MEMBRANE-BOUND HYDROGENASE AND SULFUR REDUCTASE OF THE HYPERTHERMOPHILIC AND ACIDOPHILIC ARCHAEON ACIDIANUS AMBIVALENS

Simone Laska,3 Friedrich Lottspeich4 and Arnulf Kletzin

Correspondence
Arnulf Kletzin
Kletzin@bio.tu-darmstadt.de

Institute of Microbiology and Genetics, Darmstadt University of Technology, Schnittspahnstrasse 10, 64287 Darmstadt, Germany

A sulfur reductase (SR) and a hydrogenase were purified from solubilized membrane fractions of anaerobically grown cells of the sulfur-dependent archaeon Acidianus ambivalens and the corresponding genes were sequenced. The SR reduced elemental sulfur with hydrogen as electron donor \([45 \text{ U (mg protein)}^{-1}]\) in the presence of hydrogenase and either 2,3-dimethylnaphthoquinone (DMN) or cytochrome c in the enzyme assay. The SR could not be separated from the hydrogenase during purification without loss of activity, whereas the hydrogenase could be separated from the SR. The specific activity of the hydrogenase was \(170 \text{ U (mg protein)}^{-1}\) with methyl viologen and \(833 \text{ U (mg protein)}^{-1}\) with DMN as electron acceptors.

Both holoenzymes showed molecular masses of 250 kDa. In SDS gels of active fractions, protein bands with apparent masses of 110 (SreA), 66 (HynL), 41 (HynS) and 29 kDa were present. Enriched hydrogenase fractions contained \(14 \mu\text{mol Fe and 2 } \mu\text{mol Ni (g protein)}^{-1}\); in addition, \(2-5 \mu\text{mol Mo (g protein)}^{-1}\) was found in the membrane fraction. Two overlapping genomic cosmid clones were sequenced, encoding a five-gene SR cluster (\(sre\)) including the 110 kDa subunit gene (\(sreA\)), and a 12-gene hydrogenase cluster (\(hyn\)) including the large and small subunit genes and genes encoding proteins required for the maturation of NiFe hydrogenases. A phylogenetic analysis of the SR amino acid sequence revealed that the protein belonged to the DMSO reductase family of molybdenoenzymes and that the family showed a novel clustering. A model of sulfur respiration in Acidianus developed from the biochemical results and the data of the amino acid sequence comparisons is discussed.

INTRODUCTION

The hyperthermophilic and acidophilic crenarchaeote Acidianus ambivalens grows at 80°C and pH 2-5 in a mineral medium supplemented with elemental sulfur under a gas phase of either CO₂/H₂ or CO₂-enriched air (Zillig et al., 1985, 1986). The micro-organism is an obligate chemolithoautotroph, thus the reduction of elemental sulfur with hydrogen as electron donor is the only energy-yielding reaction under anaerobic growth conditions (S/H autotrophy; Fischer et al., 1983; Zillig et al., 1986). At least two membrane-bound enzymes are required for this reaction, a hydrogenase and a sulfur reductase (SR), both coupled with carriers mediating the electron transfer (reviewed, for example, in Hedderich et al., 1999).

An electron transport chain with a similar function has been well characterized from the anaerobic bacterium Wolinella succinogenes (reviewed, for example, in Hedderich et al., 1999). The two enzymes, a NiFe hydrogenase and a molybdenum (Mo)-containing polysulfide reductase (PSR), are composed of three structural polypeptides each: a catalytic subunit, an iron–sulfur protein and a membrane anchor (for reviews of NiFe hydrogenases see, for example, Fontecilla-Camps et al., 2001; Robson, 2001a). An electrochemical gradient is generated during electron transfer from the hydrogenase via menaquinone to the PSR; however, the mechanisms of proton pumping are not well understood. The catalytic and the iron–sulfur subunits of the enzymes are oriented towards the periplasm (Hedderich et al., 1999).

The only membrane-bound electron transport multienzyme complex with similar catalytic properties (H₂:sulfur oxidoreductase) known from Archaea has been purified...
from the chemolithoautotrophic and hyperthermophilic strain Pyrodictium abyssi. This complex is composed of nine different subunits containing a NiFe hydrogenase, SR, and cytochromes b and c (Dirmeier et al., 1998). Neither Memon tungsten b and c (Dirmeier et al., 1998). Neither Memon tungsten was found in the purified complex or in the membrane fraction. Presumably, the complex contains all the constituents necessary for the electron transport from hydrogen to sulfur. Quinones were not required for activity. It is so far the only example of a fully functional hydrogenase/SR complex from sulfur-dependent and hyperthermophilic archaea. In contrast, the two Wolinella enzymes are present separately in the membrane and do not form a multienzyme complex (Dietrich & Klümmer, 2002). A functionally similar electron transport chain from the closely related archaeon Pyrococcus brockii (Pihl & Maier, 1991; Pihl et al., 1992) consists of at least two separate enzymes as in Wolinella. The hydrogenase consists of two subunits. The subunit structure of the SR is not known. A c-type cytochrome and a specific quinone presumably mediate the electron transfer. The orientation of both of the Pyrococcus enzymes, the reaction and electron transport mechanisms and the genes encoding the proteins are unknown.

Different enzymes are present in the heterotrophic archaeon Pyrococcus furiosus, which gains energy by fermentation of carbohydrates or amino acids. In the absence of sulfur, hydrogen is produced among other products. In the presence of sulfur, H2S is produced and significantly higher growth rates are observed (Schicho et al., 1993). Inducible transcripts and proteins have been identified in Pyrococcus furiosus when sulfur is added to the medium, but no specific SR (Adams et al., 2001; Schütz et al., 2001). However, several hydrogenases purified from soluble extracts of Pyrococcus furiosus and the closely related Thermococcus litoralis showed sulfur or polysulfide-reducing activity (Ma et al., 1993, 2000; Rakheley et al., 1999). Pyrococcus furiosus also possesses a soluble sulfide dehydrogenase with a broad substrate range, which also can reduce polysulfide to H2S with NADPH as electron donor (Ma & Adams, 1994), and a membrane-bound hydrogenase, which does not reduce sulfur (Sapra et al., 2000). It is thought that the main function of these enzymes is the reoxidation of an excess of reducing power rather than the generation of an electrochemical gradient over the membrane. It is unknown whether sulfur reduction is coupled to a membrane-bound electron transport chain.

