A Membrane-bound Multienzyme, Hydrogen-oxidizing, and Sulfur-reducing Complex from the Hyperthermophilic Bacterium *Aquifex aeolicus*<sup>§¶<sup>

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*Aquifex aeolicus* is a hyperthermophilic, chemolithoautotrophic, hydrogen-oxidizing, and microaerophilic bacterium growing at 85 °C. We have shown that it can grow on an H$_2$/S° medium and produce H$_2$S from sulfur in the later exponential phase. The complex carrying the sulfur reducing activity (electron transport from H$_2$ to S°) has been purified and characterized. It is a membrane-bound multiprotein complex containing a [NiFe] hydrogenase and a sulfur reductase connected via quinones. The sulfur reductase is encoded by an operon annotated *dms* (dimethyl sulfoxide reductase) that we have renamed *sre* and is composed of three subunits. Sequence analysis showed that it belongs to the *M*$_{e}$SO reductase molybdoenzyme family and is similar to the sulfur/polysulfide/thiosulfate/tetrathionate reductases. The study of catalytic properties clearly demonstrated that it can reduce tetrathionate, sulfur, and polysulfide, but cannot reduce *M*$_{e}$SO and thiosulfate, and that NADPH increases the sulfur reducing activity. To date, this is the first characterization of a supercomplex from a bacterium that couples hydrogen oxidation and sulfur reduction. The distinctive feature in *A. aeolicus* is the cytoplasmic localization of the sulfur reductase, which is in accordance with the presence of sulfur globules in the cytoplasm. Association of this sulfur-reducing complex with a hydrogen-oxygen pathway complex (hydrogenase I, *bc* complex) in the membrane suggests that subcomplexes involved in respiratory chains in this bacterium are part of supramolecular organization.

The production of biomass in extreme light-independent environments is energized by chemolithoautotrophic oxidation and reduction of inorganic compounds like elemental sulfur (S°),<sup>2</sup> hydrogen, and nitrate (1, 2). Sulfur and sulfur compounds are the most abundant sources of both electron acceptors and electron donors in extremophilic environments (like volcanic environments) and are used by many microorganisms to support growth (3–6). Reduction and oxidation of sulfur compounds (sulfate, sulfite, thiosulfate, organic sulfoxide, elemental sulfur, polysulfides, and organic disulfide) are vital processes for many bacteria and essential steps in the global sulfur cycle. Because of the multiple oxidation states of sulfur, the biochemistry and chemistry of this cycle are complex and still not completely understood (7, 8). This problem is exacerbated by the reactivities of the sulfur species at various oxidation states toward each other (7). The ability to reduce elemental sulfur is mostly found among hyperthermophilic micro-organisms (Archaea and Bacteria) (8), the majority of which depend on S° reduction for optimal growth. They use either molecular hydrogen or organic compounds as electron donors. It has been suggested that S° respiration is one of the earliest mechanisms for energy conservation, because the hyperthermophiles are the forms of life evolving the most slowly (9).

The processes by which micro-organisms reduce S° to H$_2$S are still unclear for most of them, and only few of the enzymes involved have been purified. The respiratory systems described so far differ significantly in the properties of their terminal reductases. In some organisms, the activity of sulfur reduction has been found to be associated with soluble proteins (10–14). However, some of these enzymes (12–14) are not implicated in energy production, and the reduction of sulfur to sulfide is proposed to be a mechanism for the disposal of excess reductant generated by fermentation. In other organisms, the S° or polysulfide reduction activity has been shown to be membrane-associated (15–18). So far, the best understood membrane electron transport chain to sulfur is in the mesophilic bacterium *Wolinella succinogenes*, which grows with H$_2$ or formate as electron donors, and S° or polysulfide as electron acceptors (8, 16). Supercomplexes involved in hydrogen-sulfur respiration have until now been detected only in hyperthermophilic Archaea. From the chemolithoautotrophic and hyperthermophilic archaeon *Pyrodictium abyssi*, an extremely thermostable membrane-bound sulfur-reducing enzyme complex, composed of nine different subunits, has been detected. It appears to contain the entire electron transport chain required for the reduction of S° with H$_2$ to H$_2$S, including a hydrogenase, a sulfur reductase, and an electron-transferring component (17). More recently, a complex containing a sulfur reductase and a hydrogenase has been purified from the membrane fraction of the hyperthermophilic and acidophilic archaeon *Aciditrix ambivalens* (18), and an electron transfer from hydrogenase to sulfur reductase, mediated by quinones, has been suggested.

