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Thiol:fumarate reductase (Tfr) from *Methanobacterium thermoautotrophicum*
Identification of the catalytic sites for fumarate reduction and thiol oxidation

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Most methanogenic Archaea contain an unusual cytoplasmic fumarate reductase which catalyzes the reduction of fumarate with coenzyme M (CoM-S-H) and coenzyme B (CoB-S-H) as electron donors forming succinate and CoM-S-S-CoB as products. We report here on the purification and characterization of this thiol:fumarate reductase (Tfr) from *Methanobacterium thermoautotrophicum* (strain Marburg). The purified enzyme, which was composed of two different subunits with apparent molecular masses of 58 kDa (TfrA) and 50 kDa (TfrB), was found to catalyze the following reactions: (a) the reduction of fumarate with CoM-S-H and CoB-S-H (150 U/mg); (b) the reduction of fumarate with reduced benzyl viologen (620 U/mg); (c) the oxidation of CoM-S-H and CoB-S-H to CoM-S-S-CoB with methylene blue (95 U/mg); and (d) the reduction of CoM-S-S-CoB with reduced benzyl viologen (250 U/mg). The flavoprotein contained 12 mol non-heme iron and approximately the same amount of acid-labile sulfur/mol heterodimer. The genes encoding TfrA and TfrB were cloned and sequenced. Sequence comparisons with fumarate reductases and succinate dehydrogenases from Bacteria and Eucarya and with heterodisulfide reductases from *M. thermoautotrophicum* and *Methanosarcina barkeri* revealed that TfrA harbors FAD-binding motifs and the catalytic site for fumarate reduction and that TfrB harbors one [2Fe-2S] cluster and two [4Fe-4S] clusters and the catalytic site for CoM-S-H and CoB-S-H oxidation.

Keywords: fumarate reductase; succinate dehydrogenase; heterodisulfide reductase; iron-sulfur protein; flavoprotein.

Most methanogenic Archaea, with exception of the *Methanosarcinales*, generate 2-oxoglutarate for biosynthesis of glutamate from pyruvate via oxaloacetate, malate, fumarate, succinate and succinyl-CoA as intermediates (Zeikus et al., 1977; Weimer and Zeikus, 1979). These methanogens contain a cytoplasmic fumarate reductase, designated thiol:fumarate reductase (Tfr), which catalyzes the reduction of fumarate with coenzyme M (CoM-S-H) and coenzyme B (CoB-S-H) as electron donors (Bobik and Wolfe, 1989).

$$\text{Fumarate} + \text{CoM-S-H} + \text{CoB-S-H} \rightarrow \text{succinate} + \text{CoM-S-S-CoB}$$

The heterodisulfide CoM-S-S-CoB is also formed in the last step of methanogenesis, the reduction of methyl-coenzyme M with CoB-S-H (Bobik et al., 1987; Ellermann et al., 1988).

$$CH_3\text{-S-CoM} + \text{CoB-S-H} \rightarrow CH_4 + \text{CoM-S-S-CoB}$$

The strictly anaerobic organisms also contain a heterodisulfide reductase (Hdr), which catalyzes the re-reduction of the heterodisulfide CoM-S-S-CoB (Hedderich et al., 1990).

$$\text{CoM-S-S-CoB} + [2H] \rightarrow \text{CoM-S-H} + \text{CoB-S-H}$$

The latter reaction has been shown *in vivo* to be coupled with ADP phosphorylation via the chemiosmotic mechanism (Deppenmeier et al., 1996). Thus the anabolic reduction of fumarate is indirectly coupled with energy conservation.

The heterodisulfide reductases from *Methanobacterium thermoautotrophicum* and from *Methanosarcina barkeri* have extensively been characterized (Hedderich et al., 1990; Setzke et al., 1994; Heiden et al., 1993, 1994). They are composed of two or three different subunits and contain besides several iron-sulfur clusters either FAD or heme *b*. The encoding genes have been cloned and sequenced (Hedderich et al., 1994; Künkel et al., 1997). From sequence comparisons it was deduced that the active-site-harboring subunit is an iron-sulfur protein. A catalytic mechanism similar to that of ferredoxin:thioredoxin reductases from plants (Staples et al., 1996) was proposed (Künkel et al., 1997).

Much less is known about the thiol:fumarate reductase from methanogens. Only conflicting reports on the properties of this enzyme have been published. Evidence was presented by Khandekar and Eirich (1989) that the fumarate reductase from *M. thermoautotrophicum* (strain ΔH) is composed of four identical subunits of apparent molecular mass of 22 kDa and by Bobik and Wolfe (1989) that the thiol:fumarate reductase from the

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Abbreviations. Tfr, thiol:fumarate reductase; *tfr*, gene encoding Tfr; Frd, fumarate reductase; Sdh, succinate dehydrogenase; Hdr, heterodisulfide reductase; CoM-S-H, coenzyme M or 2-mercaptoethane sulfonate; CoB-S-H, coenzyme B or 7-mercaptoheptanoylthreonine phosphate; CoM-S-S-CoB, heterodisulfide of CoM-S-H and CoB-S-H; CoM-S-S-CoM, homodisulfide of CoM-S-H; CoB-S-S-CoB, homodisulfide of CoB-S-H.

Enzymes. Thiol:fumarate reductase (EC 1.3.4.1); fumarate reductase (EC 1.3.99.1); succinate dehydrogenase (EC 1.3.5.1); heterodisulfide reductase (EC 1.99.4.−); methyl-coenzyme M reductase (EC 1.8.99.−).

