intensities of these bands showed that in cells grown with sulfite, the methyl-coenzyme M reductase subunits and Fsr were expressed at comparable levels. This observation is consistent with a detoxification role for Fsr. However, this hypothesized link between a need to protect the methyl-coenzyme M reductase and the induction of Fsr needs to be justified by the Kᵢ value of the enzyme for sulfite. Although, the inhibition of this enzyme with sulfite has been reported (3,4), the corresponding Kᵢ value is yet to be determined.

**A H₂F₄₂₀ dehydrogenase domain in Fsr** - Fsr polypeptide contained 620 amino acid residues. The N-terminal half of this polypeptide (residues 1-311; called Fsr-N) possessed the sequence features of FqoF and FpoF (Fig. 5A) (31,32) and exhibited a close phylogenetic relationship to FqoF (Fig. 7A). In *A. fulgidus* FqoF, a H₂F₄₂₀ dehydrogenase, introduces electrons into a membrane-based electron transport system (Fig. 6A). The process involves transfer of electrons from H₂F₄₂₀ to 1 electron...
carrying Fe-S clusters by using protein-bound flavin as a 1-electron/2-electron switch (31). FpoF performs a similar task in M. mazei (Fig. 6A) (32). Therefore, we propose that Fsr-N is the H\textsubscript{2}F\textsubscript{420} dehydrogenase domain of Fsr. Our data showed that Fsr had a H\textsubscript{2}F\textsubscript{420} dehydrogenase activity. In addition to performing sulfite dependent H\textsubscript{2}F\textsubscript{420} oxidation, Fsr was able to transfer electrons from H\textsubscript{2}F\textsubscript{420} to methylviologen, a one electron-restricted carrier. FqoF and FpoF also catalyze the latter reaction (31,50). Since FqoF and FpoF are flavoproteins (31,50), Fsr was expected to carry flavin, but, the spectrum in Fig. 4 does not show a clear indication for a protein-bound flavin in Fsr. It is possible that Fsr lost a major amount of the bound flavin and the 300-550 nm region in the spectrum represented a superimposition of the spectra of siroheme and a small amount of flavin (Fig. 4); a major loss of protein-bound flavins during the purification has been observed with both FqoF and FpoF (31,50). However, this explanation was not supported by our data on the effect of added flavin coenzymes on the activity of homogeneous Fsr. Either the presence of FAD, FMN or FAD+FMN in the assay mixture or incubation of purified Fsr with these compounds did not increase the specific activity of the enzyme. Rather such treatments inhibited the enzyme slightly. A detailed assessment on the role of flavins in Fsr reaction, if any, will come from our future work on the prosthetic groups of Fsr.

A dissimilatory sulfite reductase-type domain in Fsr - Although our observation described Fsr as an anabolic enzyme, in primary structure this protein was unrelated to the assimilatory sulfite reductase from E. coli (Fig. 7B). Rather, the C-terminal half of Fsr (residues 325-620) or Fsr-C was closely related to the siroheme-containing dissimilatory sulfite reductases (DSR) of the archaea and bacteria (Figs. 5B and 7B). We hypothesize that Fsr-C carried siroheme, where the electrons derived from the 1-electron restricted iron-sulfur centers of the H\textsubscript{2}F\textsubscript{420} dehydrogenase domain or Fsr-N were used for the reduction of sulfite to sulfide. Our experimental data showed that Fsr contained the elements of a DSR. First, the 280, 395 and 590 nm peaks in the UV-visible spectrum of Fsr were typical of a sulfite reductase hemeprotein (Fig. 4) (51). This spectrum did not show a 714 nm peak which is a signature for a high spin ferric complex of isobacteriochlorin that is found in the assimilatory enzymes from E. coli and Desulfovibrio vulgaris (51,52). Second, Fsr was able to utilize MV\textsuperscript{0}, a one-electron donor, for the reduction of sulfite into sulfide. This reaction has been observed with the bacterial and archaeal dissimilatory sulfite reductases (53,54).

Fsr, a novel enzyme with a unique chimeric structure. – As shown above, the homologs of two halves of Fsr have been encountered previously as independent entities (Figs. 5A and B, 6A and 8C). However, Fsr presents the first example where the structural and functional attributes of these units have been brought together (Figs. 5, 6B and 8D). Although, assimilatory sulfite reductase from E. coli was found to be structurally unrelated to Fsr, these two enzymes share some of the functional characteristics (Figs. 8A and D). In the E. coli enzyme, a flavoprotein subunit (SirFP) derives electrons from NADPH, a 2e-restricted donor, and passes those via FAD, FMN, and [4Fe-4S] centers to the siroheme of a hemoprotein subunit (Sir-HP), the site of sulfite reduction (Fig. 8A) (37). This role of NADPH is similar to that of H\textsubscript{2}F\textsubscript{420} in the Fsr reaction, but the electron transfer route between H\textsubscript{2}F\textsubscript{420} and siroheme in Fsr is currently unknown.