The most hyperthermophilic bacteria known to date are members of the genus *Aquifex* and grow at optimal temperatures of 85 °C (19). Phylogenetic analyses based on 16 S rRNA sequence comparisons showed that *Aquifex* represents the earliest branching order in the bacterial domain (20). *Aquifex* is a hyperthermophilic, hydrogen-oxidizing,
microaerophilic, and obligate chemolithoautotrophic bacterium capable of growing under an H2/O2/CO2 atmosphere in a medium containing only inorganic compounds. It gains energy for growth from hydrogen and oxygen and uses the reductive trichloroacetic acid cycle for fixing CO2. Because of the outstanding phylogenetic position and physiological properties of the Aquificales, we have focused our interest on the energetic metabolism of *Aquifex aeolicus*, whose genome has been completely sequenced (21). We have recently characterized two of the three membrane-bound and soluble hydrogenases from this organism, and we proposed a model for the metabolic roles of the three [NiFe] hydrogenases present in the cell (22). Several other components of the electron transfer chain in *A. aeolicus* have been studied as follows: two very similar membranous and soluble cytochromes *f* (23, 24), a [2Fe-2S] ferredoxin (25), the sulfide:quinone oxidoreductase (26), and the NADH:ubiquinone oxidoreductase (complex I) (27). More recently, we have reported the biochemical and biophysical properties and the implication of the cytochrome *bc* complex in the energy-conserving electron transport chain (28). *Aquifex pyrophilus* can use S2− and thiosulfate (S2O3−2) as electron donors, and in some growth conditions, H2S is formed from S2− and H2O, suggesting a role for S2− as an electron acceptor (19). The sulfur metabolism of *A. aeolicus* has not been studied, and no data are available in the literature. Here we describe the membrane-bound supercomplexes involved in energy metabolism and the purification and biochemical characterization of a membrane-bound multienzyme complex transferring electrons from H2 to S2−. Its possible metabolic roles in the *A. aeolicus* bioenergetic pathways are also discussed.

### EXPERIMENTAL PROCEDURES

#### Organism and Growth Conditions

*A. aeolicus* was grown at 85 °C in 2-liter bottles under 1.4 bars of H2/CO2 (80:20) in SME medium, modified according to Ref. 23, at pH 6.8 in the presence of thiosulfate (1 g/liter) or S2− (7.5 g/liter) and harvested in the late exponential growth phase. Typical yields were about 400 mg of cell material per liter of culture. The amount of dissolved oxygen in the medium was estimated by using the Henry equation to be in the range 0.5−0.8%. As controls, *A. aeolicus* was also cultivated first without sulfur or thiosulfate and second by using the Henry equation to be in the range 0.5–0.8%. As controls, *A. aeolicus* was also cultivated first without sulfur or thiosulfate and second in the presence of sulfur, without O2. Replacement of H2 by N2 was also performed first without sulfur or thiosulfate and second in the presence of sulfur, without O2. As controls, *A. aeolicus* was also cultivated first without sulfur or thiosulfate and second in the presence of sulfur, without O2.

#### Preparation and Solubilization of the Membrane Fraction

Cells (30 g) in 50 mM Tris/HCl, pH 7.6, 5 mM EDTA, 10 μg/ml DNase I, 5% glycerol (saturated with argon), and protease inhibitors mixtures (Roche Applied Science) were broken at 18,000–20,000 p.s.i. in a French press cell. Unbroken cells and debris were removed by centrifugation at 10,000 × g for 15 min. Membranes were pelleted by centrifugation at 43,000 rpm (rotor 45 Ti, Beckman) for 1 h. The pellet was resuspended and solubilized in 50 mM Tris/HCl, pH 7.6, 5% glycerol, 0.5% aminocaproic acid (ACA), and 1% deoxycholic acid sodium salt (buffer A) and stirred for 1 h at 25 °C under argon. The resulting suspension was centrifuged at 43,000 rpm (rotor 45 Ti, Beckman) for 1 h. At this point the supernatant contained the majority of both H2S forming activity and hydrogenase activity.

