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Dissimilatory Oxidation and Reduction of Elemental Sulfur in Thermophilic Archaea

Arnulf Kletzin,[1,4] Tim Urich,[1] Fabian Müller,[1] Tiago M. Bandeiras,[2] and Cláudio M. Gomes[2,3]

The oxidation and reduction of elemental sulfur and reduced inorganic sulfur species are some of the most important energy-yielding reactions for microorganisms living in volcanic hot springs, solfataras, and submarine hydrothermal vents, including both heterotrophic, mixotrophic, and chemolithoautotrophic, carbon dioxide-fixing species. Elemental sulfur is the electron donor in aerobic archaea like *Acidianus* and *Sulfolobus*. It is oxidized via sulfite and thiosulfate in a pathway involving both soluble and membrane-bound enzymes. This pathway was recently found to be coupled to the aerobic respiratory chain, eliciting a link between sulfur oxidation and oxygen reduction at the level of the respiratory heme copper oxidase. In contrast, elemental sulfur is the electron acceptor in a short electron transport chain consisting of a membrane-bound hydrogenase and a sulfur reductase in (facultatively) anaerobic chemolithotrophic archaea *Acidianus* and *Pyrodictium* species. It is also the electron acceptor in organoheterotrophic anaerobic species like *Pyrococcus* and *Thermococcus*, however, an electron transport chain has not been described as yet. The current knowledge on the composition and properties of the aerobic and anaerobic pathways of dissimilatory elemental sulfur metabolism in thermophilic archaea is summarized in this contribution.

KEY WORDS: Sulfur oxygenase reductase; thiosulfate:quinone oxidoreductase; sulfite:acceptor oxidoreductase; heme copper oxidase; sulfur reductase; hydrogenase; Rieske ferredoxin; *Acidianus*; *Pyrodictium*; *Pyrococcus*.

INTRODUCTION

Hydrothermal vents, solfataras (Fig. 1), hot springs, and other habitats of volcanic origin are found in large numbers all over the world. They are populated by heat-adapted communities of bacteria and archaea despite the often extreme and seemingly adverse growth conditions (Barns *et al.*, 1994; Reysenbach *et al.*, 1994; Stetter, 1992). The production of biomass in these light-independent environments is energized by chemolithoautotrophic oxidation and reduction of inorganic compounds like elemental sulfur (S°), H_2, nitrate, various metal oxides and sulfides, and others (Amend and Shock, 2001; Schönheit and Schäfer, 1995). Heterotrophic microorganisms oxidize the biomass with oxygen or with the same inorganic compounds as electron acceptors.

Sulfur derivatives, mostly in the form of SO_2, are one of the most abundant components in volcanic gases, second in dry mass only to CO_2 (Montegrossi *et al.*, 2001; Stoiber, 1995; Symonds *et al.*, 1994). Other compounds usually present in minor but varying amounts are HCl, HF, S° vapor, H_2, N_2, CO, carbonyl sulfide (COS) and, especially in hydrothermal systems, H_2S (Stoiber, 1995; Xu *et al.*, 1998). The proportion of H_2S and SO_2 depend largely on the original gas composition, the rate of precipitation as sulfides and sulfates, and the thermodynamic equilibrium. Both can easily react with each other to form deposits of S° (SO_2 is usually more abundant in younger, active volcanoes; Holland, 2002; Stoiber, 1995). The direct precipitation from S° vapors and the oxidation of H_2S with metal ions in solution or with oxygen are other mechanisms of S° deposit formation (Steudel, 1996; Xu

[1] Institute of Microbiology and Genetics, Darmstadt University of Technology, Schnittspahnstraße 10, D-64287 Darmstadt, Germany.
[2] Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, R Quinta Grande 6, Apt 127, 2780 Oeiras, Portugal.
[3] Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2825-114 Caparica, Portugal.
[4] To whom correspondence should be addressed; e-mail: Kletzin@bio.tu-darmstadt.de.

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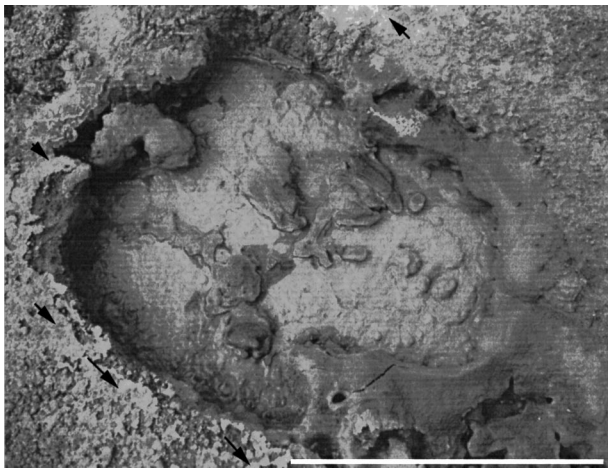


Fig. 1. Small boiling pool from Furnas solfatara field (São Miguel, Açores, Portugal), heated by a stream of hot gas from the underground. These small holes with the grayish or brown turbid liquid and S°-containing yellow precipitants (arrows) are typical habitats of the Sulfolobales. Bar: 10 cm, Photo: A. Kletzin.

et al., 2000). There are also sulfidic ores in the surrounding rock, which can be mobilized either chemically or by microbiological attack. As a consequence, S° and sulfur compounds are the most abundant sources both of electron acceptors and electron donors in volcanic environments and are used by a plentitude of microorganisms to support growth (Amend and Shock, 2001; Blöchl *et al.*, 1995; Schönheit and Schäfer, 1995; Stoiber, 1995; Xu *et al.*, 1998, 2000).

This review summarizes what is known on reactions, enzymes and mechanisms of dissimilatory S° oxidation and reduction in thermophilic archaea. We will focus on the pathways starting with S° as a growth substrate and omit what is known about dissimilatory sulfate reduction (Dahl *et al.*, 2001; Dahl and Trüper, 2001; Sperling *et al.*, 2001) or sulfur assimilation (Daniels *et al.*, 1986; Le Faou *et al.*, 1990).

Acidianus ambivalens AS MODEL ORGANISM FOR CHEMOLITHOAUTOTROPHIC "SULFUR-DEPENDENT" ARCHAEA

Acidianus ambivalens, *A. infernus*, and *A. brierleyi* are chemolithoautotrophic archaea from the Sulfolobales order of the Crenarchaeota kingdom. All of the Sulfolobales grow at high temperatures (range: 40–97°C) and under very acidic conditions, ranging from below pH 1 to a maximum of 5.5–6. The optimum is usually around pH 2.5–3.5.

The early *Sulfolobus* isolates have all been described as sulfur-dependent, facultative chemolithoautotrophic aerobes (Brierley and Brierley, 1973; Brock *et al.*, 1972; Shivvers and Brock, 1973). Interestingly, the best-studied isolates *Sulfolobus acidocaldarius* and *S. solfataricus* grow rather poorly under these conditions and the question remains whether they are true sulfur oxidizers or rather heterotrophic or mixotrophic "consumers." It is possible that they have either lost the ability to grow chemolithoautotrophically or have been selected with improved plating techniques from what has been originally mixed cultures (Grogan, 1989). However, this question has not been finally resolved.

Some of the Sulfolobales like the *Acidianus* species are true chemolithotrophs and, in addition, facultative anaerobes growing either by hydrogen oxidation with S° as electron acceptor, forming H_2S, or by S° oxidation with oxygen, forming sulfuric acid (Fuchs *et al.*, 1996; Segerer *et al.*, 1985; Zillig *et al.*, 1985, 1986). The latter property is probably responsible for the low pH in many solfataric sites, where pH 1–2 is common at ambient boiling temperature and cell counts in excess of 1×10^8 mL^{-1} are observed (Kletzin, unpublished). The model organism for most of the studies summarized here is *A. ambivalens* ($pH_{opt} = 2.5$, $T_{opt} = 72$–86°C; Fuchs *et al.*, 1996; Zillig *et al.*, 1985, 1986). In addition, we will cover what is known from S° reduction reactions in other archaea.