Ecological relevance and a possible energy production role for Fsr- Our findings are consistent with the following environmental data and deductions on the hydrothermal vents. As mentioned above, both M. jannaschii and M. kandleri are inhabitants of the deep-sea hydrothermal vents (8,55). Cold seawater that permeates through the chimney wall brings the temperature of the nutrient rich vent fluid down from 350 °C to a level where life can exist (56,57). This process also brings oxygen into the vent. Sulfide, that is present at a high level in the vent fluid (56,57), reacts with this oxygen and helps to establish anaerobic conditions in the cooled zones, thereby providing conditions conducive for the growth of a methanogen. However, this reaction has the potential of producing sulfite. A lack of free oxygen in the vent water and a decrease of H\textsubscript{2}S...
concentration with temperature within the vents have been thought to indicate that sulfur species with oxidation states between −2 and +6 (representing sulfide and sulfate) are present in the vent fluid (57); sulfate is the predominant oxidation product. The sulfite concentration in vent water is expected to be rather low and this condition is consistent with the observed low $K_m$ value of Fsr for sulfite. It should be noted that the literature on the deep-sea hydrothermal vent does not provide a value for the sulfite level in vent water. We have considered the possibility for Fsr providing an alternate non-methanogenic energy production route to the vent methanogens. The existence of a sulfite-based energy metabolism in a hydrothermal vent has been reported previously. *Archaeoglobus veneficus*, which was isolated from the walls of hydrothermal vents, derives energy from sulfite oxidation (58). It is a close relative of the late evolving methanogens such as the *Methanosarcina* species. However, thus far we have been unable to grow *M. jannaschii* with H$_2$ and sodium sulfite as the energy production substrates in a 2-(N-morpholino)ethanesulfonic acid or MES-NaOH-buffered medium containing sodium acetate as the sole carbon source.

**Evolutionary implications** – We propose two equally possible hypotheses on the evolution of Fsr: 1. Fsr is the ancestor of FqoF or FpoF and Dsr. 2. Fsr originated from a fusion of laterally transferred dsrA and fgoF genes. The fusion could have preceded the lateral transfer. Since Dsr and FqoF/FpoF have been studied in the context of the evolution of metabolism (59,60), the above two possibilities are discussed here. *M. jannaschii*, *M. kandleri* and *M. thermautotrophicus* are strictly hydrogenotrophic autotrophs (8,55,61), a characteristics that fits the conditions of early earth (62). Their positions in the 16S rRNA based tree of life are closer to the root than that of *Methanosarcina* and *A. fulgidus* (63). *M. jannaschii*, *M. kandleri* and *M. thermautotrophicus* carry Fsr homologs (Fig. 5), but they do not possess a complete sulfate reduction pathway or an Fqo/Fpo-type membrane energy transduction system (9,10,29,32). *A. fulgidus* does not contain Fsr. Instead it carries homologs of both Fsr domains as separate polypeptides (33). This archaeon employs FqoF, a Fsr-N homolog, in energy transduction (Fig. 6A) (31) and DsrA and DsrB, the Fsr-C homologs, in a dissimilatory sulfate reduction pathway (Fig. 8C) (33); DsrA and DsrB are also used by sulfate reducing bacteria (60). *Methanosarcina* species possess FpoF (Fsr-N homolog) (Fig. 6A) (32) and express a small size (23 kDa) siroheme sulfite reductase (45). Based on these pieces of information it could be hypothesized that fsr is an ancestral gene. The 5’-half of fsr gave rise to the input domain (fgoF/fpoF) of the membrane-based energy transduction systems in certain late evolving archaea and the 3’-half yielded bacterial and archaeal dissimilatory siroheme sulfite reductases (dsrA and dsrB). The catabolic type highly active nature of Fsr fits this hypothesis. One could imagine that fsr provided a selective advantage to an ancestral methanogen for surviving sulfite exposure when oxygen appeared on earth. Recent reports suggest that the development of a fully oxic atmosphere followed a protracted oxygenation period (64-66), where a small supply of oxygen was quickly and fully sequestered in a process that could have generated sulfite, an incomplete oxidation product of sulfide. At a later time fsr allowed the development of the sulfate reduction pathway within a methanogen. The *M. jannaschii* genome bears more signs of this possibility. The ORFS MJ0066 and MJ0973 show some sequence similarities to a 3’-phosphoadenosine-5’-phosphosulphate reductase and sulfate adenylyltransferase or ATP sulfurylase (9) which participate in the reduction of sulfate to sulfite in certain bacteria and archaea (67). On the other hand, the limited distribution of fsr and a wide distribution of dsr could question the hypothesized ancestral nature of fsr. Since *M. jannaschii* and *M. kandleri* are hydrothermal vent associated organisms (8,55), a search for additional fsr homologs in the vent environment might shed some light on the evolution of fsr. The generation of fsr from a fusion of fgoF or fpoF and dsr is a clear possibility, because the latter two genes have been found to co-exist in a cell (31,33). Nevertheless, our results bring new thoughts to the study of the evolution of early metabolisms on earth, especially in the field where DsrA and DsrB sequences of cultured and uncultured microorganisms and sulfur isotope records have been extensively analyzed in search of
the evolutionary origin of energy metabolisms of extant prokaryotes (40,60,68).