All the organisms described so far in this report grow at near-neutral pH. Polysulfides as soluble sulfur derivatives are stable under these conditions and available in sufficient concentration for growth (Schauer & Kröger, 1993). It has been speculated that these sulfur derivatives are also the true substrates for the SR in hyperthermophiles. However, polysulfides rapidly dissociate to sulfur and H2S under conditions supporting the growth of extreme acidophiles like Acidianus, suggesting that S is the actual substrate of the enzyme (Ringel et al., 1996). In addition, there is a steep pH gradient over the membrane. In the related aerobic archaeon Sulfolobus acidocaldarius, the cytoplasmic pH is 6.5 and it is assumed that this should also be true for A. ambivalens (Moll & Schäfer, 1988). In consequence, it would be interesting to learn whether the proteins forming the Acidianus electron transport chain for sulfur reduction are different from known systems or modulations of a common theme and whether the genes differ significantly from what is known for Wolinella.

We have described a protocol for the purification of the membrane-bound hydrogenase and SR from A. ambivalens and also from Thermoproteus neutrophilus (Laska & Kletzin, 2000). Here we describe the first combined biochemical and molecular characterization of the membrane-bound hydrogenase and SR from an archaeon. The data are used to develop a phylogeny of molybdoenzymes oxidizing or reducing inorganic sulfur compounds and a model of sulfur respiration in Acidianus.

**METHODS**

**Growth of A. ambivalens.** A. ambivalens (DSM 3772) was grown at 75 °C in a 15 l fermenter according to published procedures in mineral medium without addition of organic substrates under anaerobic conditions with a H2/CO2 gas phase (Zillig et al., 1986).

**Cell fractionation, purification and enzyme assays.** The lysis and the cell fractionation of A. ambivalens cells, the purification of the hydrogenase and SR, and the enzyme assays were performed essentially as described by Laska & Kletzin (2000). The subunit composition of the hydrogenases was determined by ‘two-dimensional’ gel electrophoresis. For the first dimension, active fractions from the density gradient were applied to a blue native PAGE gel (Schaäger, 1994). The hydrogenase was detected after electrophoresis by activity staining of the gel. The gel was incubated in a solution of 5 mM methyl viologen (MV) and 2.5 mM triphenyltetrazolium chloride in 100 mM KPi (pH 8.0) at 75 °C under a hydrogen atmosphere until red-coloured bands appeared (Kletzin, 1994). After excision from the gel and electroelution, the resulting protein solution was concentrated by ultrafiltration (Nanosep and Microsep; Pall Gelman), denatured and loaded on an SDS gel in the second dimension.

Western blotting was performed using standard procedures (Lottspeich & Zorbas, 1998) and a polyclonal antiserum against the Pyrococcus abyssi hydrogenase/SR complex was used. This antiserum was kindly provided by R. Dirmeier (Regensburg, Germany). The detection reaction was performed using horseradish-peroxidase-linked anti-rabbit IgG and the ECL detection reagents (Amersham Pharmacia). In addition, a polyclonal serum against the W. succinogenes PSR was used. The serum was kindly provided by O. Klümmer & A. Kröger (Frankfurt, Germany).

Hydrogenase activity was measured by the reduction of MV as described by Laska & Kletzin (2000). In addition, the reduction of horse heart cytochrome c (50 μM) was followed spectrophotometrically at 550 nm and at 75 °C under a hydrogen atmosphere. The reduction of NAD+ and NADP+ (0.5 mM each) was recorded photometrically at 340 nm and 75 °C under a hydrogen atmosphere. The oxidation of NADH and NADPH was followed under the same conditions under a nitrogen atmosphere. For the calculation of the enzyme activities the following specific absorption coefficients ε were used: MV, 12,000 M⁻¹ cm⁻¹; benzyl viologen (BV), 9000 M⁻¹ cm⁻¹;
2,3-dimethyl-naphthoquinone (DMN), 15 000 M⁻¹ cm⁻¹. The pH, temperature and salt profiles of hydrogenase activity were recorded with MV as electron acceptor and hydrogenase fractions enriched over density-gradient centrifugation. The temperature profile was measured in 100 mM KPi buffer (pH 8-0). The salt dependence of activity was measured in 100 mM KPi (pH 8-0) with 0–2-5 M NaCl in 0-5 M increments. Three different buffer substrates were used for the pH profile (100 mM each): pH 5-5–6-5, KPi; pH 6-5–8-5, KOH/EPPS; pH 8-5–13-5, KOH/glycine.

The SR activity was routinely determined by incubating samples with sulfur-containing buffer under hydrogen atmosphere for 45 min as described by Laska & Kletzin (2000). Electron carriers and reductants were used in the concentrations given in Table 1 (see later). Hydrogen sulfide was determined colorimetrically with the methylene blue method (King & Morris, 1967; Kletzin, 1989). PSR activity was determined at 75 °C with DMN as artificial electron acceptor according to published procedures (Fauque et al., 1994; Schroeder et al., 1988).

For the inactivation of quinones in intact membranes the washed membrane fraction was irradiated with UV light (256 nm) for 60 min in an anaerobic chamber as described by Pihl et al. (1992). For the reconstitution of the SR activity in the UV-treated membranes, H₂S production was assayed in the presence of 200 μM DMN or 100 μM cytochrome c. For a control, the SR activity was determined with untreated membranes in the presence of DMN or cytochrome c and with irradiated membranes without additional electron carriers.