AEROBIC ELEMENTAL SULFUR OXIDATION AND DISPROPORTIONATION: *Acidianus* SULFUR OXYGENASE REDUCTASE

The oxidation of elemental sulfur proceeds in at least two steps, often more. S° is usually oxidized to sulfite by a sulfur oxygenase (Suzuki, 1965; Suzuki and Silver, 1966) or a sulfur dehydrogenase (Rother *et al.*, 2001). In a second step, sulfite is oxidized to sulfate catalyzed by sulfite:acceptor oxidoreductases or dehydrogenases. Alternative intermediates may be formed like thiosulfate, tetrathionate, trithionate, etc. (Kelly, 1982, 1988). The thiosulfate or tetrathionate oxidation in bacteria is much better studied because the soluble substrates render laboratory investigations easier. The pathways of thiosulfate, tetrathionate, and S° oxidation in bacteria have been repeatedly reviewed (e.g., Friedrich, 1998; Friedrich *et al.*, 2001; Kelly, 1982, 1988; Kelly *et al.*, 1997; Kelly and Wood, 1994; Pronk *et al.*, 1990).

The only enzyme known to directly oxidize S° from archaea is rather unique. It is a soluble enzyme, most probably localized in the cytoplasm. It simultaneously oxidized and reduced S° when incubated with the substrate under air at high temperature, therefore, it has been termed sulfur oxygenase reductase (SOR). The reaction products

were sulfite, thiosulfate, and hydrogen sulfide (Kletzin, 1989). The enzyme activities could not be separated. The SOR did not require external cofactors for activity.

A similar enzyme activity has been found once in a mesophilic bacterium, but the findings reported by Tano and Imai (1968) have never been confirmed independently: the simultaneous production of thiosulfate and H_2S from sulfur by a cell-free extract of *Thiobacillus thiooxidans* was reported, but the enzyme(s) involved were not isolated. Other sulfur oxygenases, mostly glutathione-dependent or other S° oxidizing enzymes, have been described from mesophilic bacteria, especially from thiobacilli. Only very few of these enzymes have been purified and none of them bore any similarity to the *A. ambivalens* SOR (reviewed by Friedrich, 1998; Kelly, 1982, 1988).

The SOR was first described and purified from *A. ambivalens* (Kletzin, 1989) and the *sor* gene encoding the enzyme was sequenced (Kletzin, 1992). Even before, an enzyme described as a sulfur oxygenase with very similar properties had been purified from a phylogenetically not classified isolate termed "Sulfolobus brierleyi" (Emmel *et al.*, 1986). Judged from its physiological properties, "S. brierleyi" must be assumed to be an *Acidianus* species. The SOR and the sulfur oxygenase were very similar regarding the sizes of the holoenzymes and single subunits, the enzyme assays, the reaction products sulfite and thiosulfate, and other properties (Table I, Kletzin, 1994), but an S° reducing activity of the sulfur oxygenase was not reported (Emmel *et al.*, 1986). It was concluded from the similarities that both the SOR and the oxygenase actually catalyze the same reaction and that the sulfur reducing activity of the "S. brierleyi" enzyme has been overlooked (Kletzin, 1994). A moderate incorporation of ^{18}O into sulfite was demonstrated with the sulfur oxygenase (Emmel *et al.*, 1986) as with a sulfur oxygenase from *Thiobacillus thiooxidans* (now *Acidithiobacillus thiooxidans*, Suzuki, 1965). The *A. ambivalens* SOR was active only under air but not under H_2 or N_2 atmosphere (Kletzin, 1989). Both experiments showed that the enzymes are real oxygenases. It was concluded from all of the results that the SOR catalyzes an S° disproportionation coupled to an oxygenase reaction.

The same coupled oxygenase and disproportionation reaction has been found for a third enzyme, the SOR from the *Acidianus* strain S5 (He *et al.*, 2000). The recombinant S5 SOR had a lower pH optimum (pH 5) than the other two (pH 6.5–7.5) and a much higher specific activity (Sun *et al.*, 2003). The temperature optima of the S5 and the "S. brierleyi" enzymes were 65–70°C and 85°C for the *A. ambivalens* SOR (T_{max}:108°C; Kletzin, 1989), consistent with the growth temperatures of the organisms (Table I). The holoenzymes had a high apparent molecular mass, ~550 kDa, and were each composed of a single 35–36 kDa subunit (Emmel *et al.*, 1986; He *et al.*, 2000; Kletzin, 1989). Hollow globular particles of 15.6 nm in diameter appeared in EM pictures of the *A. ambivalens* SOR, which resembled bacterial ferritins (Kletzin, 1989; Urich *et al.*, submitted for publication).

Three other *sor* genes have been identified in the genome sequences of the related archaeon *Sulfolobus tokodaii*, of the euryarchaeote *Ferriplasma acidarmanum*

Table I. Some Properties of the SOR and Sulfur Oxygenase

	SOR *A. ambivalens*	S-oxygenase "S. brierley"	SOR *Acidianus* sp. S5
Holoenzyme apparent mol. mass	560,000	550,000	n.r.
Subunit mol. mass	35,617[a]	35,000	35,172[a]
pH range	4–8	n.r.	3,5–9
pH_{opt}	7-7.4	6.5-7.5	5
T_{opt}	85°C	65°C	70°C
T_{max}	108°C	>80°C	>90
Specific oxygenase activity at optimal temperature	10.6 U/mg[b] / 6.0 U/mg[c]	0.9 U/mg[b]	186.7 U/mg[c]
Specific reductase activity at optimal temperature	2.6 U/mg[b] / 1.4 U/mg[c]	n.r.	45.2 U/mg[c]
^{18}O-incorporation	n.r.	+	n.r.
Diameter	15.6 nm	n.r.	n.r.
Reference(s)	Kletzin (1989), Urich *et al.* (submitted for publication)	Emmel *et al.* (1986)	He *et al.* (2000), Sun *et al.* (2003)

[a] From sequence.
[b] Wild type enzyme.
[c] Recombinant enzyme.

and of the hyperthermophilic bacterium *Aquifex aeolicus* (Urich *et al.*, submitted for publication). Interestingly, the gene was missing in the *S. solfataricus* genome, supporting the hypothesis that this organism might have lost or never possessed the ability to oxidize S°. The deduced amino acid sequences shared 35% (*A. aeolicus*) 88% identical residues (S5; all compared to the *A. ambivalens* SOR) and formed a unique and novel protein family with not even remotely similar relatives. The mRNA transcript had approximately the length of the ORF in *A. ambivalens* (Kletzin, 1992). This result and the production of active SOR from *Escherichia coli* cells expressing the S5 or *A. ambivalens sor* genes showed that the enzyme is made without other subunits and no specific helper proteins (He *et al.*, 2000; Urich *et al.*, submitted for publication).

The SOR activity was inhibited by thiol-binding reagents like iodoacetic acid and zinc ions pointing to the involvement of one or several cysteines in the catalytic process (Kletzin, 1989; Urich *et al.*, submitted for publication). Three conserved cysteine residues were identified in the SOR sequences. It could be speculated that one of the cys residues might bind S° in a similar way as it has been described for the sulfide binding residue in *Rhodococcus* sulfide:quinone oxidoreductase (Griesbeck *et al.*, 2002) or for the thiosulfate binding protein in the *Paracoccus* periplasmatic thiosulfate oxidizing multienzyme complex (TOMES, Friedrich *et al.*, 2001; Rother *et al.*, 2001) but this has to be demonstrated yet.