#### Purification of the Hydrogenase-Sulfur-reducing Complex

The supernatant was applied to a DEAE-52 column (4 × 20 cm, Whatman) equilibrated in buffer B (identical to buffer A except for 0.2% deoxycholic acid sodium salt). Proteins were eluted with a 50 mM step gradient of 0–350 mM NaCl. The 50–100 mM NaCl fraction contained the hydrogenase and the sulfur reductase activities. This fraction was then loaded onto a hydroxyapatite column (2 × 12 cm, Bio-Gel; Bio-Rad) equilibrated in buffer A containing NaCl at a concentration of 75 mM. The column was washed with the same buffer, and proteins were then eluted with a 50 mM step gradient of 0–300 mM potassium phosphate. The 250 mM phosphate fraction was dialyzed overnight and loaded on MonoQ column (FPLC, Amersham Biosciences). Proteins were eluted by a gradient of NaCl from 0 to 500 mM. Active fractions were concentrated, stored anaerobically, and frozen in liquid nitrogen. All steps were performed at room temperature under argon.

#### N-terminal Sequence Determination

The N-terminal amino acid sequences were determined from the enzymatic complex preparation after separation of the subunits by SDS-PAGE. After electrophoresis on 10% polyacrylamide gel under denaturing conditions, proteins were transferred onto polyvinylidene difluoride membrane for 45 min at a current intensity of 0.8 mA/cm2 using a semi-dry electrophoretic transfer unit. Sequence determinations were carried out with an Applied Biosystems Procise 494 microsequencer. Quantitative determination of phenylthiodyantoin derivatives was done by high pressure liquid chromatography (Waters) monitored by a data and chromatography control station (Waters).

#### Enzyme Activities

The sulfur reductase (SR) activity was followed by the evolution of the H2S formed by the reduction of elemental sulfur with H2. The reaction mixture contained 10 mM Tris/HCl, pH 7.6, and 5% sulfur flower, 40 μM 2-OH-1,4-naphthoquinone, and 20 μM NADPH. Assays were performed in 5-ml vials sealed with rubber stoppers under argon atmosphere. After 10 min of incubation under H2, the enzyme was injected, and the vials were incubated at 85 °C under H2 for 20 min. H2S concentration was then determined by the following method: first, 100 μl of 30 mM FeCl3 in 1.2 mM HCl were added to the vial to stop the reaction, second, 50 μl of 20 mM N,N-dimethyl-p-phenylene diamine dihydrochloride in 7.2 mM HCl were added to generate the methylene blue. After mixing, the test tube was incubated 20 min at room temperature in the dark. Samples were centrifuged to remove solid sulfur, and spectrophotometric measurements of the methylene blue formed were done at 670 nm. A calibration curve had been made previously using Na2S (30). As this method does not take account the loss of sulfide through reactions with sulfur, the activities determined are underestimated. One unit of SR activity corresponds to the uptake of 1 μmol of H2S/min.

The standard assay mixture without sulfur was also used to assay for reduction of alternative electron acceptors: TMAO, Me2SO, S2O3−2, S4O6−6, and polysulfide. NADPH and NADH were used also as electron donors. The following extinction coefficients (mM−1·liter−1) were used: methyl viologen (MV), 13.6 at 604 nm; NADH and NADPH, 6.2 at 340 nm.

#### Hydrogenase activity was determined at 80 °C by the hydrogen consumption and production assays as previously described (22). One unit of hydrogenase activity corresponds to the uptake of 1 μmol of H2/min.

#### Analytical Procedures

ICP-MS-Analyses of metals (molybdenum, iron, sulfur, and nickel) were performed using inductively coupled plasma-mass spectrometry (ICP-MS) on purified complex hydrogenase/SR.

### Molybdenum Cofactor Analysis

Purified complex hydrogenase/SR was demolybdenated with 1% SDS followed by boiling for 45 min. The SDS was precipitated with KCl (0.25 M final concentration). The cofactor was separated from the protein utilizing microcon Amicon (3000 molecular weight cut-off). The presence of cofactor was determined by fluorescence spectroscopy using a Cary Eclipse spectrofluorometer. Excitation spectra were obtained using an emission wavelength of 460 nm, and emission spectra were obtained using an excitation wavelength of 370 nm with a slit of 5 nm. Similar experiments were done with TMAO.
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reductase from *Escherichia coli* purified as described previously (31) and used as a control.

**Low Temperature Spectra**—Low temperature spectra were recorded in the range of 350–700 nm at liquid nitrogen temperature (77 K) using a dual wavelength 2000 SLM-Amicon spectrophotometer with a slit of 0.2–0.4 nm. The experiments were performed with 0.4 mg/ml proteins in 50 mM Tris/HCl, pH 7.6. Reduction of the samples was achieved by addition of sodium ascorbate and sodium dithionate.