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FOOTNOTES

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Abbreviations used: ASR and DSR, assimilatory and dissimilatory sulfite reductase, respectively; DsrA and DsrB, a and b subunits of DSR, respectively; Fsr, coenzyme F420-depent sulfite reductase; Fsr-C and Fsr-N, C- and N-terminal half of Fsr, respectively; H2F420, reduced coenzyme F420; Fqo, H2F420:quinone oxidoreductase; Fpo, H2F420:phenazine oxidoreductase; FqoF and FpoF, H2F420 dehydrogenase subunit of Fqo and Fpo, respectively; KP, potassium phosphate; MV+, methylviologen; MV0, reduced methylviologen.

Keywords: novel enzyme, coenzyme F420, sulfite reductase, methanogenic archaea, Methanocaldococcus jannaschii, sulfite detoxification, evolution
FIGURE LEGENDS

Fig. 1. Growth and methane formation in *M. jannaschii* cultures with sulfide or sulfite as sole sulfur source. Methanogenesis data represent accumulated methane. Each experiment was done in duplicate and both sets are shown. The bottles were re-pressurized to $3 \times 10^5$ Pa with H$_2$-CO$_2$ (80:20, v/v) prior to each sampling. At each of the times indicated, 50-100 µl headspace gas and 3 ml culture sample were removed for methane assay and optical density (600 nm) measurement. The concentration of dissolved sulfide was less than 1 mM and most of the added sulfide existed as H$_2$S in the headspace gas, because the pH of the medium was 6.0 and the pK$_a$ for H$_2$S/HS$^-$ is 7.04 (69).

Fig. 2. Gel electrophoresis of *M. jannaschii* cell lysates and purified coenzyme F$_{420}$-dependent sulfite reductase (Fsr). MW, molecular mass standards. A. SDS-PAGE for lysates of cells grown with either sulfite (SO$_3^-$) or sulfide (S$^-$). A lysate was prepared by boiling cells in a solution containing 62.5 mM Tris-HCl buffer pH 6.8, 10% glycerol, 5% 2-mercaptoethanol. The gels were stained with coomassie blue. The over-expressed band in the SO$_3^-$ lane is for MJ0870 or Fsr. The arrows with labels A, B, and C point to the suggested locations of α, β, and γ subunits of methyl-coenzyme M reductase. B. Non-denaturing PAGE for homogenous Fsr. C. SDS-PAGE for homogenous Fsr. Identities from mass spectrometry data: 2XFsr, linked dimer; Fsr, one polypeptide; Fsr’, degradation product.

Fig. 3. Identification of the sulfite induced 70 kDa polypeptide of *M. jannaschii* as MJ0870. A sample of the 70 kDa polypeptide contained within a SDS-PAGE gel band (see Fig. 2A) was digested with trypsin. The resulting tryptic peptide fragment mixture was analyzed via MALDI-TOF mass spectrometry. A search of the NCBI’s protein database matched 17 of the mass spectrometry derived mass values to the regions of ORF MJ0870 that are shown in Red; the corresponding trypptic fragments are shown Underlined. The regions of MJ0870 that were not represented in the mass spectrometry data are shown in Black. See Material and Methods for the experimental details and Table 1 for an analysis of the mass spectrometry data. *Cysteines modified with iodoacetamide to form carbamidomethyl cysteine.