Note. The DNA sequences published here have been submitted to the EMBL sequence data bank and are available under accession number TfrA, AJ000941; TfrB, AJ000942.

same organism is composed of two non-identical subunits of apparent molecular masses of 59 kDa and 50 kDa, respectively. In both publications information on the possible presence of cofactors was not given.

Fumarate reductases from Bacteria and Eucarya have been shown to be flavoproteins. They can be divided in two classes: the membrane-bound fumarate reductases involved in anaerobic respiration, which are iron-sulfur flavoproteins composed of three or four different subunits, the largest containing a covalently bound FAD (Ackrell et al., 1992; Cole et al., 1985; Kröger et al., 1992; van Hellemond and Tielens, 1994); and the cytoplasmic fumarate reductases, which are flavoproteins composed of only one type of subunits, harboring a non-covalently bound FAD and lacking iron-sulfur clusters (Muratsubaki et al., 1994).

In the following communication evidence is presented that in the thiol:fumarate reductase from methanogens structural features of fumarate reductases from Bacteria and Eucarya and of heterodisulfide reductases from methanogenic Archaea are combined. The results were obtained for the thiol:fumarate reductase from *M. thermoautotrophicum* (strain Marburg), a methanogenic archaeon growing on H_2 and CO_2 as the sole carbon and energy sources with a growth temperature optimum of 65 °C.

MATERIALS AND METHODS

Coenzyme M was from Sigma. Coenzyme B and CoM-S-S-CoB were synthesized as described (Ellermann et al., 1988). Endoproteinase Lys-C, *Taq* DNA polymerase, digoxigenin DNA labeling kit and digoxigenin luminescent detection kit for nucleic acids were from Boehringer Mannheim. All DNA-modifying enzymes and the DNA sequencing kit were from Amersham. Nucleotide pyrophosphatase type II from *Crotalus adamenteus* was from Sigma. FPLC columns were from Pharmacia. *M. thermoautotrophicum* (strain Marburg) (DSM 2133) was obtained from the Deutsche Sammlung für Mikroorganismen in Braunschweig. The organism was grown in a 10-l fermenter on 80% H_2/20% CO_2/0.1% H_2S in a completely mineral salt medium (Schönheit et al., 1980).

Enzyme purification. All steps were performed under strictly anaerobic conditions under a gas phase of 5% H_2/95% N_2. Cell extracts were prepared by suspending 60 g cells (wet mass) in 100 ml 50 mM Tris/HCl, pH 7.6, containing 2 mM dithiothreitol (buffer A). Cells were disrupted by sonication at 4 °C in intervals of 3×5 min using an energy output of 200 W (Bandelin sonifier). Undisrupted cells and cell debris were removed by centrifugation at 15 000×*g* for 25 min. The supernatant was designated as cell extract.

After a heat treatment (75 °C for 2 h) the cell extract was centrifuged at 90 000×*g* for 30 min. The 90 000×*g* supernatant was supplemented with $(NH_4)_2SO_4$ to a final concentration of 2.4 M. Precipitated protein was pelleted by centrifugation at 90 000×*g*. The pellet was dissolved in 50 ml buffer A. This solution, which contained more than 90% of the fumarate reductase activity, was supplemented with $(NH_4)_2SO_4$ to a final concentration of 1.6 M. Precipitated protein was removed by centrifugation at 90 000×*g*. The supernatant contained about 95% of the fumarate reductase activity. The supernatant of the second $(NH_4)_2SO_4$ precipitation was applied to a SOURCE 15 ISO column (2.6 cm×15 cm) equilibrated with 1.6 M $(NH_4)_2SO_4$ in buffer A. Protein was eluted in a step gradient with decreasing $(NH_4)_2SO_4$ concentrations in buffer A: 80 ml 1.6 M; 80 ml 1.4 M; 80 ml 1.3 M; 80 ml 1.2 M; 80 ml 1.1 M; 80 ml 1.0 M; and 80 ml 0 M. The fractions containing fumarate reductase activity eluted at an $(NH_4)_2SO_4$ concentration of 1.2 M. After desalting and concentrating via ultrafiltration using an Amicon chamber equipped with an YM30 ultrafiltration membrane (30-kDa cut-off; Amicon) protein was diluted with 10 ml 5 mM potassium phosphate, pH 7.0, and applied to a hydroxyapatite column (1 cm×20 cm). Protein was eluted in a step gradient with increasing potassium phosphate concentrations: 40 ml 30 mM; 40 ml 40 mM; 40 ml 55 mM; 40 ml 65 mM; 40 ml 75 mM; and 40 ml 90 mM. The fractions containing fumarate reductase activity eluted at a phosphate concentration of 55 mM. The desalted hydroxyapatite fractions were applied to a Resource Q column (6 ml). Adsorbed protein was eluted with a linear gradient of NaCl in buffer A (0 to 0.5 M, 250 ml). Thiol:fumarate reductase activity was recovered in fractions that eluted with an NaCl concentration of 0.42 M. Protein was concentrated (Centricon 30 microconcentrators) to a final concentration of 0.5 mg/ml and stored at −20 °C.

Determination of enzyme activities. The assays were routinely performed at 60 °C either in 8-ml serum bottles or in 1.5-ml cuvettes under N_2 as gas phase.