**Analytical procedures.** A metal analysis of *A. ambivalens* solubilized membrane fraction and from partially purified enzyme fractions was performed by X-ray fluorescence spectroscopy (TXRF-analysis) with an EXTRAIIA instrument (Atomika Instruments) at the Institute of Inorganic and Analytical Chemistry in Frankfurt/Main (Germany). Cytochrome spectra (dithionite-reduced minus oxidation) with an EXTRAIIA instrument (Atomika Instruments) was determined. For the determination of the N-terminal amino acid sequences of the 41 kDa subunit of the hydrogenase was determined by H. Wilson (1990). A size-selected genomic DNA library was constructed of the 41 kDa subunit by PCR (oligonucleotides hynS1, GTKAA TATTG TKTGG TTYGA; and hynS2-rev, GGATC KGTKG CTTGW ATWAT). The amplified fragment was ligated into the TA cloning vector pCR2.1 (Invitrogen), transformed into Escherichia coli XL-1 Blue cells (Stratagen), and sequenced. The fragment was labelled with digoxigenin for the identification of the hydrogenase gene cluster by PCR (Roche Diagnostics) and used for Southern and colony hybridization with T4 polynucleotide kinase according to the manufacturer’s recommendations followed by phenol/chloroform extraction and ethanol precipitation. Modified DNA (200 ng) was ligated into the cosmid vector pXPII without previous size selection according to the manufacturer’s protocol. Two microtites of the ligation reaction was packaged in vitro into lambda heads using the packaging extract supplied with the kit (the cloning capacity of the vector is 16–26 kbp). After transfection of *E. coli* DH5α cells with the phage particles and plating on ampicillin-containing LB agar plates, 560 colonies were screened by colony hybridization with the 71 bp hynS probe leading to the identification of the cosmid clone cHyd-2.

**Sulfur reductase.** The N-terminal amino acid sequences of four peptide fragments of the 110 kDa subunit of the SR were determined after proteolytic cleavage with the endoproteinase Lys-C and separation of the fragments using HPLC. Using the sreA 110_34 (++) (GCKTT TACKG AYGTN CA) and sreA 110_44 (−) (GTATC TTGKG TTTGN ACYTG) oligonucleotides derived from two different N-terminal amino acid sequences from Lys-C fragments of the protein, an 827 bp fragment of the sreA gene encoding the 110 kDa subunit of the SR was amplified, cloned and sequenced. The fragment was labelled with digoxigenin by random priming and used for Southern and colony hybridization of the cosmid library according to standard procedures, leading to the identification of the cosmid clone cSR4 (Sambrook et al., 1989).

For the determination of the nucleotide sequences of the partially overlapping plasmid and cosmid inserts (see later, Fig. 4), fragments were subcloned using different strategies. Suitable 2–7 kbp fragments of cHyd-2 were subcloned into pBluescript II SK− (Stratagen). From the plasmid constructs various ‘deletion clones’ were constructed by deleting subfragments specifically with suitable restriction endonucleases, relaxation of the plasmid, and transformation of *E. coli* Novablu cells (Novagen). In addition, a shotgun library of a 9770 bp Swl subfragment of cSR4 was constructed by partial digestion of the fragment with the restriction endonuclease Trul (MBI Fermentas) and subsequent cloning of 1–2 kb large DNA fragments in the plasmid vector pGemT (Promega). The nucleotide sequences were determined by cycle sequencing using the SequiTherm ExcellI DNA Sequencing Kit-LC (Epicentre Technologies), standard primers, and a LICOR automated sequencer according to the manufacturer’s recommendations. Gaps in the assembled sequences were closed by the synthesis of specific primers, PCR-amplification of the missing fragments, cloning of the amplified products into pGemT, pStBlue or pCR2.1 (Invitrogen) vectors and sequencing. The nucleotide sequences of the cosmid ends were determined using the T7 and a modified M13 − 20 sequencing primer (CCCGT AAAAC GACGG CCAGT) at a concentration of 20 pmol and 2500 ng cosmid DNA as template in the reaction mixture.

**Sequence analysis and phylogeny programs.** The assembly and analysis of the sequences was performed with the Wisconsin package (GCG; Accelrys, Untheraching, Germany) and additional programs available at the ‘Heidelberg UNIX Sequence Analysis Source’ server (HSV, German Cancer Research Center). For phylogenetic analysis the six to eight top hits from aBLAST search of the database were used. The sequences were aligned using PILEUP (GCG) and the alignment was manually corrected. The resulting multiple alignment was fed into HMMERsearch and HMMERalign to create and compare a hidden Markov model. The model was used to align 50–70 top hits from the BLAST searches with HHMalign. The resulting HMM-based multiple alignment was again manually corrected and afterwards shortened to the approximately 300 (SreA), 130 (SreB), 250 (HynL) or 130 (HynS) most conserved positions. With these, phylogenetic analyses were performed using the programs DISTANCES, GROWTREE, PAUPSEARCH and PAUPDISPLAY from the GCG package. The hydrogenase and SR subunits deduced from the genome sequence of
Acidithiobacillus ferrooxidans obtained from TIGR prior to publication were also included in the analysis. The two sub-sequences of the large subunit of the Pyrobaculum aerophilum SR homologue resulting from a frame-shift in the genomic nucleotide sequence were treated as a single polypeptide (GenBank identifiers gi 18313625 and 18313626).

RESULTS

Subunit structure of the hydrogenase and sulfur reductase

The membrane-bound hydrogenase, the SR and the soluble hydrogenase from anaerobically grown cells of *A. ambivalens* were purified by density-gradient centrifugation and column chromatography as described by Laska & Kletzin (2000). After SDS-PAGE of active hydrogenase and SR fractions, four distinct bands with molecular masses of 110, 66, 41 and 29 kDa were visible on SDS gels (Fig. 1). However, the preparation was not electrophoretically homogeneous. Three further bands with apparent molecular masses of 53, 42 and 22 kDa appeared on the gels in less intensity. The hydrogenase could be further purified leading to preparations which were free of SR activity but not vice versa. SR-free hydrogenase preparations lacked the 110 kDa band in SDS gels. The hydrogenase purified from the soluble fraction had bands with apparent molecular masses of 66 and 41 kDa in SDS gels (not shown).

The active fraction after density-gradient centrifugation was subjected to a blue native PAGE. The hydrogenase band was excised after detection of the enzyme using activity staining. After separation by SDS-PAGE and silver staining, five major bands were visible with apparent molecular masses of 66, 41, 29, 22 and 13 kDa, as well as several minor bands (Fig. 1). Again, the hydrogenase from the soluble fraction showed bands with apparent molecular masses of 66 and 41 kDa with this technique.

Western blotting experiments using antiserum against the *Pyrodictium abyssi* hydrogenase/SR complex resulted in a very faint cross-reaction with the 41 kDa subunit of the hydrogenase. Other subunits did not hybridize (data not shown). Western blotting using antiserum against the *W. succinogenes* PSR gave no cross-reaction.