Fig. 4. UV-visible spectrum of *M. jannaschii* Fsr. The full spectrum (insert) and an expanded version (350-750 nm) are shown. A 300 µl anaerobic solution containing 38 µg homogenous enzyme (as isolated), 25 mM potassium phosphate buffer pH 7, and 440 mM NaCl was analyzed.

Fig. 5. Comparative analysis of Fsr or MJ0870 amino acid sequence. A. N-terminal half of Fsr or MJ0870 (residues 1-311); B. C-terminal half of Fsr or MJ0870 (residues 325-620). Symbols: MJ-Fsr-N and MJ-Fsr-C, MTH280-N and MTH280-C, and MK0799-N and MK0799-C, N-terminal and C-terminal halves of *M. jannaschii* ORF MJ0870, *M.thermautotrophicus* ORF MTH280, and *M. kandleri* ORF MK0799, respectively; AF-FqoF, *A. fulgidus* H$_2$F$_{420}$ dehydrogenase subunit of H$_2$F$_{420}$:quinone oxidoreductase or Fqo (ORF AF1833); MM-FpoF, *Methanosarcina mazei* H$_2$F$_{420}$ dehydrogenase subunit of H$_2$F$_{420}$:phenazine oxidoreductase or Fpo (ORF MM0627); MV-FruB and MV-FrhB, *Methanococcus voltae* β subunits of two coenzyme F$_{420}$-reducing hydrogenases (accession number Q00391 and CAA43503); MTF-FdhB, *Methanobacterium formicicum* β subunit of F$_{420}$-reducing formate dehydrogenase (accession number P06130); AF-DsrA and AF-DsrB, *A. fulgidus* α and β subunits of dissimilatory sulfite reductase (ORFs AF0423 and AF0424); DV-DsrA and DV-DsrB, *Desulfovibrio vulgaris* α and β subunits of dissimilatory sulfite reductase (accession number AAA70107 and AAA70108); ST-AsrC, *Salmonella enterica* hemoprotein subunit of small size anaerobic sulfite reductase (accession number CAD02753). Black and teal bullets, conserved Cys and Pro in MJ0870, respectively; Grey bullets, Cys and Pro conserved elsewhere. +, for sulfite binding Arg residues. Following colors were used to show sequence identity (shading) and functional conservation (colored letters): D, E, Red; R, K, H, Blue; F, Y, W, H, Yellow; I, L, V, Grey; A, G, S, T, Olive; Q, N, Green; M, Purple; P, Teal; C, White.
**Fig. 6.** Complexes and enzymes with an $H_2F420$ dehydrogenase unit. A. Fqo and Fpo complexes (31,32). These are similar to respiratory Complex I of *E. coli* and mitochondria (32). MQ, menaquinone. FqoF or FpoF, $H_2F420$ dehydrogenase subunit. FqoF and FpoF use protein-associated flavin for transferring electrons from $H_2F420$ to [4Fe-4S] centers. The input module of complex I catalyzes similar transfer from NADH. B. Coenzyme $F_{420}$-dependent sulfite reductase. Fsr-N, residues 1-311 of Fsr; Fsr-C, residues 325-620 of Fsr. It is not known whether Fsr contains bound flavin. The indication for the presence of [Fe-S] clusters in Fsr came from a primary sequence analysis.

**Fig. 7.** Phylogenetic tree for the N-terminal and C-terminal halves of *M. jannaschii* Fsr and its homologs. The bar indicates number of substitutions per site. The values appearing near the branches are for the bootstrap confidence levels. A. N-terminal half of Fsr or MJ0870 (residues 1-311) and its homologs. B. C-terminal half of Fsr or MJ0870 (residues 325-620) and its homologs. Symbols: Same as in Fig. 4 in addition to the following (accession or ORF number): AT-SirHP, *Arabidopsis thaliana* sulfite reductase (CAA89154); AV-DsrA and AV-DsrB, *Allochromatium vinosum* DsrA and DsrB (AAC35394 and AAC35395); DT-DsrA and DT-DsrB, *Desulfotomaculum thermocisternum* DsrA and DsrB (AAC96107 and AAC96108); EC-SirFP and EC-SirHP, *E. coli* sulfite reductase hemoprotein and flavoprotein subunits (AAA23650 and AAA23651); MJ-FrhB and MTH-FrhB, $\beta$ subunits of *M. jannaschii* and *M. thermautotrophicus* coenzyme F$_{420}$-reducing hydrogenases (MJ0032 and MTH1297); MJ-FdhB, *M. jannaschii* formate dehydrogenase $\beta$ subunit (MJ0005).