Reduction of fumarate with CoM-S-H and CoB-S-H. The activity was determined by following the decrease of CoM-S-H and CoB-S-H with Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid)] (Zahler and Cleland, 1968). The 1-ml assay mixture in 8-ml serum bottles contained 50 mM Tris/HCl, pH 7.6, 4 mM CoM-S-H, 4 mM CoB-S-H and 10 mM sodium fumarate. The reaction was started by addition of the enzyme solution. At 1-min intervals aliquots were withdrawn and analyzed for thiols with Ellman's reagent. The method was calibrated with CoM-S-H as standard. 1 U enzyme activity refers to 1 μmol thiol oxidized/min.

Reduction of fumarate with reduced benzyl viologen. The activity was determined by following the oxidation of reduced benzyl viologen with fumarate at 578 nm photometrically. The 0.8-ml assay mixture contained 50 mM Tris/HCl pH 7.6, 2 mM benzyl viologen and 2 mM sodium fumarate. Benzyl viologen was reduced with sodium dithionite to an absorbance at 578 nm of about 1. The reaction was started by the addition of protein (2.5−150 μg). 1 U enzyme activity refers to 1 μmol reduced benzyl viologen oxidized/min.

Reduction of CoM-S-S-CoB with benzyl viologen as electron donor. The activity was determined by following the oxidation of reduced benzyl viologen with CoM-S-S-CoB at 578 nm. The 0.8-ml assay mixture contained 50 mM Tris/HCl pH 7.6, 2 mM benzyl viologen and 2 mM CoM-S-S-CoB. Benzyl viologen was reduced with sodium dithionite to an absorbance at 578 nm of about 1. The reaction was started by the addition of protein (2.5−150 μg). 1 U enzyme activity refers to 1 μmol reduced benzyl viologen oxidized/min.

Oxidation of CoM-S-H plus CoB-S-H with methylene blue. The activity was determined by following the reduction of methylene blue at 578 nm. The 0.8-ml assay mixture contained 50 mM Tris/HCl, pH 7.6, 0.15 mM methylene blue, 0.5 mM CoM-S-H, and 0.5 mM CoB-S-H. The reaction was started by the addition of protein (2.5−150 μg). 1 U enzyme activity refers to 1 μmol methylene blue reduced/min.

Oxidation of succinate with phenazine methosulfate. The activity was determined by following the reduction of phenazine methosulfate at 387 nm. The 0.8-ml assay mixture contained 50 mM Tris/HCl, pH 7.6, 0.4 mM phenazine methosulfate and 2 mM sodium succinate. The reaction was started by the addition of 2.5 μg purified protein. 1 U activity refers to 1 μmol phenazine methosulfate reduced/min.

Protein was determined by the method of Bradford (1976) using the BioRad dye reagent.

Determinaton of cofactors. Non-heme iron was determined colorimetrically with neocuproin (2,9-dimethyl-1,10-phen-

anthroline) and ferrozine [3-(2-pyridyl)-5,6-bis(4-phenylsulfonate)-1,2,4-triazine] (see Hedderich et al., 1990). Acid-labile sulfur was analyzed as methylene blue (see Hedderich et al., 1990).

For the analysis of non-covalently bound flavins, purified thiol:fumarate reductase (0.5 mg in 1 ml; 4.6 μM) was air oxidized. Protein was denatured by adding trichloroacetic acid to a final concentration of 5% and stored on ice for 15 min. Precipitated protein was removed by centrifugation. The pellet was washed once with 50 μl 5% trichloroacetic acid. The supernatants were pooled and adjusted to pH 5.0 with 2 M K_2HPO_4. For the identification of the flavin cofactor the supernatant was applied to an HPLC column (Lichrospher 100 RP-18, 5 μm; 4 mm×12.5 cm). Flavins were eluted in a linear gradient of 2% to 60% acetonitrile containing 0.1% trifluoroacetic acid (elution volume 60 ml). Flavins were detected at 520 nm with a Sykam S 3400 fluorescence detector (excitation 445 nm). FAD (retention time 2.7 min), FMN (retention time 4.3 min) and riboflavin (retention time 7.8 min), each at a concentration of 1 μM, were used as standards.

For the analysis of covalently bound flavins the 5% trichloroacetic acid pellet (0.5 mg protein) was resuspended in 0.1 ml 50 mM Tris/HCl, pH 8.5, containing 50 μg endoproteinase Lys-C and incubated for 12 h at 37°C to digest the protein. The clear solution was then adjusted to pH 7.0. To determine the pH dependence of fluorescence intensity the obtained flavin peptide was converted to the riboflavin level via incubation with 2 U nucleotide pyrophosphatase and with 5 U shrimp alkaline phosphatase (2 h, 37°C, pH 7.0). To determine whether the covalently bound flavin is an FAD or an FMN, nucleotide pyrophosphatase (2 U) was added to the flavin peptides and the increase in relative fluorescence at 520 nm was monitored (Fox and Walsh, 1982). Fluorescence emission and excitation spectra were recorded on a Shimadzu RF 540 spectrofluorophotometer.

Determination of amino acid sequences. For determination of N-terminal amino acid sequences the two subunits of thiol:fumarate reductase were separated by SDS/PAGE (Fig. 1) and blotted onto a ProBlott membrane (Applied Biosystems). The blot was stained with a solution of Commassie brillant blue (0.1%) in 40% methanol and 1% acetic acid. The areas containing the protein bands were excised. Sequence determination was performed by Dr D. Linder (University of Gießen) on a 4774 protein/peptide sequencer (Applied Biosystems) using the protocol given by the manufacturer.