The membrane-bound hydrogenase is quinone-specific

The hydrogenase activities were routinely measured with MV or BV as electron acceptors. Membrane-bound hydrogenase also exhibited activity with the artificial quinone DMN. The specific hydrogenase activity of density gradient fractions prepared from the solubilized membrane fraction was 138·7 U (mg protein)$^{-1}$ with MV, 567 U mg$^{-1}$ with BV, and 345·8 U mg$^{-1}$ with DMN as electron acceptor. The purified hydrogenase had a specific activity of 114 U mg$^{-1}$ with MV and of 624 U mg$^{-1}$ with DMN. It was previously shown that H$_2$ is evolved from reduced MV with specific activities of 0·19 U (mg protein)$^{-1}$ in the soluble and 1·9 U mg$^{-1}$ in the membrane fraction (Kletzin, 1994). The purified enzyme did not show SR activity with hydrogen or NADPH as electron donors.

The specific MV-dependent hydrogenase activity in the soluble fraction was 11·9 U (mg protein)$^{-1}$. DMN was not

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Fig. 1. Preparative blue native polyacrylamide gel of the *A. ambivalens* hydrogenase before (a) and after (b) activity staining in the gel. (c) Silver-stained SDS gel of the membrane-bound hydrogenase eluted from a blue native gel. M, Molecular mass standard; arrows indicate the 66, 41, 29, 22 and 13 kDa bands. (d) SDS-polyacrylamide gel of different purification steps of the membrane-bound hydrogenase and SR preparation of *A. ambivalens*. M, Marker proteins; DG, fractions after density-gradient centrifugation; D, fractions after DEAE chromatography. Subunits confirmed by N-terminal sequencing are shown in bold.
reduced. Both enzymes reduced BV but neither NAD\(^+\), NADP\(^+\), FMN nor cytochrome c was reduced. Neither enzyme oxidized NADH or NADPH. The hydrogenase enriched from membrane fractions had the highest activity at pH 10.5 (data not shown). Below pH 6.0 no activity was recorded. The temperature optimum exceeded 90 °C.

**Table 1.** SR activity with various electron donors and carriers in solubilized membrane and UV-treated intact membrane fractions

<table>
<thead>
<tr>
<th>Addition of solubilizate</th>
<th>Gas phase</th>
<th>Electron carrier and reductant</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>100 μM</td>
<td>1 mM</td>
</tr>
<tr>
<td>+</td>
<td>H(_2)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>–</td>
<td>H(_2)</td>
<td>NADPH, Ti-citrate</td>
<td>≤1</td>
</tr>
<tr>
<td>–</td>
<td>H(_2)</td>
<td>NADPH, NaBH(_4)</td>
<td>≤1</td>
</tr>
<tr>
<td>+</td>
<td>N(_2)</td>
<td>NADPH</td>
<td>≤1</td>
</tr>
<tr>
<td>+</td>
<td>H(_2)</td>
<td>Cytochrome c</td>
<td>100</td>
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<tr>
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<td>H(_2)</td>
<td>Cytochrome c</td>
<td>≤40</td>
</tr>
<tr>
<td>+</td>
<td>H(_2)</td>
<td>Ubiquinone</td>
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<tr>
<td>+</td>
<td>H(_2)</td>
<td>Ubiquinol(^*)</td>
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<tr>
<td>+</td>
<td>H(_2)</td>
<td>Plastoquinone</td>
<td>59</td>
</tr>
<tr>
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<td>H(_2)</td>
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<td>+</td>
<td>H(_2)</td>
<td>Plastoquinol(^†)</td>
<td>66</td>
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<td>Plastoquinol(^†)</td>
<td>28</td>
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<td>N(_2)</td>
<td>Cytochrome c</td>
<td>3</td>
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<td>N(_2)</td>
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<td>≤1</td>
</tr>
<tr>
<td>+</td>
<td>N(_2)</td>
<td>DMN</td>
<td>≤1</td>
</tr>
</tbody>
</table>

**Reconstitution of SR activity in UV-treated membrane fractions**

| UV | H\(_2\) | Cytochrome c | NADPH | 8 |
| UV | H\(_2\) | DMN          | NADPH | 45 |

\(^*\)Reductant titanium citrate.

\(^†\)Reductant NaBH\(_4\).
No activity was detectable below 40 °C. No significant differences in hydrogenase activity were found at NaCl concentrations in the range between 0 and 2.5 M. The purified hydrogenases did not show SR activity with hydrogen or NADPH as electron donors. The hydrogenases were insensitive to the presence of oxygen during the purification procedure, and also they did not show a lag or activation phase in the enzyme assay.

**Sulfur reductase activity depends on electron carriers**

SR activity was routinely measured under a hydrogen atmosphere in an assay mixture of sulfur powder in a detergent-containing buffer. In the intact membrane fraction, the specific SR activity was between 2 and 16 U (mg protein)^{-1} depending on the individual batch of cells used for the purification of the enzyme (not shown). In contrast, in the solubilized membrane fraction no activity was found without the addition of electron carriers to the reaction mixture. Upon addition of DMN, plastoquinone, plastoquinone or horse heart cytochrome c the activities were up to 18 U (mg protein)^{-1} when using a hydrogen atmosphere (Fig. 2, Table 1). The addition of NADPH increased the activity (Fig. 2, Table 1). With isolated Caldariellaquinone or ubiquinone no SR activity was measurable. Neither of the fractions had PSR activity (Fauque *et al.*, 1994). Under a nitrogen atmosphere a specific activity of up to 8 U (mg protein)^{-1} was measured in the presence of electron donors like plastoquinone or NADPH but not of NADP^+ (Table 1, Fig. 2).

UV-irradiation of the membrane fraction resulted in a decrease of the specific SR activity to 2.2 U (mg protein)^{-1}, 8% of the activity in the untreated fraction. After addition of DMN to the reaction mixture containing the irradiated membrane fraction a specific activity of 13 U (mg protein)^{-1} was observed. The activity with cytochrome c instead of DMN was again 2 U (mg protein)^{-1}. No SR activity was observed below pH 6.0. The specific activities gradually increased up to pH 8.0. Above pH 8.0, the rate of non-enzymic sulfur disproportionation reactions, barely measurable at pH 7.5, was higher than the enzymatic rate.