The *A. ambivalens sor* gene expressed in *E. coli* resulted in two forms of the protein (Urich *et al.*, submitted for publication). The smaller amount was active and remained in the soluble fraction after breaking of the cells, whereas the major part precipitated as insoluble inclusion bodies. The SOR from inclusion bodies could be dissolved in 8 M urea and refolded to the active and near-native state, but only when ferrous iron was present in the refolding solution, thus demonstrating that iron was essential for enzyme activity. Iron quantitation of the wildtype enzyme resulted in a stoichiometry of one Fe per subunit. EPR spectroscopy and redox titration showed that the wildtype, the recombinant, and the refolded SOR all contain a mononuclear non-heme iron core with a low redox potential ($E_0' = -268$ mV). The signal disappeared upon reduction of the enzyme with dithionate or incubation with substrate at elevated temperature (Urich *et al.*, submitted for publication). In the UV/Visible spectrum no feature was visible besides the 280 nm tryptophan peak. Therefore, it was concluded that the iron is most probably coordinated by histidines and/or carboxylate residues and not by cysteines. This type of iron coordination is usually found in dioxygenases, hydoxylases, and superoxide dismutases. It was intriguing to find that the redox potential was more than 300 mV lower than usually found for this type of iron centers, but it is low enough to explain the S° reducing activity of the enzyme (E_0' [H_2S/S°] $= -270$ mV; Thauer *et al*., 1977). It will be subject of future studies to analyze the underlying structural features of this type of iron center.

From the results of the spectroscopic and inhibition studies and the sequence comparisons, one can hypothesize that S° is bound to the enzyme by one or several cysteine residues and that a mixed reaction takes place consisting of the formation and breakage of disulfides and a redox reaction on the iron core. However, the exact reaction mechanism remains to be elucidated.

AEROBIC ELECTRON TRANSPORT CHAINS IN THE OXIDATION OF SULFIDE, THIOSULFATE, AND SULFITE

The lack of cofactors besides iron and the localization of the enzyme in the cytoplasm make it impossible that the SOR reaction couples S° oxidation to electron transport or to substrate level phosphorylation. Therefore, other reactions must be involved in the process. Enzymes that oxidize all three products of the SOR reaction, H_2S, sulfite and thiosulfate, and transfer the electrons either to quinones or *c*-type cytochromes are known from several bacteria and eukaryotes. For example, many bacteria like *Rhodobacter capsulatus* and *Aquifex aeolicus*, and eukaryotes like the yeast *Schizosaccharomyces pombe* and the lugworm *Arenicola marina* possess a sulfide quinone oxidoreductase (SQR), a flavoprotein oxidizing H_2S and transferring the electrons to the respective quinone (reviewed, for example in Theissen *et al.*, 2003). SQR activities have so far not been reported in *A. ambivalens* or in other archaea, although homologs can be found in many archaeal genomes. The fate of the sulfide is therefore not clear at present.

Thiosulfate oxidation to sulfuric acid is the best-studied pathway of sulfur compound oxidation at all. Most studies have been conducted with *Paracoccus pantotrophus* and *Paracoccus versutus*. Different periplasmatic complexes are present in both bacteria and archaes (recently reviewed in Friedrich, 1998; Friedrich *et al.*, 2001; Kelly *et al.*, 1997). Thiosulfate is bound covalently to one of the subunits of the complex and both S atoms are oxidized to sulfate without the presence of free intermediates (Friedrich, 1998; Friedrich *et al.*, 2001; Quentmeier and Friedrich, 2001). The electrons are transferred to cytochrome *c* and then to the terminal oxidase (Friedrich *et al.*, 2001; Rother *et al.*, 2001). A different type of periplasmatic or soluble thiosulfate oxidase or

dehydrogenase is found in other, mostly chemolithoautotrophic and acidophilic species. These enzymes oxidize thiosulfate to tetrathionate while reducing either artificial electron acceptors like ferricyanide or *c*-type cytochromes (Nakamura *et al.*, 2001; Visser *et al.*, 1997). They differ considerably from each other in subunit composition and molecular mass and some even contain *c*-type hemes themselves.

The first membrane-bound thiosulfate:quinone oxidoreductase (TQO) known was purified from aerobically grown *A. ambivalens* cells (Müller *et al.*, submitted for publication). It oxidized thiosulfate with tetrathionate as the product and ferricyanide and decyl ubiquinone as artificial electron acceptors. The reaction could be reversed when reduced methylene blue was used as electron donor. Optimal activity was observed at 85°C and pH 5. There was no end product inhibition by tetrathionate or by sulfate. The 102 kDa holoenzyme consists of 28 and 16 kDa subunits, in an as yet unknown topology. Caldariella quinone was found to be noncovalently bound to the protein and is the likely natural electron acceptor, as the pure protein is capable of reducing the analogous quinone decylubiquinone. Further, cyanide sensitive oxygen consumption was measured in membrane preparations upon the addition of thiosulfate, thus showing electron transport to molecular oxygen via a heme copper oxidase from a reduced sulfur component for the first time in an archaeon (Fig. 2). The TQO subunits were found to be identical to DoxA and DoxD, previously described as part of the cytochrome aa_3 terminal quinol:oxygen oxidoreductase (see below, and Purschke *et al.*, 1997).

DoxD and DoxA were encoded in a bicistronic operon. Five other similar *dox*DA operons were identified in the databases. Two of them from *Sulfolobus solfataricus* and *S. tokodaii* were clearly orthologous (>70% identity in the deduced amino acid sequences). A second gene pair paralogous to *dox*DA (termed *dox*XY, was found in *A. ambivalens* adjacent to the sulfur reductase genes (37% aa identity, see Fig. 6) Laska *et al.*, 2003; Müller *et al.*, submitted for publication), other were found in *S. solfataricus*, *S. tokodaii*, and the mesophilic bacterium *Bacteroides thetaiotaomicron* (Müller *et al.*, submitted for publication). It is not known what their physiological function is, since the *A. ambivalens* TQO is so far the only protein from this enzyme family with biochemical data available. Secondary structure prediction programs showed that DoxD most likely forms four transmembrane helices and DoxA one. In the multiple alignment, a single conserved cysteine residue was identified. Inhibition studies showed that the TQO is only moderately inhibited by thiol-binding reagents like *N*-ethylmaleiimide and Zn^{2+}. The thiosulfate and quinone binding sites and the reaction mechanism of the TQO are therefore not clear at present.

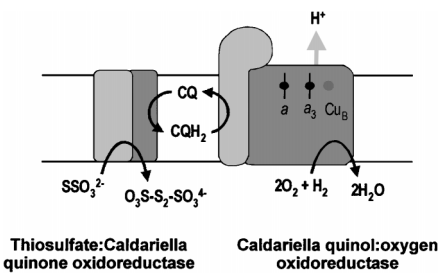
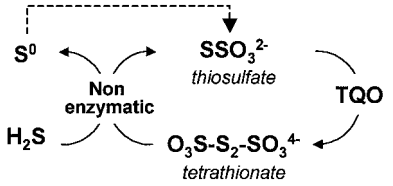


Fig. 2. Schematic cartoon illustrating the interaction between TQO and the terminal oxygen reductase, highlighting the role of the caldariella quinone (CQ) in the process. At the present stage, the topology and membrane attachment mode of TQO are not yet fully elucidated (after Müller *et al.*, submitted for publication). The arrow emerging from the terminal oxidase indicates proton pumping.

The fate of the tetrathionate formed by the TQO has not been investigated yet. However, there is a possibility that a thiosulfate/tetrathionate cycle exists (Scheme 1, Müller *et al.*, submitted for publication). Tetrathionate is unstable in the presence of H_2S and other strong reductants and is reduced to thiosulfate *in vitro* especially at high temperatures (Xu *et al.*, 1998, 2000). Therefore, the H_2S formed by the SOR might be able to re-reduce tetrathionate made by the TQO and thus feed electrons indirectly from the S° disproportionation reaction catalyzed by SOR into the quinone pool (Müller *et al.*, submitted for publication).

A different electron entry point into the quinone pool exists with the oxidation of sulfite. Sulfite:acceptor oxidoreductases (SAOR) or dehydrogenases directly oxidizing sulfite to sulfate are known from many organisms (reviewed by Kappler and Dahl, 2001). There are three main pathways and functions of sulfite oxidation:

(1) The oxidation and detoxification of sulfite generated during cysteine and methionine degradation. The best-studied examples are the chicken liver and mammalian sulfite oxidases, where crystal structures are known. The chicken liver sulfite



Scheme 1. Hypothetical thiosulfate/tetrathionate cycle depicting the reduction of tetrathionate formed by the TQO with H_2S.

oxidase is a molybdopterin enzyme containing a b_5 heme (Kisker *et al.*, 1997).