For determination of internal peptides the two subunits of purified thiol:fumarate reductase were separated by SDS/PAGE (Fig. 1). After staining the gel with Commassie brillant blue the two protein bands were excised and treated with endoproteinase Lys-C in the polyacrylamide matrix. The peptides thus obtained were separated by HPLC on a Vydac 218TP54 column (250 mm×4.6 mm) (Eckerskorn and Lottspeich, 1989). The amino acid sequences were determined as described above.

Generation of hybridization probes for *tfr* genes and cloning of the *tfr* genes. The hybridization probes for the *tfrA* and *tfrB* genes were obtained by PCR using genomic DNA from *M. thermoautotrophicum* strain Marburg as template which was isolated as described by Jarrell et al. (1992). The heterologous oligonucleotides GATTTGAGCCGGGTGTAG (sense) and GGTTTCTCCATTTCAACG (antisense) were derived from the N-terminal amino acid sequence and the internal peptide shown in Table 2 and used as primers for amplifying the 5′ terminal region of the *tfrB* gene. The 5′ terminal region of the *tfrA* gene was amplified by using the heterologous oligonucleotides TCATAATAGGCTCAGGTGG (sense) and GTAACCTCACCGGCTGC (antisense) derived from the N-terminal amino acid sequence and an internal peptide (Table 2). The PCR products obtained were cloned into the pCRII vector using the Invitrogen TOPO TA cloning kit. The identity of the PCR products was determined by DNA sequencing. The sequences allowed the construction of homologous oligonucleotide probes that were labeled with digoxigenin-dUTP by PCR following the protocol given by the manufacturer. The specificity of the oligonucleotide probes was tested by Southern blot hybridizations with genomic DNA from *M. thermoautotrophicum* completely digested with *Eco*RI, *Bam*HI and *Eco*RV restriction endonucleases. Under the employed conditions hybridizations with both oligonucleotide probes resulted each in only one hybridization signal.

The digoxigenin labelled probes (Dig-tfrA and Dig-tfrB) were used to screen the λZAP genomic library (*Sau*3A) of *M. thermoautotrophicum* strain Marburg. The λZAP Express II library was plated according to the protocol of Stratagene. Positive phage clones were identified by Southern hybridization. Excision and recircularization of positive clones generated the phagmids pBK-tfrA (containing the *tfrA* gene) and pBK-tfrB (containing the *tfrB* gene).

For DNA sequencing the didesoxynucleotide method was used (Sanger et al., 1977).

Table 1. Purification of thiol:fumarate reductase from *M. thermoautotrophicum*. Cell extract was prepared from 60 g (wet mass) of freshly harvested cells. The activities were determined as described in Materials and Methods. Fumarate reductase and heterodisulfide reductase activity were determined with reduced benzyl viologen as electron donor. The activity yield is given for fumarate reductase activity.

Purification step	Fumarate reductase activity	Heterodisulfide reductase activity	Thiol:fumarate reductase activity	Yield
	U/mg			%
Cell extract	0.8	2.1	0.5	100
Heat treatment	1.4	2.1	0.7	90
2.4 M $(NH_4)_2SO_4$ precipitate	1.8	1.1	1	73
1.6 M $(NH_4)_2SO_4$ supernatant	2.1	1.2	1.1	66
SOURCE 15 ISO	30	7.8	17	51
Hydroxyapatite	85	29	28	31
Resource Q	620	250	150	22

RESULTS

The following methanogenic Archaea representing the different phylogenetic branches were screened for thiol:fumarate reductase activity: *M. thermoautotrophicum* strain Marburg (0.7 U/mg) and *ΔH* (0.6 U/mg), *Methanococcus thermolithotrophicus* (0.09 U/mg) and *Methanococcus voltae* (0.03 U/mg), *Methanopyrus kandleri* (0.03 U/mg), *Methanosarcina barkeri* (< 0.01 U/mg) and *Methanogenium liminatans* (0.18 U/mg). From the organisms tested the Marburg strain of *M. thermoautotrophicum* contained the highest activity levels. Cell extracts of this organism also showed the highest fumarate reductase activity as determined from the rate of reduced benzyl viologen oxidation with fumarate. In all organisms, which contained fumarate reductase activity, CoM-S-H and CoB-S-H were found to be the physiological electron donors for fumarate reduction.

Thiol:fumarate reductase from *M. thermoautotrophicum* (strain Marburg) turned out to be a labile enzyme rapidly inactivated by dioxygen even when present in only trace amounts. Under anaerobic conditions the enzyme was stable at high pro-

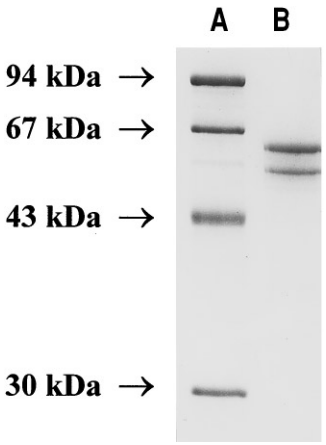

Fig. 1. Analysis of purified thiol:fumarate reductase from *M. thermoautotrophicum* by SDS/PAGE. Protein was denatured by SDS and separated in a 12% slab gel (8 cm×7 cm) which was subsequently stained with Coomassie brillant blue R250 (Laemmli, 1970). Molecular-mass standards (lane A); 10 μg purified thiol:fumarate reductase (lane B).