**Iron, nickel and molybdenum**

The solubilized membrane fraction contained 2.5 μmol Mo besides 134 μmol Fe and 2.5 μmol Ni (g protein)^{-1}. A hydrogenase preparation enriched by sucrose density-gradient centrifugation contained 14 μmol Fe and 2 μmol Ni (g protein)^{-1}. The W content was below the level of detection. The amounts of the purified enzymes were too low to detect any trace metals. Haem staining of the solubilized membrane fraction, the soluble fraction or enriched proteins separated by SDS-PAGE did not give any result, showing that c-type cytochromes were not present.

**N-terminal amino acid sequences**

The N-terminal amino acid sequence of the 41 kDa subunit of the membrane-bound hydrogenase was VNIIVW FEAQA XEGNT TAIQA TDPX. Attempts to determine the N-termini of the 66 and 29 kDa subunits of the membrane-bound and of the 66 and 41 kDa subunits of the soluble enzyme were not successful.
The N-terminus of the 110 kDa SR subunit was blocked. The N-terminal sequences determined for four different peptide fragments after proteolytic cleavage were LCARG RSGTL ITYNK (110_28), AFTDV QSQII AQK (110_34), ATPDE ISEYT QLAQV QTQDT (110_44) and AAAAF ALFPD AGIYX AMQGQ (110_62).

Cloning of the genes and sequence analysis

A plasmid with a 7 kbp insert (pHyd-7) and a cosmid with a 22 kbp insert (cHyd-2) were identified in genomic A. ambivalens plasmid and cosmid libraries, respectively, using a 71 bp probe amplified with degenerate oligonucleotides derived from the N-terminal amino acid sequence of the 41 kDa hydrogenase subunit (Fig. 3). Degenerate oligonucleotides derived from the N-terminal amino acid sequence of the 110_34 and 110_44 fragments were used to amplify an 827 bp genomic DNA fragment. Using this fragment as a probe, the cosmid cSR4 with a 19 kbp insert was identified (Fig. 3). Sequencing of the cosmid ends showed that cSR4 overlapped partially with the hydrogenase-encoding plasmid and cosmid inserts pHyd-7 and cHyd-2.

A total of 28–614 bp of DNA sequence from cHyd-2, pHyd-7 and cSR4 were determined (accession nos AJ320523 and AJ345004). They encoded a hydrogenase gene cluster 10 kbp in length containing 12 ORFs and the SR gene cluster 7–5 kbp in length containing five ORFs. The hydrogenase and SR gene clusters were 9–5 kbp apart with 14 unrelated genes separating them. Southern hybridization of genomic DNA digested with several restriction enzymes and hydrogenase- or SR-specific probes resulted in single bands, showing that no second copy of these or highly similar operons was present in the genome.

The hydrogenase gene cluster: 12 genes encoding a NiFe hydrogenase

The gene cluster started with the hynS gene encoding the 41 kDa subunit of the hydrogenase (Fig. 3 and Table 2). The N-terminal amino acid sequence determined from the purified subunit matched exactly the deduced amino acid sequence of the hynS ORF (position 41–64 of 421 aa). A twin-arginine motif was found in the 40 aa leader peptide sequence. The deduced amino acid sequence of hynS shared 41% identical residues with the small subunit of an uncharacterized hydrogenase from Streptomyces avermitilis, and up to 39% to small subunits of other bacterial Group I uptake hydrogenases (Table 2; Vignais et al., 2001). The previously unassigned N-terminus of the 42 kDa subunit of the Pyrodictium abyssi hydrogenase/SR complex (Dirmeier et al., 1998) matched in the HynS alignment at a similar position as the A. ambivalens N-terminus (not shown).

The hynS ORF was followed by the ORFs isp1 encoding a hypothetical membrane protein (269 aa) and isp2 encoding a hypothetical FeS protein (454 aa; Table 2, Fig. 3). The gene hynL was located 53 bp downstream of isp2. The deduced amino acid sequence (621 aa) showed 39% identical amino acid residues to the large subunit of the hypothetical Streptomyces avermitilis hydrogenase and 25–36% identical residues to other NiFe uptake hydrogenases from bacteria like Helicobacter pylori and Ralstonia eutropha. A phylogenetic tree reconstruction using different algorithms was performed, which included the unpublished hydrogenase sequences from Acidithiobacillus ferrooxidans (Fig. 4). Both the HynL (not shown) and the HynS subunits of the Acididnus paired with the Streptomyces avermitilis hydrogenase. They form a deeply rooting branch in Group I of the NiFe hydrogenases (Fig. 4; Vignais et al., 2001). Ten out of 17 residues of the N-terminus of the 66 kDa subunit of the Pyrodictium abyssi hydrogenase/SR complex were identical to the Acididnus HynL sequence (not shown).

Immediately downstream of hynL an ORF encoding a small Rieske ferredoxin (113 aa) was found, sharing 40% identical residues with a similar protein from Pseudomonas putida and E. coli (hynl; Fig. 3, Table 2). The four ORFs h ypD, hypC, hypE and hoxM encoded putative proteins with significant similarities to hydrogenase maturation proteins from R. eutropha and E. coli (Hyp; Table 2). HypA, B and F homologues were absent. For the remaining ORFs database matches were not found. A gene cluster, tatABC,
### Table 2. Properties of the amino acid sequences deduced from the *A. ambivalens* hydrogenase and SR gene clusters, similarities of the deduced amino acid sequences and inferred functions

<table>
<thead>
<tr>
<th>Gene</th>
<th><em>A. ambivalens</em> deduced proteins</th>
<th>Homologues/most similar proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length of ORF (aa)</td>
<td>Deduced molecular mass (Da)</td>
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<tr>
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<tr>
<td>sreE</td>
<td>178</td>
<td>21 562</td>
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*By similarity.
†Hypothetical: calculated without the peptide presumably cleaved from the C-terminal end (by similarity).
‡A. Kletzin, unpublished results.
§Most similar excluding *Sulfolobus solfataricus* Dsr proteins.
||Second percentage/accession number from *S. solfataricus* putative Sre (=Dsr) proteins.
encoding membrane proteins required for the TAT pathway of protein translocation was found 306 bp upstream of hynS and translated in the opposite direction. Using the amino acid sequences of the A. ambivalens hydrogenase gene cluster as BLAST probes, no homologous genes were found in the genome sequences of the crenarchaeotes Sulfolobus solfataricus, Sulfolobus tokodaii and Aeropyrum pernix.