(2) Different dissimilatory SAORs or sulfite dehydrogenases have been described from bacteria, most of them periplasmatic or soluble enzymes, only one was membrane-bound (Kappler and Dahl, 2001). Sulfite dehydrogenase activity is included in the periplasmatic complexes in *Paracoccus pantotrophus* and *Paracoccus versutus*. All of them are part of the electron transport chain and transfer electrons mostly via *c*-type cytochromes to the terminal oxidase. They are also mostly molybdenum enzymes.

(3) An alternative pathway existing in parallel to the SAOR enzymes has been identified in *Paracoccus denitrificans* and *A. ambivalens* involving the indirect oxidation using the enzymes adenylylsulfate (APS) reductase and adenylylsulfate:phosphate adenyltransferase (APAT) with APS as an intermediate (Brüser *et al*., 2000; Zimmermann *et al.*, 1999). This pathway allows substrate level phosphorylation. The enzymes are localized in the cytoplasm of the microorganisms. The APS reductase catalyzes the reversible 2-electron reduction of APS to sulfite and AMP. APS reductases from a sulfate-reducing archaeon (*Archaeoglobus fulgidus*) and three different bacteria have been purified; they are flavoproteins with remote similarity to fumarate reductases (Fritz *et al*., 2002). The APAT, formerly termed "ADP sulfurylase" catalyzes the synthesis of ADP from APS and phosphate, thus allowing substrate-level phosphorylation. ATP and AMP are formed by adenylate kinase from two ADP molecules (Brüser *et al*., 2000; Zimmermann *et al.*, 1999).

The activities of a SAOR, of the two APS pathway enzymes, and of adenylate kinase have been measured in *A. ambivalens* (Fig. 4; Zimmermann *et al.*, 1999), showing that both pathways exist in the archaeon, similarly as in the mesophilic bacterium *Paracoccus denitrificans* (Brüser *et al*., 2000). The enzymes have not yet been purified. The activity of the membrane-bound SAOR had a pH optimum of 6 and a temperature optimum of $>90^{\circ}$C. The SAOR oxidized sulfite with ferricyanide and decyl ubiquinone as artificial electron acceptors (Müller, Gomes, and Kletzin, unpublished; Zimmermann *et al.*, 1999). The genes and the proteins are not yet known.

In the genome sequences of the *S. solfataricus*, *S. tokodaii*, and other microorganisms ORFs of approximately 600 nucleotides in length were identified whose deduced amino acid sequences shared significant similarity to the molypdopterin-binding central domain of the chicken liver sulfite oxidase. In most cases the proteins had a twin arginine protein translocation pathway motif and were twinned with a hypothetical membrane protein in a bicistronic operon (Kletzin, unpublished), suggesting that the soluble subunit of these proteins containing the molybdopterin domain sits on the outside of the cytoplasmatic membrane attached to a membrane anchor. The results suggest that these proteins might be membrane-bound sulfite oxidases or sulfite:quinone oxidoreductases. However, it remains to be demonstrated whether this hypothesis is true and whether the protein is responsible for the observed SAOR activity in *A. ambivalens*.

COUPLING BETWEEN OXIDATION OF SULFUR COMPOUNDS AND DIOXYGEN REDUCTION BY COMPONENTS OF THE MEMBRANE BOUND AEROBIC RESPIRATORY CHAIN

As discussed before, the novel thiosulfate:quinone oxidoreductase (TQO) elicited in *A. ambivalens* provided, for the first time, direct evidence for the coupling between sulfur and oxygen metabolism (Müller *et al.*, submitted for publication). Altogether with the reported SAOR activity (Zimmermann *et al.*, 1999), it is now clear that there are enzymes capable of reducing caldariella quinone while oxidizing sulfur compounds. This coupling allows that the electrons made available by the successive oxidation of reduced sulfur compounds are funneled to the aerobic respiratory chain, feeding the pool of caldariella quinone. These electrons will be used to drive the high energy yielding dioxygen reduction reaction, catalyzed by the terminal quinol:oxygen oxidoreductase. Ultimately this will contribute to the build up of the proton gradient and subsequent ATP formation.

In the last years, the aerobic membrane-bound respiratory chain of *A. ambivalens* has been extensively characterized in respect to the structural, biophysical, and functional features of its basic components (e.g., Das *et al.*, 1999; Gomes *et al*., 1999, 2001a,b). These studies have elicited that, under the aerobic growth conditions (see Teixeira *et al*., 1995, for details), this organism expresses the simplest membrane-bound aerobic respiratory chain known so far (Fig. 3, Gomes, 1999). Briefly, this minimal respiratory chain is composed by a noncanonical type-II NADH dehydrogenase (Bandeiras *et al.*, 2002; Gomes *et al.*, 2001a) and by an atypical succinate dehydrogenase (Gomes *et al*., 1999; Lemos *et al.*, 2001), both having the ability to reduce caldariella quinone, the major quinone from aerobically grown *A. ambivalens* cells

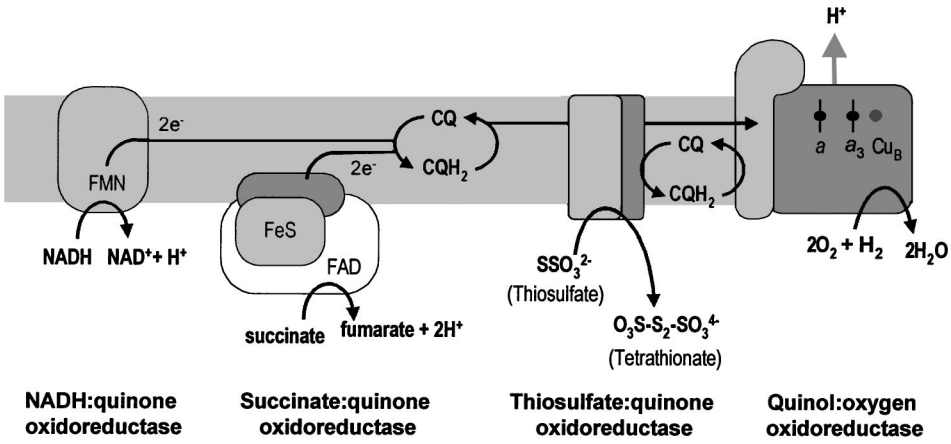


Fig. 3. Scheme outlining the *A. ambivalens* respiratory chain and the so far known coupling point with sulfur metabolism. All components depicted in the cartoon have been isolated and characterized (see references throughout the text). The cofactors from the respiratory complexes are indicated as follows: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; FeS, Iron sulfur clusters; Cu, copper atoms; *a*, heme a; CQ, caldariella quinone; CQH_2, caldariella quinol. The arrow emerging from the terminal oxidase indicates proton pumping. See text for details.

(Trincone *et al.*, 1989). This pool of caldariella quinol is then used to reduce the only terminal oxygen reductase expressed in the studied conditions, a proton-pumping (Gomes *et al.*, 2001b) aa_3-type quinol oxidase belonging to the heme-copper superfamily, which is the major heme-containing protein present in the membranes. The latter protein is unique in respect to several functional features (e.g., see Aagaard *et al.*, 1999; Gomes *et al.*, 2001b; Hellwig *et al.*, 2003) and it was originally reported to be encoded in two different *loci (doxBCE* and *doxDA)*, which were duplicated in the genome. This oxidase is a quite divergent member from this superfamily of enzymes (Gomes, 1999; Purschke *et al.*, 1997). The large 587 amino acid DoxB peptide is the homologue of terminal oxidases subunit I. Although it contains the set of histidines required for binding the redox cofactors (heme *a* and heme $a3$-Cu_B), it has a very low sequence identity in respect to other oxygen reductases (<28%). The other subunits even had less similarity to biochemically characterized terminal oxidases and their function is not clear. The large subunits (DoxB and DoxC) and one of the smaller subunits (DoxE) were encoded together in the *doxB* operon (Purschke *et al.*, 1997). Two other subunits, DoxD and DoxA were encoded separately as described above. It has been postulated that DoxD represents the functional analog of the subunit II of standard terminal oxidases (Purschke *et al.*, 1997). This assumption has been proven wrong by the identification of the DoxDA subunits as the novel TQO enzyme (Müller *et al.*, submitted for publication). The terminal oxidase of *A. ambivalens* is therefore even simpler than previously thought.