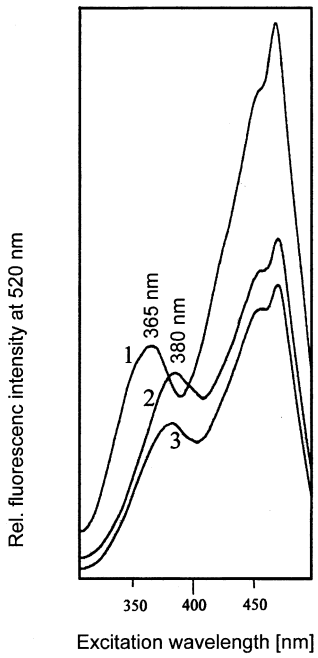

Fig. 2. Fluorescence excitation spectrum of the flavin covalently bound to TfrA in comparison to that of FAD. (1) Covalently bound flavin from 0.5 mg Tfr protein; (2) FAD standard (0.5 μM); (3) flavin extracted from 0.5 mg Tfr protein with 5% trichloroacetic acid. Before determination of the spectra the solutions were adjusted to pH 5.0. The relative emission was determined at 520 nm.

Table 2. N-Terminal amino acid sequence of the two subunits TfrA and TfrB of thiol:fumarate reductase from *M. thermoautotrophicum* (strain Marburg) and of internal peptides.

Subunit	Amino acid sequence
TfrA:	
N-Terminus	M E V E L Y E K D V K I I G S G G A G 19
internal peptide	A A G E V S G G V H G A N
TfrB:	
N-Terminus	M I N V K V L R F E P G V D E K P V L E 20
internal peptide	I L N S I P G L E F V E M E K

tein concentrations at 4°C. Diluted solutions of the enzyme rapidly lost activity. Despite these finding we achieved a purification of the enzyme of 300-fold in a 22% activity yield in amounts sufficient to characterize most of its molecular and catalytic properties.

Purification of the thiol:fumarate reductase. The enzyme was purified from freshly harvested cells of *M. thermoautotrophicum* mainly according to the procedure described by Bobik and Wolfe (1989; Table 1). To obtain higher activity yields a few alterations had to be made. Hydrophobic chromatography on phenyl-Sepharose was replaced by chromatography on the less hydrophobic SOURCE 15 ISO material which, in our hands, gave much better results. The purification procedure outlined in Table 1 yielded a preparation with a specific thiol:fumarate reductase activity of 150 U/mg and an apparent molecular mass of 90 kDa as revealed by native polyacrylamide gradient gel electrophoresis. From 60 g cells (wet mass) approximately 0.5 mg of purified thiol:fumarate reductase was obtained.

Molecular properties. SDS/PAGE revealed that the purified thiol:fumarate reductase was composed of two different subunits with apparent molecular masses of 58 kDa (TfrA) and 50 kDa (TfrB; Fig. 1). The N-terminal amino acid sequence of the two polypeptides and of internal peptides obtained by endoproteinase Lys-C digestion are shown in Table 2.

A polypeptide with an apparent molecular mass of approximately 22 kDa, which was postulated to be the only subunit of fumarate reductase from *M. thermoautotrophicum* (Khandekar and Eirich, 1989), was not observed in any of our preparations.

The purified enzyme exhibited a light brown colour with an ultraviolet/visible spectrum characteristic for iron-sulfur flavoproteins (Fig. 3). Analysis for iron-sulfur clusters showed that the enzyme preparations contained approximately 12 mol non-heme iron and 10 mol acid-labile sulfur/mol heterodimer. An analysis for flavins indicated that the thiol:fumarate reductase contained 0.4 mol non-covalently bound FAD/mol heterodimer and also covalently bound flavin.

The existence of a covalently bound flavin is based on the following findings: (a) after extraction of the enzyme with 5% trichloroacetic acid the redissolved protein fraction still exhibited a fluorescence emission spectrum characteristic for flavins. The fluorescence was found to be associated with the subunit TfrA after separation of the two polypeptides by SDS/PAGE; (b) after proteolysis of the trichloroacetic acid-extracted enzyme the released flavin was not FAD, FMN or riboflavin as revealed by HPLC on a Vydac 218TP54 column. The fluorescence excitation spectrum was indicative of an 8α-substituted flavin: the near ultraviolet/visible maximum was hypsochromically shifted from 380 nm (free FAD) to 365 nm (Fig. 2; Singer and Edmondson, 1974). The 8α-substituted flavin is probably an 8α-substituted FAD as indicated by a relative fluorescence increase at 520 nm after pyrophosphatase treatment (Fox and Walsh, 1982). The substituent is not a histidine residue as indicated by the finding that, after conversion of the substituted flavin to the riboflavin level by pyrophosphatase and alkaline phosphatase treatment, the fluorescence intensity at 520 nm was independent of the pH (Decker, 1992).

Fumarate reductases with covalently bound FAD contain a highly conserved histidine residue (His44 in *Escherichia coli* FrdA; Cole et al., 1985), which forms a covalent linkage to the FAD prosthetic group. As will be discussed below, this histidine residue is replaced by a cysteine residue in TfrA. Therefore it