**Fig. 8.** Assimilatory and dissimilatory sulfite reductases. SR, or Sir, sulfite reductase; HP, heme containing protein; FP, flavoprotein; Fd, ferredoxin; PS I, photosystem I; Cyt, cytochrome. Most dissimilatory sulfite reductases are $\alpha_2\beta_2$ proteins, although $\alpha_2\beta_2\gamma_2$ structures have also observed, where the function of the $\gamma$ subunit is unknown. The style of this figure has been adopted in part from Reference (70). The quaternary structure for *M. jannaschii* Fsr is not known. ? in D indicates that it is not known whether Fsr contains bound flavin.
Table 1. Identity of a 70 kDa protein induced by sulfite (To be published as supplemental data in JBC On-line)

| MALDI mass for tryptic fragments, Da (MH⁺, average) | The peptide sequence consistent with observed mass† (start-end position in the parent polypeptide) {calculated average size, Da} | ∆Da (observed mass - calculated mass) | Polypeptide identified (Calculated mass, Da) {pI} Accession number |
|---------------------------------------------------|---------------------------------------------------------------------------------------------------------------|----------------------------------------|------------------------------------------------------------------|
| 2894.20                                           | AKPGGWYKPEEIKEILDAEYNAK (358-382) {2893.265}                                                                 | 0.935                                  |                                                                  |
| 2371.22                                           | RENNEYSVSFTADYGGIGK (327-346) {2370.539}                                                                   | 0.681                                  |                                                                  |
| 2371.22                                           | ENNEYVSYWTADYGGIGKR (328-347) {2370.539}                                                                  | 0.681                                  |                                                                  |
| 2215.20                                           | ENNEYVSYWTADYGGIGK (328-346) {2214.352}                                                                  | 0.848                                  |                                                                  |
| 2092.09                                           | AGYELHGISEGFDVEDILR (389-407) {2091.328}                                                                  | 0.762                                  |                                                                  |
| 1571.54                                           | EVKEEGYLVYVGK (540-553) {1570.782}                                                                        | 0.758                                  |                                                                  |
| 1540.27                                           | AKPGGWYKPEEIK (358-370) {1503.741}                                                                        | 0.529                                  | MJ0870                                                          |
| 1365.40                                           | KGHGMC*FEVC*PR (45-56) {1364.641}                                                                         | 0.759                                  | (69793.89)                                                      |
| 1200.34                                           | GLLTGGEGPLVR (412-423) {1199.392}                                                                         | 0.948                                  | {8.19}                                                          |
| 1177.24                                           | YAEKPQRER (586-594) {1177.305}                                                                           | -0.065                                 | Q58280                                                          |
| 1168.25                                           | HGIDEKEVK (217-226) {1168.335}                                                                            | -0.085                                 |                                                                  |
| 1124.14                                           | YAISTLDALR (134-143) {1123.294}                                                                           | 0.846                                  |                                                                  |
| 1100.06                                           | RADGTYFIR (347-355) {1099.234}                                                                            | 0.826                                  |                                                                  |
| 974.74                                            | FRYDNMK (205-211) {974.122}                                                                               | 0.618                                  |                                                                  |
| 943.96                                            | ADGTYFIR (348-355) {943.0466}                                                                             | 0.913                                  |                                                                  |
| 892.90                                            | YAEKPQER (586-592) {892.002}                                                                              | 0.898                                  |                                                                  |
| 861.83                                            | LIDEC*LIR (38-44) {862.031}                                                                               | 0.201                                  |                                                                  |

Peptide matching was done by use of the programs MS-FIT (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm) and ProFound (http://129.85.13.70/prowl/profound/profound_E_adv.html) with the parameters described in references 11 and 13.