One yet unanswered question concerns the enzymatic reduction of NAD^+ in chemolithoautotrophic archaea oxidizing or reducing S° or other sulfur compounds, as so far no enzyme capable of coupling S° oxidation and NAD^+ reduction was found. In fact, the same question is open for all the other archaea using autotrophic nitrate respiration, metal leaching, or other metabolic traits. There are few candidates judged from the standard redox potentials. The oxidation of H_2 should allow NAD^+ reduction, however, no hydrogenase activity has been identified from a chemolithoautotroph yet with NAD^+ as co-substrate. Of the sulfur compounds with a sufficiently negative redox potential, only sulfite is usually found in biological systems ($E'_0 = -515$ mV; Thauer *et al.*, 1977). A ferredoxin:NAD^+ oxidoreductase activity has been detected in *A. ambivalens* soluble fraction, but the corresponding enzyme was never isolated (Gomes, unpublished). It will be interesting to elucidate whether enzyme(s) exist coupling both reactions or whether reverse electron transport is used as in many other microorganisms.

From the presently available data, a sketch of the putative pathways of S° oxidation present in *A. ambivalens* can be outlined (Fig. 4). Some of the enzymes involved have been purified or at least the enzyme activity has been demonstrated. Sulfur is oxidized by the SOR, the products thiosulfate and sulfite are oxidized by membrane-bound oxidoreductases. There is also the alternative APS oxidation pathway available for sulfite. Still, there are many gaps open. One of the most important unsolved questions is, how the sulfur gets into the cell and

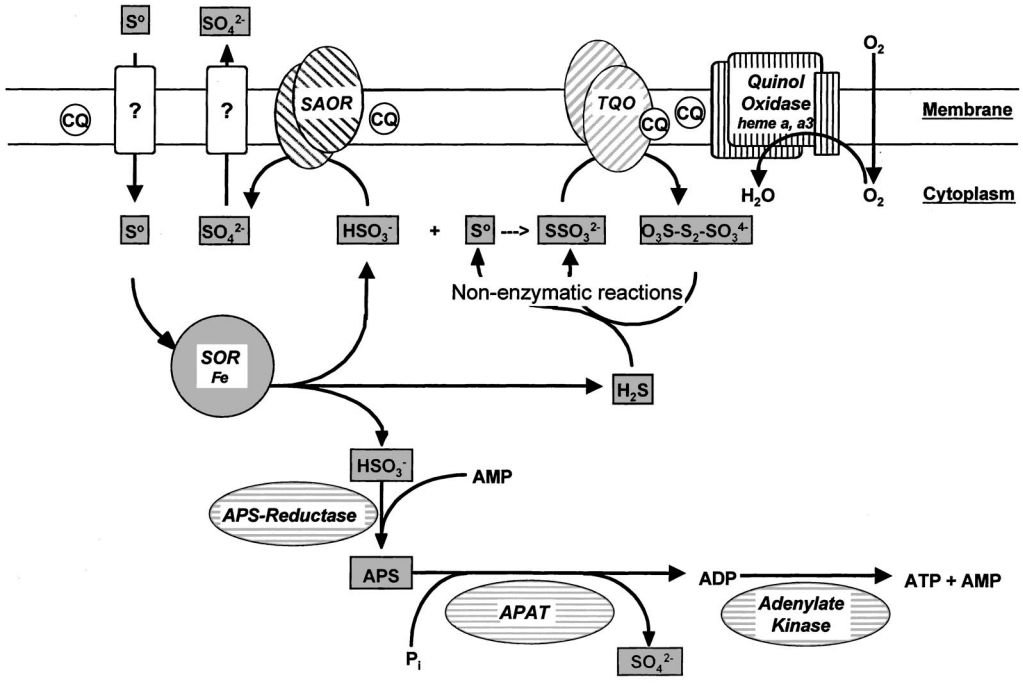


Fig. 4. Hypothetical model of S° oxidation in *A. ambivalens*. The model was derived from known enzymes and enzyme activities and other probably nonenzymatic reactions in *A. ambivalens*. Italics: enzymes; SAOR, sulfite:acceptor oxidoreductase; TQO, thiosulfate:quinone oxidoreductase; SOR, sulfur oxygenase reductase; APS, adenylylsulfate; APAT, adenylylsulfate phosphate adenyltransferase. See text for details.

the sulfate or other intermediates get out. Also, it is still unsolved whether additional membrane-bound enzymes exist that catalyze sulfur compound oxidation at the outside of the membrane, whether a tetrathionate-oxidizing pathway exists and how is NAD^+ reduction coupled to sulfur metabolism. Some of these gaps, however, will be hopefully filled in the near future.

ANAEROBIC HYDROGEN OXIDATION AND SULFUR REDUCTION

Reduction of elemental sulfur is one of the most common reactions by which many different isolates of thermophilic archaea can be grown in the laboratory (Amend and Shock, 2001; Blöchl *et al.*, 1995; Schönheit and Schäfer, 1995). Four different types of S° reduction can be distinguished with respect to substrates and products (Table II).

(1) The obligately chemolithoautotrophic S° reducers like *A. ambivalens, Pyrodictium occultum, P. abyssi*, and *Tp. neutrophilus* gain energy from H_2 oxidation with S° as electron acceptor. Some of them, like *Thermoproteus tenax*, are facultatively chemolithoautotrophic (Fischer *et al.*, 1983; Schönheit and Schäfer, 1995; Stetter *et al.*, 1983; Zillig *et al.*, 1981). They use ATP and reduction equivalents for CO_2 fixation with a reverse tricarboxylic acid or a modified hydroxypropionate cycle (Hugler *et al.*, 2003; Ishii *et al.*, 1996; Schäfer *et al.*, 1986; Strauss *et al.*, 1992).

(2) Some archaea like *Thermoproteus tenax* completely oxidize organic substrates to CO_2, thus enhancing the energy efficiency of the oxidation reaction. This type of metabolism absolutely requires a terminal electron acceptor like S°, and as a consequence, these microorganisms are strictly S°-dependent and require a membrane-bound electron transport chain (Selig and Schönheit, 1994).

(3) Most heterotrophic, fermentative S° reducers require the addition of S° to the medium, although there are exceptions like *Pyrococcus furiosus* and *Thermococcus litoralis*. These organisms do not fully oxidize the substrate to CO_2; the main products are hydrogen and small organic compounds like acetate and alanine (Kengen and Stams, 1994).

Table II. Some Examples of Thermophilic S° Metabolizing Archaea Referred To in This Contribution[a]

Species	e-Donor	e-Acceptor	References
Acidianus ambivalens, A. infernus	H_2, S°	S°, O_2	Zillig *et al.* (1986), Huber *et al.* (1992), Fuchs *et al.* (1996)
A. brierleyi	S°, H_2, pyrite, organics	S°, O_2, MO_4^{2-}	Brierley and Brierley (1982), Larsson *et al.* (1990), Huber *et al.* (1992), Fuchs *et al.* (1996)
Sulfolobus acidocaldarius/S. solfataricus	Various organic molecules	O_2	Brock *et al.* (1972), Zillig *et al.* (1980), Grogan (1989)
Pyrodictium occultum, P. brockii, P. abyssi	H_2	S, $S_2O_3^{2-}$	Stetter *et al.* (1983), Pley *et al.* (1991)
Pyrobaculum islandicum	H_2, peptides,	S°, $S_2O_3^{2-}$—, HSO_3—	Selig and Schönheit (1994), Huber *et al.* (1987)
Pyrobaculum aerophilum	peptides	O_2, NO_3^-, NO_2^-	Völkl *et al.* (1993)
Thermoproteus tenax	H_2, carbohydrates, peptides, alcohols	S°	Selig and Schönheit (1994), Stetter and Zillig (1985)
Archaeoglobus fulgidus	H_2, various organic molecules	SO_4^{2-}, HSO_3^-	Stetter (1988), Zellner *et al.* (1989), Dahl and Trüper (2001)
Pyrococcus furiosus	Sugars, peptides	S°, H^+, organic molecules	Schicho *et al.* (1993), Kengen and Stams (1994), Adams *et al.* (2001)

[a]More comprehensive overviews of the metabolic properties of thermophilic and hyperthermophilic archaea were published in Schönheit and Schäfer (1995), Hedderich *et al.* (1999), and Amend and Shock (2001).