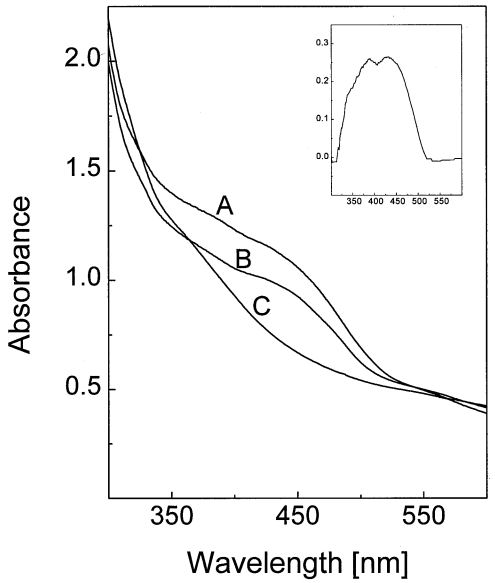

Fig. 3. Absorption spectrum of purified Tfr in the oxidized and reduced state. The solution contained purified Tfr (1.8 mg protein/ml) in 50 mM Tris/HCl, pH 7.6 under N_2 as gas phase. (A) After oxidation of the sodium dithionite-reduced enzyme with sodium fumarate (80 μM). (B) After reduction of the sodium fumarate-oxidized enzyme with coenzyme M (100 μM) and coenzyme B (100 μM). (C) After reduction of the fumarate-oxidized enzyme with sodium dithionite (100 μM). The inset shows the difference spectrum of spectrum A and spectrum B.

has to be considered that the flavin in TfrA is covalently linked to the enzyme via this cysteine residue. All attempts to prove this hypothesis, until now, have failed. The amounts of enzyme available were not sufficient to obtain conclusive results.

The amount of covalently bound flavin was estimated from the relative fluorescence at 520 nm to be approximately 0.4 mol/mol heterodimer assuming that the covalently bound flavin has the same fluorescence yield as free FAD.

The existence of both covalently and non-covalently bound flavin was also observed when the enzyme was denatured at neutral pH by incubation with 0.2% SDS (15 min at 40 °C) instead of the trichloroacetic acid method.

When, as a control, fumarate reductase from *Wolinella succinogenes*, an enzyme with covalently bound FAD, was extracted with trichloroacetic acid, 100% of the fluorescence remained associated with the protein fraction and the fluorescence properties were identical to those reported for 8α-histidine substituted FAD (Kenney and Kröger, 1977). Under the same conditions the flavin cofactor was completely released from glucose oxidase, an enzyme which contains non-covalently bound FAD.

The prosthetic groups in Tfr could be completely oxidized with fumarate (as compared with O_2 as oxidant; Fig. 3). The fumarate-oxidized enzyme could be reduced with coenzyme M and coenzyme B. This reduction was not complete compared with that with sodium dithionite as reductant. No reduction of the enzyme was observed with either of the two thiol-coenzymes alone. The ox-red difference spectrum clearly indicates that both the flavin and the Fe/S clusters changed their redox state. Oxidation or reduction rates were to fast to be determined.

Catalytic properties. The purified thiol:fumarate reductase catalyzed the reduction of fumarate with CoM-S-H and CoB-S-H with a specific activity of 150 U/mg protein; the apparent K_m for fumarate was 0.6 mM ([CoM-S-H] and [CoB-S-H] each 4 mM). The enzyme also catalyzed the reduction of fumarate with reduced benzyl viologen (620 U/mg; apparent K_m for fumarate = 0.3 mM) but not the oxidation of succinate with methylene blue or phenazine methosulfate. The enzyme also catalyzed the oxidation of CoM-S-H and CoB-S-H with methylene blue as electron acceptor (95 U/mg) and the reduction of CoM-S-S-CoB with reduced benzyl viologen (250 U/mg; apparent K_m for CoM-S-S-CoB = 0.6 mM). The two latter reactions are also catalyzed by heterodisulfide reductase from *M. thermoautotrophicum* (Hedderich et al., 1990).

The thiol:fumarate reductase did not catalyze the reduction of fumarate when only CoM-S-H or CoB-S-H were present as electron donor. Also, the enzyme did not mediate the reduction of CoM-S-S-CoM or CoB-S-S-CoB with reduced benzyl viologen. The enzyme thus showed the same specificity for the two thiol compounds as the heterodisulfide reductase from *M. thermoautotrophicum* (Hedderich et al., 1990).

Cloning of the *tfrA* and *tfrB* genes from *M. thermoautotrophicum* strain Marburg. Since the biochemical characterization of Tfr was performed with the enzyme from *M. thermoautotrophicum* strain Marburg we also determined the primary structure of the enzyme from the Marburg strain via cloning and sequencing of the encoding genes (see Materials and Methods). The sequences obtained for TfrA and TfrB were compared with the respective sequences derived from the genome sequence of *M. thermoautotrophicum* strain ΔH (Smith et al., 1997) and *Methanococcus jannaschii* (Bult et al., 1996). TfrA has 82% sequence identity to TfrA from *M. thermoautotrophicum* strain ΔH and 52% sequence identity to TfrA from *M. jannaschii*. Likewise TfrB shows 85% and 50% sequence identity to the respective protein from *M. thermoautotrophicum* strain ΔH and *M. jannaschii*. The two genes *tfrA* and *tfrB* are located at different loci within the genome as it is also the case for the two genes in *M. thermoautotrophicum* strain ΔH and *M. jannaschii*.

DISCUSSION

Thiol:fumarate reductase is a unique enzyme which catalyzes the reduction of fumarate with the two thiol coenzymes, coenzyme M and coenzyme B. In this study we have characterized the prosthetic groups of this enzyme and have defined the functions of the two individual subunits.