Sulfur reductase: similarity to molybdoproteins

The SR gene cluster encompassed five ORFs with a total length of 7389 bp (sreABCD; Fig. 3). It started with the sreA gene encoding the 110 kDa subunit of the SR (1018 aa). The four N-terminal amino acid sequences determined from the protease LysC fragments matched exactly the deduced amino acid sequence of the sreA ORF (not shown). A typical twin-arginine motif was present in the amino acid sequence of SreA at position 14–19. The deduced amino acid sequence showed 25–39 % identical positions to molybdopterin oxidoreductases of the DMSO reductase family from various archaea and bacteria (Table 2, Fig. 5) and 56 % to a 1050 aa ORF from Sulfolobus solfataricus (see later). The previously unassigned N-terminus of the Pyrococcus abyssi 85 kDa subunit of the hydrogenase/SR complex (Dirmeier et al., 1998) could be aligned to the A. ambivalens SreA sequence, albeit with low similarity and confidence (not shown). The sreA gene was followed by sreB, encoding an FeS protein with 18 cysteine residues and 35–48 % identity to the β-subunits of various molybdeno-enzymes from the DMSO reductase family (227 aa; Table 2, Fig. 3). The N-terminus of the Pyrococcus abyssi 24 kDa subunit of the hydrogenase/SR complex matched the other SreB proteins in a multiple alignment (not shown).

The following ORF, sreC, encoded a hydrophobic protein with 10 predicted transmembrane helices (464 aa). The sreD gene encoded a putative hydrophilic FeS protein (473 aa) with 26 cysteine residues and of unknown cluster composition. The sreE gene encoded a protein (178 aa) with similarity to reductase assembly proteins (chaperones; TorD, COG3381.1, and NarJ, COG2180.1) required either for the assembly of the Mo-containing large subunit of DMSO reductase or nitrate reductase (Blasco et al., 1998; Ray et al., 2003) or for binding to the TAT signal sequence (Oresnik et al., 2001).

A highly similar gene cluster was found in the genome of Sulfolobus solfataricus P2 (Table 2; She et al., 2001). The five genes of the Sulfolobus cluster were arranged in the same order as in A. ambivalens. The deduced amino acid sequences of the Sulfolobus proteins shared 50–80 % identical residues with A. ambivalens and contained all of the putative metal-binding amino acid residues.

To determine whether the A. ambivalens SR shares common sequence motifs with other molybdoenzymes, the A and B subunits were aligned with 62 paralogous and orthologous sequences from the DMSO reductase/formate dehydrogenase family. The SreA sequences differed considerably in length (mean sequence length 860 aa, range 657–1426 aa). Using a subset of eight sequences as a training set, the alignment procedure using HMMERalign resulted in the identification of conserved regions in SreA of a total length of 280–320 aa residues separated by very long gaps. In SreB, 130 residues were identified (mean length 256 aa, range 182–531). The calculated dendrograms were reasonably stable with these aligned and conserved regions, when using different algorithms or variations of these parameters. The Acidimitus and Sulfolobus SR sequences always branched with the putative PSR or SRs from Pyrococcus aerophilum, Archaeoglobus fulgidus, Chlorobium tepidum and Acidithiobacillus ferrooxidans. In a related subclade sharing a common root, the W. succinogenes PSR, the Salmonella typhimurium thioulate reductase and the Azorarcus evansii phenylacetate-CoA reductase were grouped. The Rhodovulum DMSO reductase, the Thauera selenatis selenate reductase and the Salmonella tetrahydrogenate
reductase were grouped in another subclade with a deeper gap, but all of them shared a common root separating them from most of the DMSO reductases, formate dehydrogenases and nitrate reductases (Fig. 5). A multiple alignment and a dendrogram calculation using the putative chaperone SreE with TorD, DmsD, NarJ and related proteins gave essentially similar results. The Archaeoglobus (GenBank accession no. NP_069012) and the Chlorobium homologues were the most similar (NP_661393; <30% identity) and the nitrate reductase chaperones NarJ and relatives the most distant.

**DISCUSSION**

**Hydrogenase and sulfur reductase subunits and activities**

In this report, we describe the purification and the molecular characterization of a hydrogenase and an SR from membrane fractions of the hyperthermophilic and acidophilic archaean *A. ambivalens* grown under anaerobic conditions. Hydrogenase activity was also found in the cytoplasmic fraction; however, only the hydrogenase from the membrane fraction reduced the artificial quinone...
DMN. The electrophoretic mobilities of the two major subunits found both in the membrane and cytoplasmic fractions were nearly identical (66 and 41 kDa). The soluble hydrogenase was probably liberated during disintegration of the cells and the washing procedure, thereby losing the membrane anchor and the ability to reduce DMN.

Active hydrogenase could be purified at room temperature without protection from oxygen. An activation effect in the enzyme assay was not observed (e.g. a lag phase; not shown). This remarkable stability might be due to the ability of the organism to grow both aerobically and anaerobically. The high optimum pH of the enzyme cannot be explained presently. It might be an effect of the interaction between hydrogenase and SR or the substrate affinity of the artificial viologen dyes for the enzyme. Similar observations have been made with the only other hydrogenase purified from an acidophile, *Acidithiobacillus ferrooxidans* (Fischer et al., 1996).

The SR reduced elemental sulfur to hydrogen sulfide with H₂ and, with a reduced activity, with NADPH₂ as electron donors, as long as hydrogenase was present. No additional electron carrier was necessary when intact membrane fractions were used. After washing and membrane solubilization, the addition of external electron carriers, like horse heart cytochrome c or quinones, was necessary to reconstitute full enzymic activity [with both ≈ 29 U (mg protein)⁻¹]. We concluded that the membrane-associated physiological electron carriers were lost during the washing and solubilization step. The addition of NADPH increased the specific activity. The same enzyme assay performed under a N₂ atmosphere resulted in lower but recognizable activities when NADPH or plastoquinone was added as reductant. The most likely explanation for these results is that the observed SR activity is due to an electron transport reaction from hydrogen to sulfur mediated by hydrogenase and SR and not equivalent to a sulfoxidase (NADPH : sulfur oxidoreductase) reaction like that observed with the soluble hydrogenases from *Pyrococcus furiosus* (Ma et al., 1993). The effect of NADPH might be due to a NADPH : acceptor oxidoreductase activity not identified so far.