(4) Methanogenic archaea and especially the thermophilic and hyperthermophilic members of the genera *Methanopyrus, Methanobacterium, Methanothermus*, and *Methanococcus* produce significant amounts of H_2S in the presence of S°, while the rate of methanogenesis is reduced (Stetter and Gaag, 1983). Details about the molecular mechanisms of this pathway (excessive sulfur reduction) have not been reported yet.

We will focus here mostly on the chemolithoautotrophic S° reduction with H_2 as electron donor. Several studies were performed with *Acidianus* and *Pyrodictium* as model organisms. In addition, we will briefly summarize what is known about S° reduction in *P. furiosus*, the best studied fermentatively growing H_2 and H_2S producer among hyperthermophilic archaea.

The chemolithoautotrophic H_2 oxidation with S° as electron acceptor requires at least two enzymes in a very short electron transport chain — a hydrogenase and a sulfur or polysulfide reductase (SR/PSR), both membrane-bound. The hydrogenase/PSR system from the mesophilic bacterium *Wolinella succinogenes* is paradigmatic for this type of metabolism (Fig. 5, reviewed, among others, by Hedderich *et al.*, 1999). The PSR couples the oxidation of hydrogen or formate to polysulfide reduction using free menaquinone as intermediate. Menaquinone can be directly reduced by the hydrogenase or the formate dehydrogenase (Hedderich *et al.*, 1999). It is thought that a proton motive force is generated by the reduction of the quinone at the hydrogenase membrane anchor coupled to proton uptake from the cytoplasm. The proton(s) are believed to be released into the periplasm upon reoxidation of the menaquinol by the PSR.

Hydrogenases have been purified from different archaea, many of them from methanogens, which we will not cover here. Hydrogenases from S°-dependent archaea are known from *P. furiosus* (Bryant and Adams, 1989; Ma *et al.*, 2000; Ma and Adams, 2001; Sapra *et al.*, 2000), four different *Thermococcus* species (for a comparison of the soluble hydrogenases see Kanai *et al*., 2003), *P. abyssi*, *P. brockii*, and *A. ambivalens* (Dirmeier *et al.*, 1998; Laska *et al.*, 2003; Laska and Kletzin, 2000; Pihl and Maier, 1991). Enzymes with S° reducing activity include the soluble sulfhydrogenases identical to the soluble hydrogenases from *P. furiosus* (Arendsen *et al.*, 1995; Ma *et al.*, 1993), the sulfide dehydrogenases from *P. furiosus* (Ma and Adams, 1994), and finally the membrane-bound sulfur or polysulfide reductases (SR/PSR) from *A. ambivalens* and two *Pyrodictium* species (Dirmeier *et al.*, 1998; Laska *et al.*, 2003; Laska and Kletzin, 2000; Pihl *et al.*, 1992).

ANAEROBIC ELECTRON TRANSPORT CHAINS 1: *Acidianus ambivalens* HYDROGENASE AND SULFUR REDUCTASE

A sulfur reductase (SR) purified from solubilized membrane fractions of anaerobically grown *A. ambivalens*

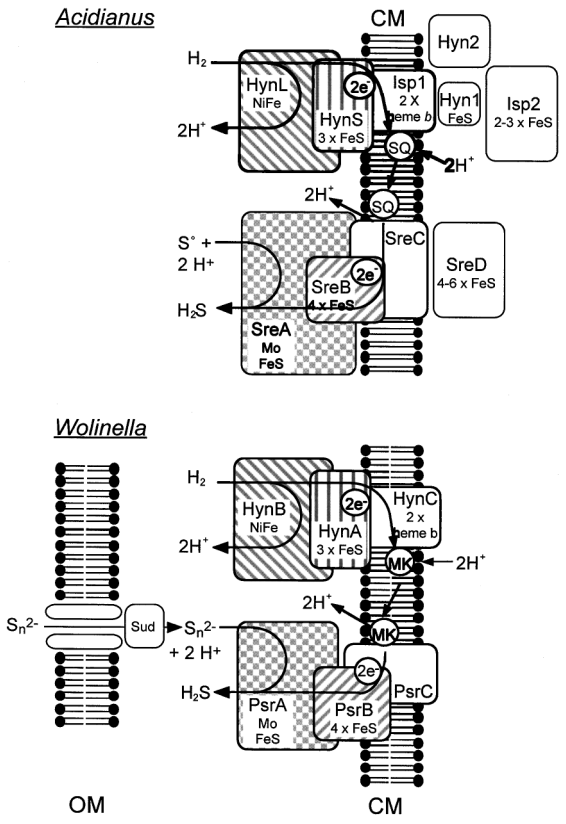


Fig. 5. Hypothetical model of S° reduction and the anaerobic electron transport chains in *A. ambivalens* and *Wolinella succinogenes.* The *A. ambivalens* model was developed from the results of the sequence comparison, the biochemical data and in analogy to the *W. succinogenes* hydrogenase/PSR system (Hedderich *et al.*, 1999; Laska *et al.*, 2003). Subunits of the hydrogenases are labeled HynL, HynS, Hyn1 an Isp1 and 2 after the *Acidianus* genes (Fig. 6; Laska *et al.*, 2003) or HynABC after the *Wolinella* genes (Hedderich *et al.*, 1999). Subunits of the SR are labeled SreA-D (Fig. 6; Laska *et al.*, 2003) and PsrA-C (Hedderich *et al.*, 1999). Homologous subunits are shown in identical shading, and predicted metal-binding sites are marked. CM cytoplasmatic membrane, OM, outer membrane, MK, menaquinone, SQ, sulfolobus quinone.

cells reduced S° with hydrogen as electron donor in the presence of a copurified hydrogenase and either 2,3-dimethyl-1,4-naphthoquinone (DMN) or cytochrome *c* as electron carriers in the enzyme assay (Laska *et al.*, 2003; Laska and Kletzin, 2000). The bi-directional and oxygen-stable hydrogenase purified separately did not have SR or sulfhydrogenase activity. Apparently, electron transfer between both enzymes involves quinones: the SR activity was diminished to ≈13% when the membrane fraction was irradiated with UV light, which could nevertheless be partially reconstituted upon addition of DMN (Laska *et al.*, 2003). The hydrogenase reduced DMN and the dyes methyl and benzyl viologen. Altogether, these data suggest that two separate enzymes are present in the *A. ambivalens* membranes as in *Wolinella* (Dietrich and Klimmek, 2002), and that the electron transfer is mediated by quinones. No *c*-type cytochromes have ever been found in any isolate of the Sulfolobales. Also, no genes encoding *c*-type cytochromes have been identified in the genome sequences.

The copurified hydrogenase and the SR showed molecular masses of 250 kDa each (Laska *et al.*, 2003). Protein bands with apparent masses of 110 (large subunit of the SR), 66, 41, and 29 kDa (hydrogenase subunits) and some minor bands were present in SDS gels. When the hydrogenase was separated over a 2D gel electrophoresis consisting of a blue native PAGE in the first dimension and a SDS PAGE in the second, additional subunits were visible (22 and 14 kDa). The hydrogenase was encoded by a 12-ORF gene cluster (*hyn*, Fig. 6) including the genes for the NiFe and FeS subunits (see below) and some but not all of the proteins required for the maturation of NiFe-hydrogenases. The genes for the NiFe and FeS subunits showed significant similarity to bacterial uptake NiFe hydrogenases (most similar: a hydrogenase from *Streptomyces avermitilis*, 41% identity) but not to archaeal ones (Laska *et al.*, 2003). The amino acid sequence of the FeS subunit (HynS) contained a 40 amino acid leader peptide with a conserved twin-arginine protein translocation motif suggesting a transport to the outside of the membrane. Metal analysis revealed the presence of Fe and Ni in enriched hydrogenase fractions, thus suggesting that the *A. ambivalens* hydrogenase could be of the NiFe type. It consists of at least three subunits and the catalytic subunits are likely to be located on the outside of the membrane as in the case of *Wolinella* (Fig. 5). The likely physiological electron acceptor is sulfolobus quinone, which predominates in anaerobically grown cells (Trincone *et al.*, 1989).