A sequence analysis of TfrA indicates that it has significant similarity to the catalytic subunit of fumarate reductases (FrdA) and succinate dehydrogenase (SdhA) from various species. The sequence identity to the well-characterized fumarate reductases from *E. coli* and *W. succinogenes* were 35% and 34%, respectively. Like other fumarate reductases TfrA contains the typical sequence motifs involved in the binding of the FAD prosthetic group, with one important exception. The highly conserved histidine residue (His44 in *E. coli* FrdA), which is covalently attached to the FAD prosthetic group (Cole et al., 1985), is not present in TfrA. The histidine residue is replaced by a cysteine residue in the enzyme from *M. thermoautotrophicum* strain Marburg and also in the enzyme from *M. thermoautotrophicum* strain ΔH (Smith et al., 1997) and *M. jannaschii* (Bult et al., 1996). Although this conserved His/Cys exchange might indicate a role of the conserved cysteine residue in the formation of a covalent linkage to the flavin in Tfr, our results are still not definite in this respect. It also remains unclear why part of the flavin is found to be covalently bound and part of the flavin is found to be non-covalently bound.

The first 215 amino acids of TfrB show high sequence similarity to the iron-sulfur cluster containing subunit of fumarate reductases (FrdB; Fig. 4). In this sequence region 11 cysteine

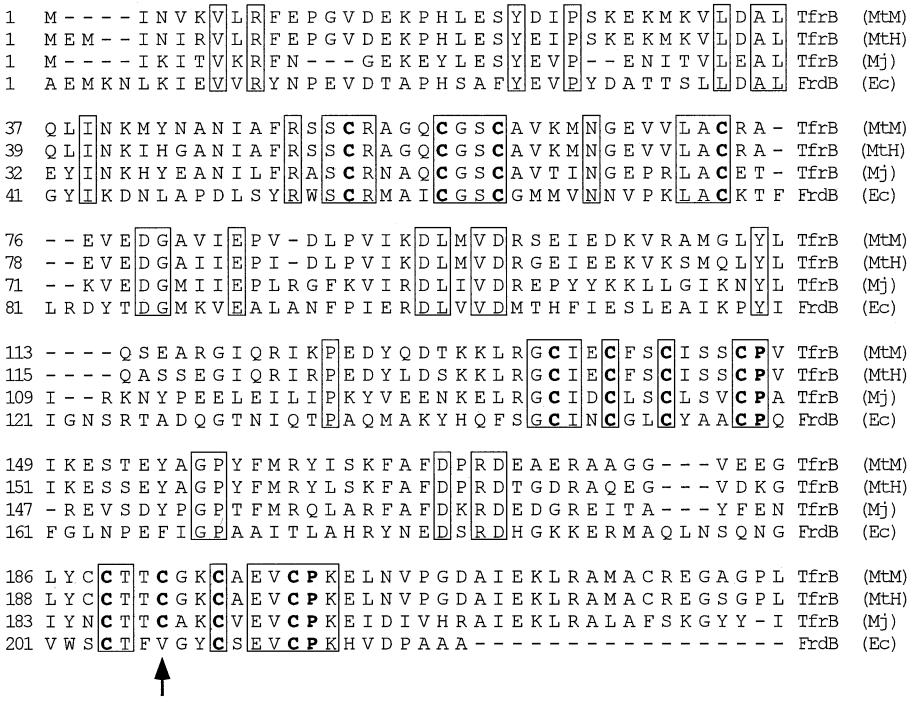

Fig. 4. Sequence comparison of the N-terminal region of TfrB from methanogens with FrdB from *E. coli.* Amino acids 53−73 in TrfB from MtM indicate a sequence region characteristic for a [2Fe-2S] cluster and amino acids 136−147 and 189−200 indicate sequence regions for two [4Fe-4S] clusters. The conserved cysteine residues are highlighted in bold face. Note the missing cysteine residue in the FrdB sequence at position 207. The *E. coli* fumarate reductase contains a [3Fe-4S] and a [4Fe-4S] cluster (Cole et al., 1985; Lauterbach et al., 1990). MtM = *M. thermoautotrophicum* strain Marburg; MtH = *M. thermoautotrophicum* strain *Δ*H; Mj = *M. jannaschii*; Ec = *E. coli;* Tfr = thiol:fumarate reductase; Frd = fumarate reductase.