†Masses not matched: 805.73, 919.93, 960.02, 1032.8, 1130.7, 1334.4, 1351.42, 1370.35, 1479.61, 1487.76, 1497.85, 1511.88, 1564.62, 1578.7, 1638.23, 1802.17, 1820.7, 2175.97, 2309.20, 2328.93, 2389.31, 2520.89, 2526.76, 2776.74, 2790.93, 2807.09, 2969.35

*Cysteines modified with Iodoacetamide to form carbamidomethyl cysteine
Table 2. Purification of coenzyme F$_{420}$-dependent sulfite reductase from *M. jannaschii*

| Purification Step            | Volume (ml) | Protein (mg) | Activity$^a$ (µmol min$^{-1}$) | Specific activity (µmol min$^{-1}$ mg$^{-1}$) | Purification$^b$ (fold) | Yield$^c$ (%) |
|-----------------------------|-------------|--------------|--------------------------------|-----------------------------------------------|-------------------------|---------------|
| Cell extract                | 32.5        | 325.4        | 423                            | 1.3                                           | 1                       | 100           |
| Ammonium Sulfate (60% saturation) | 58.7        | 75.1         | 285.4                          | 3.8                                           | 2.9                     | 67.5          |
| Phenyl-Sepharose            | 6.1         | 6.6          | 44.9                           | 6.8                                           | 5.2                     | 10.4          |
| F$_{420}$-Sepharose         | 2.4         | 1.5          | 11.6                           | 7.7                                           | 5.9                     | 2.7           |
| QAE-Sephadex                | 5.8         | 0.6          | 10.9                           | 18.2                                          | 14                      | 2.6           |

$^a$The activity was assayed in a standard reaction mixture of the following composition: 50 mM potassium phosphate buffer, pH 7, 40 µM reduced F$_{420}$ (H$_2$F$_{420}$), and 1.5 mM sodium sulfite.

$^b$The reason for apparently low value for the fold of purification: The cell extract contained a very high level of Fsr (Fig. 2A). Therefore, only a moderate fold of purification provided a homogeneous preparation of the enzyme.

$^c$The reason for poor yield at each stage of fractionation beyond the ammonium sulfate precipitation: The column fractions with Fsr activity were examined for purity via SDS-PAGE. Only those fractions with a minimum number of non-Fsr polypeptides were pooled. This strategy provided a homogeneous preparation of the enzyme, but resulted in a substantial loss of Fsr.
Fig. 1
Fig. 2
**Fig. 3**

```
MYEWKLNEIVDSGVCARCGTCTIVCPNGILTDFERPKLID\ECLRKGHGMCFEV
**\*\**
**\*\**
**\*\**
CP\RT**
VSSAKYQIKIREKFYEKYYAKSDIEGQDGGVVTAVFKYLLENGKIDADEI
VVGDECWKPVSLVQNAEDLLKTASKYAI\STLDA\RKAGEMGLEK\VAVVGLP
CQINGLRKLQYFPHYAKHD\ELELRNGKP\VKLPKIEY\LIG\LFC\TEKFRYDNMK\E
VLSKHGIDIEKVEKFDI\KKKL\LVV\VNGE\KEFDLKEFIECSG\CKMCRDFDAE
MADVSVGCVGSPDGYSTIIIRTEK\GEEIKN\AVLKE\G\V\N\L\E\EIEKLRQ\LKLKR
FKKE\\V\R\RENNE\Y\V\SF\Y\W\T\\D\Y\G\G\G\I\G\R\A\D\G\T\Y\F\I\R\V\RAKPPGWYK\P\E\E\\I\E\K\E
______________________________
ILDIAEEYNAKIKVTD\R\AG\Y\ELHG\ISG\FD\VED\IV\L\R\L\EK\GL\LT\G\SEG\GPL\\L\RA
TLACP\GG\NC\SS\GL\V\DT\TEL\AI\IED\FK\ERP\A\PY\K\\K\IA\ISG\CP\NGC\V\RP\Q\V
HDIGIAGVKYPVNEEKCNGCGRCAEVCKVE\AID\G\ET\S\TYNY\VC\V\G\GC\KC
IK\CP\NE\R\EV\KE\EG\Y\LV\V\GG\K\T\G\REV\VE\G\VK\M\L\S\\V\D\E\I\IN\F\I\DK\VL\V\V
GY\Y\AE\K\P\Q\R\RL\A\AV\M\KR\V\GY\G\FK\\L\E\E\V\K\L\MK\K\E\IC
```
Fig. 4
Fig. 6

H₂F₄₂₀:quinone oxidoreductase of A. fulgidus (Fqo) (similar to H₂F₄₂₀:phenazine oxidoreductase or Fpo of M. mazei)

Coenzyme F₄₂₀*-dependent sulfite reductase (Fsr) of Methanocaldococcus jannaschii
Fig. 7
Fig. 8
A new type of sulfite reductase - a novel coenzyme F420-dependent enzyme from the a methanarchaeon methanocaldococcus jannaschii
Eric F. Johnson and Biswarup Mukhopadhyay

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