The sequence analysis of the hydrogenase gene cluster showed some more interesting features (Fig. 6, Laska *et al.*, 2003). One of the conserved cysteine residues usually coordinating the proximal of three FeS clusters in the small subunit of the hydrogenase was replaced by an asparagine. Although it is not known whether this replacement affects the cluster composition of the subunit, it is possible that the proximal cluster may be changed from the standard [4Fe-4S] to a [3Fe-4S].

The order and composition of the structural genes in the cluster were also rather uncommon (*hyn*S-*isp*1-*isp*2-*hyn*L-*hyn*1 encoding: small subunit-membrane anchor-FeS protein with unknown function-large NiFe subunit-Rieske ferredoxin, Fig. 6). The deduced amino acid sequence of the isp1 protein showed similarity to

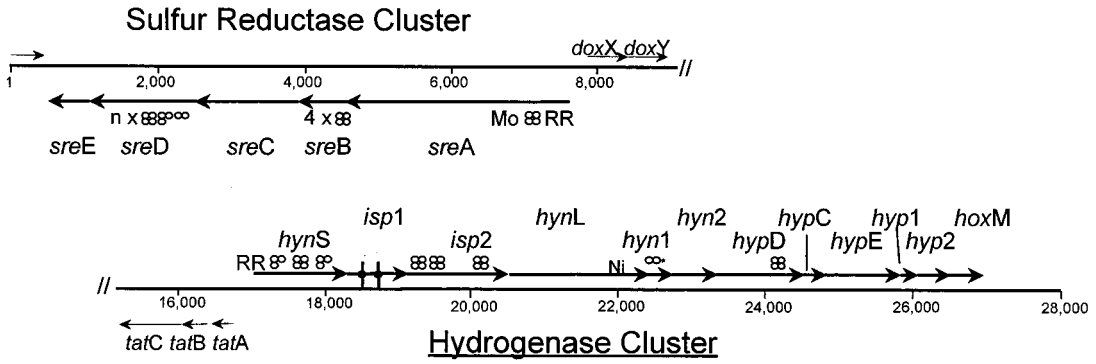


Fig. 6. Gene clusters encoding the NiFe hydrogenase and SR (bold) in *A. ambivalens*. The ruler indicates genomic DNA. Arrows indicate ORFs, for the explanation of the genes of the hydrogenase and SR cluster see text. RR twin arginine protein translocation motif, the *tat* cluster encodes proteins required for this pathway. Small black circles indicate the presence and the number of cysteine-containing sequence motifs potentially coordinating 2-, 3- or 4-iron-sulfur clusters in the deduced proteins (Laska *et al.*, 2003); Ni and Mo indicate potential metal binding sites.

heme *b* containing membrane anchors of nitrate reductases and other proteins but not to the analogous protein from *Wolinella*. However, it has to be demonstrated whether this prediction is true.

Downstream of *hyn*L a gene encoding a soluble small 113 amino acid Rieske ferredoxin was located, the first found in a hydrogenase gene cluster. The *A. ambivalens* Rieske was unique in several aspects. It clustered together with Rieske domains of larger flavoproteins from the genomes of the eukaryotes *Caenorhabditis elegans* and *Schizosachharomyces pombe* in a phylogenetic analysis and not with the Rieske ferredoxins found in the *Sulfolobus* spp. genome sequences. Also it was very different from the only other soluble Rieske ferredoxin known from an archaeon, the pink sulredoxin from *S. tokodaii* (<30% identity). The Rieske gene expressed in *E. coli* resulted in a red colored protein with the characteristic Rieske signature in EPR spectroscopy and a surprisingly positive redox potential ($E_0' = +180$ mV; Kletzin, Hechler, and Gomes, unpublished). The reduction potential might change when the Rieske is bound to other proteins like the hydrogenase in its natural environment; however, its function is not clear at present.

In consequence, many features of the *A. ambivalens* hydrogenase are similar to other uptake hydrogenases known from bacteria like *W. succinogenes*. However, the enzyme has many unique and surprising features distinguishing it from known proteins. Especially the presence of a Rieske ferredoxin and the lack of the conserved proximal FeS ligand cysteine are unique.

Investigations on the SR were somewhat hampered, because the enzyme could not be purified without the hydrogenase always being present and the overall amounts of protein were low (Laska *et al.*, 2003). However, the biochemical data available and the analysis of the first sequence of archaeal SR genes gave some interesting results. The gene cluster encoding the SR consisted of five ORFs, *sre*ABCDE (Fig. 6). The deduced amino acid sequences of *sre*A and *sre*B showed significant but limited similarity to the catalytic and the FeS subunits of molybdoproteins of the DMSO/FDH/Nitrate reductase family (Laska *et al.*, 2003). The *N*-terminal amino acid sequence of SreA again contained a twin arginine motif, suggesting that the protein is exported over the membrane by the TAT pathway. The *sre*C gene encoded a hydrophobic putative membrane protein with 10 transmembrane helices; the *sre*D gene encoded a hypothetical polyferredoxin with 26 cysteine residues. The *sre*E gene encoded a small protein with similarity to maturation proteins like NarJ within the *E. coli nar*GHJI operon (nitrate reductase) suggesting that the role of SreE might be similar to that of NarJ, which is a system-specific chaperone protein involved in the introduction of the molybdenum cofactor into the protein. Molybdenum was found in the solubilized membrane fraction but not tungsten. The results taken together suggest that SreA is the catalytic subunit and that the enzyme might be a molybdoprotein, that SreB is the electron transfer subunit and that SreC is the membrane anchor of the SR. The orientation and the molecular composition of the *A. ambivalens* SR are to some extent similar to the *W. succinogenes* PSR (Fig. 5; Laska *et al.*, 2003). Both enzymes consist of two homologs subunits each (SreAB/PsrAB) and a non-homologous membrane anchor (SreC/PsrC). The hydrogenases have a similar subunit structure, the *Acidianus* and *Wolinella* enzymes are composed of at least three subunits each, the homologous Ni-containing catalytic subunits (HynL/HynB; Fig. 5), the FeS-containing electron transfer subunits (HynS/HynA) and the non-homologous

membrane anchors (Isp1/HynC). In the case of the *Acidianus* SR the SreD protein might be an additional subunit not yet biochemically identified.

It is not known what the function of SreD, Isp2, and Rieske subunits encoded in the *A. ambivalens* hydrogenase and SR clusters is. They might form additional subunits in the holoenzymes, probably similar to the nine subunits of the *Pyrodictium abyssi* H_2:sulfur oxidoreductase complex (see below, Dirmeier *et al.*, 1998). They might be required because the organisms grow under different pH conditions and therefore, the substrate for the *Acidianus* SR is different from the substrates of the *Wolinella* PSR or the *Pyrodictium* H_2:sulfur oxidoreductase. At neutral or near-neutral pH, polysulfide is formed nonenzymatically from S° at elevated temperatures in a pH and temperature-dependent manner, whereas it decomposes under acidic conditions to H_2S and S° (Schauder and Kröger, 1993). From theoretical calculations, it was shown that at pH 5 and above, polysulfides are stable enough to be present in the media in concentrations sufficient to allow growth but not under conditions that support the growth of *Acidianus*. Therefore, the actual substrate of the *Acidianus* SR must be different from the substrates of the *Pyrodictium* and *Wolinella* enzymes. The additional subunits might play a role in the pH and substrate adaptation.

The only other homologous *sre*ABCDE gene cluster has been identified in the genome of *S. solfataricus*, another thermoacidophile (Laska *et al.*, 2003). No hydrogenase gene has been identified; therefore, *S. solfataricus* cannot grow chemolithoautotrophically by H_2 oxidation like *Acidianus*. The presence of this gene cluster suggests that *S. solfataricus* is able to reduce S° and might grow by heterotrophic anaerobic S° respiration, thus expanding the metabolic properties of the archaeon. However, this has not yet been demonstrated.