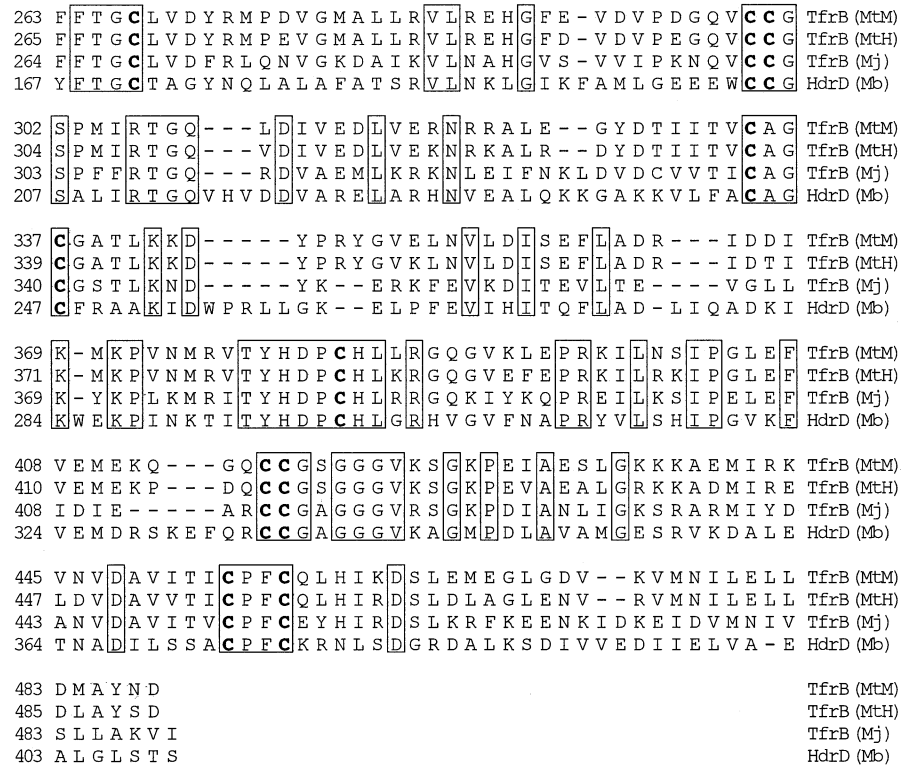

Fig. 5. Sequence comparison of the C-terminal region of TfrB from methanogens with HdrD from *M. barkeri.* The C-terminal region of TfrB from *M. thermoautotrophicum* strain Marburg, *M. thermoautotrophicum* strain *Δ*H and *M.* jannaschii was aligned with the C-terminal region of HdrD from *M. barkeri* (Künkel et al., 1997). The ten highly conserved cysteine residues are highlighted in bold face. MtM = *M. thermoautotrophicum* strain Marburg; MtH = *M. thermoautotrophicum* strain *Δ*H; Mj = *M. jannaschii*; Mb = *M. barkeri;* Tfr = thiol:fumarate reductase; Hdr = heterodisulfide reductase.

residues are highly conserved between TfrB and the iron-sulfur cluster containing subunit of FrdB (Lauterbach et al., 1990). These 11 cysteine residues are involved in the ligation of one [2Fe-2S] cluster (cluster I), one [4Fe-4S] cluster (Cluster II) and one [3Fe-4S] cluster (cluster III) in FrdB from *E. coli* and *W. succinogenes*. It is interesting to note that TfrB contains one additional cysteine residue and thus contains two sequence motifs CX2CX2CX3CP indicating the presence of two [4Fe-4S] clusters, instead of one [3Fe-4S] and one [4Fe-4S] cluster (Fig. 4). The presence of a [2Fe-2S] cluster and two [4Fe-4S] clusters has recently been found for a succinate dehydrogenase from *Sulfolobus acidocaldarius* (Janssen et al., 1997). The N-terminal part of TfrB also shows sequence similarity to the N-terminal part of the subunit HdrD of heterodisulfide reductase from *M. barkeri* and the subunit HdrC of heterodisulfide reductase from *M. thermoautotrophicum* which however ligate only two [4Fe-4S] clusters.

The C-terminal part of TfrB (amino acids 263−488) has high sequence similarity to the catalytic subunit HdrD of heterodisulfide reductase from *M. barkeri* and somewhat lower sequence similarity to the subunit HdrB of heterodisulfide reductase from *M. thermoautotrophicum*. In Fig. 5 an alignment of the three available TfrB sequences with HdrD is shown. It reveals that conserved residues are not randomly distributed, but are concentrated in several segments (boxed in Fig. 5). The three polypeptides contain 10 highly conserved cysteine residues which might ligate an additional iron-sulfur cluster and an active-site disulfide as has been previously suggested (Künkel et al., 1997).

In vivo heterodisulfide reductase catalyzes the reduction of CoM-S-S-CoB and thiol:fumarate reductase catalyzes the oxidation of CoM-S-H and CoB-S-H. *In vitro* both enzymes Hdr and Tfr catalyze both reactions. Thus the active sites in both enzymes could be similar. This is supported by the finding that the heterodisulfide reductase from *M. thermoautotrophicum* and from *M. barkeri* and the thiol:fumarate reductase from *M. thermoautotrophicum* each contain a subunit (*Mb*HdrD, *Mt*HdrB and TfrB, respectively) with similar primary structures which therefore should harbor the active site for heterodisulfide reduction or CoM-S-H and CoB-S-H oxidation. These enzymes are not related to the enzymes belonging to the family of pyridine nucleotide disulfide oxidoreductases and represent a novel class of disulfide oxidoreductases.

Our results thus suggest that thiol:fumarate reductase interacts with its substrates at separate catalytic sites, fumarate at a site located on TfrA and the two thiol compounds at a site located on TfrB. Sequence comparisons revealed that the catalytic site for fumarate reduction is similar to that of other fumarate reductases and probably involves a covalently bound FAD cofactor. Electron transfer could occur via the Fe/S centers, located in the N-terminal part of TfrB, to the catalytic site of CoM-S-H and CoB-S-H oxidation located at the C-terminal part of TfrB.

In the databases a group of proteins from non-methanogenic Archaea is emerging with high sequence similarity to HdrD, HdrB and TfrB. Examples for such proteins are YwdF from *Bacillus subtilis* (Kunst et al., 1997) AF1998 and AF0505 from *Archaeoglobus fulgidus* (Klenk et al., 1997), Hdr from *Synechocystis* (Nakamura et al., 1997) and SdhC from *Sulfolobus acidocaldarius* (Janssen et al., 1997). For none of these proteins from the non-methanogens has a function been defined. The high sequence similarity to HdrD, HdrB and TfrB however, indicates a role in disulfide reduction or thiol oxidation. It, therefore, can be speculated that Hdr and Tfr are the first-characterized members of a previously unknown family of disulfide reductases.

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