No evidence for c-type cytochromes was found in spectra of soluble or membrane fractions of the organism (this study) or in other *Sulfolobales* (Schäfer et al., 1996). In anaerobically grown *A. ambivalens* cells, only b-type cytochromes were found in low-temperature spectra (Gomes & Teixeira, 1998). UV-irradiated membrane fractions showed approximately 8% of the activity of untreated membranes, pointing to the involvement of quinones instead of cytochromes in the electron transfer. The activity could be partially reconstituted by adding DMN or plastoquinone to the mixture. The inactivity of the SR with Caldariellaquinone might be explained by its low solubility or by the finding that it is usually not found in anaerobically grown *A. ambivalens*. A derivative of Sulfolobusquinone, which has a slightly different structure (Trincone et al., 1989; a Sulfolobusquinone preparation was not available), is present in *A. ambivalens*. Taken together, the results indicate that the electron transfer between hydrogenase and SR is mediated by quinones, most probably Sulfolobusquinone (Fig. 6). The activity mediated with cytochrome c is most probably unspecific due to the high isoelectric point (10.3) of the molecule leading to unspecific direct interaction between the hydrogenase and the SR. These results contrast the findings for *Pyrodictium abyssi* where cytochromes b and c, but not quinones, are involved in electron transport from hydrogenase to the SR (Dirmeier et al., 1998).

### Hydrogenase gene cluster and sequence analysis

The 12 genes of the hydrogenase gene cluster encoded both structural proteins (e.g. HynS, HynL and Isp1) and some of the maturation proteins required for the assembly of NiFe hydrogenases (HypC, D and E, Fig. 3; see, for example Robson, 2001b). No close relatives of *Acididunus* HynS and HynL were found in the database searches. The highest pairwise identities were approximately 40%. The phylogenetic analysis showed that amino acid sequences were most similar to those of bacterial Group I NiFe hydrogenases (Vignais et al., 2001) and less similar to those of other bacteria, but not to archaea (with only one exception, *Archaeoglobus fulgidus* Vht; Table 2, Fig. 4). The HynL sequence contained the Ni-binding cysteine residues and the processing site for the HoxM/HupD protease required for Ni incorporation (e.g. Fritsche et al., 1999). The protease that putatively cleaves HynL is encoded by a gene within the hydrogenase gene cluster, which encodes additional maturation proteins (Fig. 4). Three proteins absolutely required for the maturation of NiFe hydrogenases were missing in the cluster (HypF required for CO and/or CN generation and HypA and HypB required for nickel acquisition; Robson, 2001b). However, their genes are not necessarily linked to the other genes for maturation proteins so that they might be encoded in a different locus. Taking these results together with the metal analysis, it seems reasonable to deduce that the enzyme is of the NiFe type.

The leader sequence of HynS is cleaved post-translationally. A twin-arginine motif was found within this leader, which is similar to that of most of the membrane-bound or periplasmic hydrogenases. This suggests that the entire protein is located on the outer side of the membrane, as in *W. succinogenes* (Gross et al., 1999; Wu et al., 2000). It was intriguing to find a *tatABC* gene cluster encoding the proteins required for the twin-arginine protein translocation pathway just upstream of the hynS gene, oriented in the opposite direction. The *Acididunus* HynS subunit also has a C-terminal putative transmembrane helix demonstrated by secondary structure prediction programs. This helix is similar to the one from HynA, the homologous FeS subunit of the *W. succinogenes* hydrogenase (Gross et al., 1998).

Nine of ten typically conserved cysteine residues were found...
in HynS. One normally conserved histidine residue was replaced by an asparagine (confirmed by the N-terminal amino acid sequence, see earlier). From the crystal structure of bacterial hydrogenases it is known that this cysteine residue coordinates one Fe in the proximal of three different FeS clusters (Fontecilla-Camps et al., 2001; Garcin et al., 1998; Volbeda et al., 1995). Some hydrogenase genes found in the genomes of the bacteria Acidithiobacillus ferrooxidans, Streptomyces avermitilis and some cyanobacteria showed similar replacements. At present, it is not known how this replacement affects the cluster composition but it suggests that the proximal FeS cluster is changed from 4 to 3Fe.

The protein encoded by the isp1 gene has a deduced molecular mass of 30 729 Da and five transmembrane helices can be predicted. A detailed sequence comparison showed a distant but recognizable similarity to the haem-b-containing membrane anchors of respiratory nitrate reductases NarI and NarV (Dahl et al., 1999). When the sequences were aligned so that the predicted transmembrane helices matched, the proteins shared four conserved histidine residues, along with other conserved residues (not shown). As discussed earlier, the presence of the 29 kDa subunit is required for the reduction of DMN by the Acidianus hydrogenase. These results provide additional evidence that the membrane anchor of the hydrogenase from the membrane fraction is indeed the 29 kDa subunit seen in SDS gels and might contain the b-type haems found in low-temperature spectra (Gomes & Teixeira, 1998). The role of the Isp2 protein is not known. Homologous genes showing the same unusual arrangement (hynS–isp1–isp2–hynL) were present in the gene clusters of the stable hydrogenase of the phototrophic, mesophilic bacteria Thiocapsa roseopersicina (Rakhely et al., 1998) and Allochromatium vinosum (Dahl et al., 1999), in hydrogenase 2 of E. coli (Richard et al., 1999), and in the hyperthermophilic bacterium Aquifex aeolicus, but not in archaea. This is a deviation from the typical arrangement, since in most cases the hyn/hyd/hupSL genes are adjacent. It is also interesting to note that the enzymes with the same operon organization are not the closest relatives to the Acidianus enzyme in the phylogenetic analysis (Fig. 4), pointing to a rather flexible exchange of the subunit genes. It can be concluded that the Acidianus enzyme is in its structure and molecular properties more similar to bacterial than to most archaeal hydrogenases.