ANAEROBIC ELECTRON TRANSPORT CHAINS 2: *Pyrodictium* HYDROGENASE AND SULFUR REDUCTASE

Pyrodictium strains became famous because they were the first microorganisms isolated with an optimal growth temperature above 100°C (Stetter, 1982). In addition, they grew without organic substrates (Table II). Hydrogenase and SR have been analyzed from two different *Pyrodictium* species, *P. brockii* (Pihl *et al.*, 1989, 1992; Pihl and Maier, 1991) and *P. abyssi* (Dirmeier *et al.*, 1998).

An uptake NiFe hydrogenase purified from the membranes of *P. brockii* consisted of two subunits with molecular masses of 66 and 45 kDa (Pihl and Maier, 1991). The enzyme was bidirectional, catalyzing the H_2 oxidation with various artificial dyes and also the H_2 evolution with reduced methylene blue as electron donor. It was reversibly inhibited by O_2. H_2-dependent SR (H_2:S° oxidoreductase) activity was measured in the membranes but the enzymes have not been purified besides the hydrogenase and a small *c*-type cytochrome (13 kDa; Pihl *et al.*, 1992). When the membrane fraction was irradiated with UV light, no SR activity was measured. The activity could be reconstituted upon addition of the artificial ubiquinones Q_{10} and Q_6. A quinone was identified in the membrane, but the structure was not reported. The cytochrome solubilized from the membrane fraction showed a typical visible spectroscopy signature (soret = 421 nm, $\alpha = 553$ nm and $\beta = 522$). It was reduced upon incubation of the membrane fraction with H_2. It is not known whether the cytochrome is actually involved in the electron transport from hydrogen to S° (Pihl *et al.*, 1992). The *P. brockii* electron transport chain and its enzymes have many features in common with the *A. ambivalens* enzymes, especially the molecular masses of the subunits of the NiFe hydrogenase (approx. 66 and 40–44 kDa) and the quinone-dependent activity. The most prominent difference is the presence of a cytochrome *c*.

Some more biochemical details and some interesting properties different from the *P. brockii* enzymes are known from the membrane-bound 520 kDa H_2:sulfur oxidoreductase multienzyme complex from the related hyperthermophilic isolate *P. abyssi*. The complex was composed of nine different subunits containing a NiFe hydrogenase, SR, and hemes *b* and *c* (Dirmeier *et al.*, 1998). Large amounts of iron and acid-labile sulfur were found, in addition 2.8 mol heme *b*/mol of holoenzyme, 0.3 mol heme *c*, 1.6 mol of nickel, and 1.2 mol of copper. Molybdenum and tungsten (W) were not found. Quinones were also not found or required for activity. The H_2:sulfur oxidoreductase contained all the constituents necessary for the electron transport from hydrogen to S°. It is one of the rare examples of an entire electron transport chain present in a single complex.

None of the genes encoding the *Pyrodictium* enzymes is known or sequenced. Only four N-termini of the nine subunits of the *P. abyssi* complex have been reported. The N-termini of the 65 and 42 kDa subunits showed sequence similarity to the HynL and HynS subunits of the *A. ambivalens* NiFe hydrogenase and other to hydrogenases (Dirmeier *et al.*, 1998; Laska *et al.*, 2003). The N-termini of the 85 and 24 kDa subunits showed similarity to the SreA and SreB subunits of the *A. ambivalens* SR. It was concluded that the *P. abyssi* H_2:sulfur oxidoreductase were members of the DMSO reductase family of molybdoenzymes and therefore must contain Mo or W despite the fact that none of the elements was found (Laska *et al.*,

2003). It is a known effect that those elements are often depleted upon enzyme purification, so that they cannot be measured in the elemental analysis.

In consequence, both of the *Pyrodictium* enzymes have some properties in common, especially the heme *b* and *c* contents. There are still some differences, most notably the quinone dependence of the *P. brockii* enzyme. Additional subunits (in the *P. abyssi* enzyme) and the heme *c* content separate the *Pyrodictium* enzymes from *A. ambivalens*. It will be interesting to study how *Acidianus* replaces the cytochrome *c*. Its place should be filled by one of those subunits encoded in the hydrogenase/SR gene clusters with yet unknown function.

ANAEROBIC HETEROTROPHIC SULFUR AND HYDROGEN METABOLISM: *Pyrococcus*

The Pyrococcales gain energy by fermentation of carbohydrates, amino acids, or peptides with hydrogen and small organic molecules as the major products (Table II, Adams *et al.*, 2001; Kengen and Stams, 1994; Schicho *et al.*, 1993). When S° was present, *P. furiosus* produced H_2S and higher growth rates were observed (Schicho *et al.*, 1993). Growth with S° was depending whether the organism grew on maltose or on peptides (Adams *et al.*, 2001). The addition of S° did not have an effect on the growth yields on maltose as sole energy and carbon source; however, H_2S was formed in the presence of S°. The cells grew only on peptides as sole energy and carbon source when S° was added (Adams *et al.*, 2001). A specific SR has not been identified yet and also the molecular mechanism of the S° dependency of peptide-growing cell has not been elucidated. Several enzymes have been identified, which reduce S° among other activities. Neither of them has been identified as the major S°-reducing enzyme in peptide-growing cells.

Three different NiFe hydrogenases have been purified from *P. furiosus*, two of them soluble enzymes with the ability of S° or polysulfide reduction with H_2 as electron donors (Ma *et al.*, 1993, 2000). The third hydrogenase was membrane-bound and did not reduce S° (Sapra *et al.*, 2000). In addition, a soluble polysulfide dehydrogenase was purified with a broad substrate range, reducing many different substrates including O_2 and polysulfides with NADPH as electron donor (Ma and Adams, 1994). The three S°-reducing enzymes are apparently not involved in electron transport chains; in contrast, it is rather thought that their function is the oxidation of an excess of reducing power instead of the generation of an electrochemical gradient over the membrane. S°-inducible transcripts and proteins have been identified in *P. furiosus* using microarray and 2D gel techniques in cultures grown with S°. The two genes with the highest increase in mRNA level in "sulfur cultures" were *sip*A and *sip*B (61 and 25 fold compared to cultures grown without, Schut *et al.*, 2001). The gene products had been previously identified as membrane associated proteins (Holden *et al.*, 2001). Both genes were transcribed from a common promoter region in opposite directions (Schut *et al.*, 2001). Homologs of SipA and B were found in many archaea and in a few bacteria. SipB contains eight cysteine residues arranged like in FeS proteins suggesting that the protein is involved in redox reactions. SipA contained only one. Transmembrane helices could not be predicted from the amino acid sequences. The biochemical function of the Sip proteins is not clear. It has been proposed that they are part of a membrane-associated SR complex involved in S° reduction; however, no biochemical evidence has been reported yet (Adams *et al.*, 2001). Therefore, it is not known whether there is another SR in *Pyrococcus* different from the soluble sulfhydrogenases.

The properties of the membrane-bound hydrogenase are very interesting. Recent results suggested that the hydrogenase itself is the only protein required for the proton reduction with ferredoxin as the electron donor (Sapra *et al.*, 2003). The reaction was coupled to proton translocation across the cytoplasmatic membrane and chemiosmotic ATP synthesis. The *Pyrococcus* ferredoxin is reduced by the glyceraldehyde 3-phosphate and pyruvate:ferredoxin oxidoreductases during the modified glycolysis pathway (Blamey and Adams, 1993; Mukund and Adams, 1995). This single-enzyme respiratory system allows energy conservation under anaerobic conditions by hydrogen production and explains why the growth yields of *P. furiosus* are higher than could be accounted for if ATP synthesis occurred only by substrate-level phosphorylation (Sapra *et al.*, 2003). These results have resolved the question of energy conservation of *Pyrococcus* cells growing in the absence of S°. However, it is still unknown how S° reduction is coupled to an electron transport chain.

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