**Sulfur reductase gene cluster and sequence analysis**

SreA encoding the 110 kDa subunit of the SR and SreB showed a high degree of sequence similarity to Mo–FeS enzymes of the DMSO reductase family. In the deduced amino acid sequence of SreA, four cysteine residues were

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**Fig. 6.** Hypothetical model of sulfur respiration in A. ambivalens and W. succinogenes including putative redox-active centres and the proposed redox loop mechanism of proton translocation (drawn after Hedderich et al., 1999; Jormakka et al., 2002). Homologous subunits are shown in the same shading. OM, Outer membrane; CM, cytoplasmic membrane.
conserved. These could potentially coordinate a [4Fe–4S]-cluster, as in oxidoreductases for which crystal structures are known, e.g. formate dehydrogenase and nitrate reductase, but not in DMSO reductase (Volbeda et al., 1995; Kisker et al., 1998; McMaster & Enemark, 1998). In addition, another cysteine residue was conserved, which was known to be involved in Mo coordination in these enzymes. We have found significant amounts of Mo in the spectroscopic analysis of the solubilized membrane fraction. It was concluded from both results that the SR is probably a molybdoenzyme. The sreC gene downstream of sreB encoding a putative hydrophobic 52 kDa protein apparently represents the membrane anchor of the SR. The twin-arginine motif in the leader sequence and the similarity of SreA and SreB to the molybdoenzymes suggest that the SR is located on the outer side of the membrane as is the case with the W. succinogenes PSR (Fig. 6; Hedderich et al., 1999). The function of SreD is not clear. The deduced amino acid sequence contained 26 cysteine residues similar to polyferredoxins from methanogenic archaea. The SreD, SreC and the Isp2 protein of the hydrogenase had similar molecular masses. Therefore, it could not be decided which of the proteins gave rise to the diffuse 52 kDa band observed in SDS gels (Fig. 1).

Comparison of the Acidiumus SR with the Pyrodictium nine-subunit hydrogenase/SR complex showed some clear differences between both enzymes, mostly in the structure of the complex and in the nature of the electron carrier. The Pyrodictium enzyme contains cytochrome c, whereas the Acidiumus SR and the Wolinella PSR are coupled to the respective hydrogenases using quinones. The comparison of the three enzymes also showed some clear similarities. From the limited sequence information of the Pyrodictium complex the homologous subunits could be identified (Dirmeier et al., 1998). The N-terminus of the 66 kDa subunit was already shown to be similar to bacterial and archaeal NiFe hydrogenases (Dirmeier et al., 1998). No match had been found for the other N-termini. In this study, we found that the Pyrodictium 85 kDa subunit was similar to SreA, however with only a few matching residues. The 24 kDa subunit was clearly similar to SreB. Therefore, these are most probably the catalytic and the FeS subunits of the Pyrodictium SR within the nine-subunit complex and contain a Mo or tungsten cofactor. Also, we could show that the 42 kDa subunit was similar to HynS. The faint cross-reaction with the 41 kDa subunit in the Western blotting experiment using an antiserum against the Pyrodictium abyssi hydrogenase/SR complex supported this result. Therefore, we concluded that the Pyrodictium hydrogenase/SR complex has the main constituents in common with Acidiumus but uses different electron carriers and adopts a different quaternary structure.

Several other enzymes are known in the DMSO reductase family of molybdopterin-containing proteins, which reduce inorganic or organic sulfur compounds. Using multiple alignment and phylogenetic dendrogram reconstruction, we tried to resolve whether a specific clustering of the large subunits of these enzymes within the DMSO reductase family exists. The background of 26–35 % sequence identity in pairwise comparisons between any of the SreA homologues showed that they share common features. Short conserved motifs were separated by stretches of high variability and different length without phylogenetic signal, preventing alignment tools like CLUSTAL W and PILEUP from giving reasonable results. We were successful only with HMM-based algorithms. However, for a successful tree reconstruction it was necessary to eliminate the variable regions from the alignment, leaving a set of approximately 300 residues, which are more or less conserved in all of the enzymes. The following phylogenetic analysis showed that all enzymes of the DMSO reductase family fall into several larger groups. It also showed that those enzymes oxidizing or reducing inorganic sulfur compounds group in a distinct clade, separated not only from formate dehydrogenases and nitrate reductases, but also from most of the DMSO/biotin sulfoxide reductases.

The function of the highly similar sre gene cluster found in S. solfataricus is not known. Hydrogenase genes were not identified in the genome, suggesting that S. solfataricus cannot gain energy by chemolithotrophic electron transport from H₂ to sulfur. However, there is a possibility that S. solfataricus can grow heterotrophically with sulfur respiration with organic hydrogen donors, although this has not yet been demonstrated. Attempts to grow S. solfataricus P2 anaerobically on sulfur and organic nutrients were made; however, these experiments were not successful (S. Laska & A. Kletzin, unpublished observations). No gene cluster showing the same arrangement of genes was found in the genomes of the crenarchaeota Sulfolobus tokodaii (NCBI accession no. NC_003106), Pyrobaculum aerophilum (NC_003364), Aeropyrum pernix (NC_000854) or of other micro-organisms.

From the results of this study, we propose that the Acidiumus hydrogenase and SR both possess subunits similar in structure and properties to their homologues from W. succinogenes (Fig. 6). The core enzymes are probably composed of at least three main structural proteins, a catalytic subunit, an iron–sulfur protein and a membrane anchor, the latter of which is phylogenetically unrelated to the analogous Wolinella proteins in both enzymes (Fig. 6). No bacterial counterparts are known for the additional A. ambivalens genes with the exception of Isp1 and 2 from the hydrogenase. The electron transport chain is also composed of these two enzymes, most likely connected via quinones. The net balance of protons derived from the chemical reaction on the outside of the cytoplasmic membrane is zero. Therefore, the generation of an electrochemical gradient with the proteins arranged as suggested would require a redox loop mechanism as proposed for the E. coli nitrate reductase/formate dehydrogenase system (Jones, 1980; Jormakka et al., 2002).
Protons would be taken up by Sulfolobusquinone upon reduction on the cytoplasmic side of the membrane and released to the outside upon reoxidation. At present, this model has not been proven experimentally but it fits with the data presented here. It will be the objective of future studies to resolve whether this is correct or not.

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