*Native Gel*—The purified complex was loaded on a native 4% polyacrylamide stacking, 10% running gel (Mini-Protean II; Bio-Rad). After migration, hydrogenase activity was revealed on the gel as described previously (22).

*Denaturing Gel Electrophoresis*—100 μg of purified complex was loaded on a 4% polyacrylamide stacking, 10% running SDS gel (Protean II XL cell; Bio-Rad). After migration, the gel was silver-stained as described previously (32) with the Vorum protocol slightly modified. Ten bands were excised from the gel and stored at 20 °C before mass spectrometry analysis.

**Immunoblotting**—Western blotting was performed using standard procedures. Anti-DmsA antibodies against the Me₅SO reductase chain A from *E. coli* (33) and anti-hydrogenase antibodies against the hydrogenase from *Desulfovibrio fructosovorants* (34) were used. The detection reaction was performed using goat peroxidase-conjugate anti-rabbit IgG (Sigma) and the ECL detection reagents (Amersham Biosciences).

**Tryptic Digestion, Mass Spectrometry, and Data Base Search**—Digestion of excised gel plugs was performed as described previously (22). Protein identification was performed by peptide mass fingerprinting on a MALDI-TOF-MS mass spectrometer DE-RP (Applied Biosystems) exploiting the nonredundant data base NCBInr (National Institutes of Health, Bethesda) using MASCOT search.

**Transmission Electron Microscopy Analysis**—*Aquifex* cultures (800 ml) were centrifuged (15,000 g for 10 min), and cells were rinsed once with fresh media and centrifuged again. The yellow sulfur powder was almost totally removed from the sulfur sample. Then they were fixed for 1 h in 2.5% glutaraldehyde in phosphate-buffered saline and for 1 h in 2% OsO₄ in phosphate-buffered saline (35). The pellets were dehydrated in alcohol and embedded in Epon (Embed 812). Ultrathin sections, obtained with microtome Leica EM FC/UC6, were stained with uranyl acetate and lead citrate and further observed by using a Zeiss EM109 electron microscope. Scanning transmission electron microscopy (STEM), including high angle annular dark field (HAADF) and energy-dispersive x-ray spectroscopy (EDXS), has been done on an unstained ultrathin section using a Jeol 3010F equipped with a Noran spectrometer. Acceleration voltage of 300 keV and electron probes sized down to 1 nm have been used to image the ultrathin section.

**Sequence Alignment**—Multiple sequence alignments were done using ClustalW (36) at pbil.univ-lyon1.fr/.

**RESULTS**

**Growth of *A. aeolicus* with Elemental Sulfur or Thiosulfate**—In this work, we show that *A. aeolicus* can grow in an H₂/CO₂/O₂ atmosphere with S⁰ with a high final cell concentration (A₅₆₀ = 1.2 on S⁰ against A₅₆₀ = 0.8 on S₂O₃⁻) (Fig. 1). Moreover, in *A. aeolicus* culture containing elemental sulfur, we have detected H₂S at the end of the exponential phase in the medium (data not shown) suggesting a possible sulfur respiration in this bacterium. *Aquifex* thus contains a terminal sulfur reductase (SR). H₂S was not detected when bacteria grew with thiosulfate instead of sulfur. As a control, we have cultivated *A. aeolicus* in presence of sulfur and O₂ (0.5–0.8%) under N₂/CO₂ (80:20) atmosphere. No growth was obtained in this condition. This result confirms a link between the hydrogen and S⁰ consumption in the *A. aeolicus* metabolism. No significant growth was detected under a strict anaerobic condition. This result is in line with the microaerophilic properties of most of the Aquificales. Typical growth patterns of *A. aeolicus* in SME medium with either thiosulfate or elemental sulfur are shown in Fig. 1. The doubling time is 108 min with S⁰ and 84 min with S₂O₃⁻. Sterile controls indicated no significant chemical reduction of elemental sulfur during incubation. In cultures containing sulfur, some of the cells may have attached and grown on the insoluble sulfur particles surface. We may thus have underestimated the cell number to some extent, because only suspended cells were measured in this culture. This could explain the lag phase observed when the bacterium grows on sulfur. During the stationary phase, the cell concentration is constant on S⁰ unlike growth on S₂O₃⁻, where the absorbance drops rapidly at the end of the exponential phase. Microscopic observations showed that the bacteria tend to aggregate during the stationary phase when grown on S₂O₃⁻. With 5 or 10 times more S₂O₃⁻ in the medium (5 and 10 g/liter), the same final cell concentration (A₅₆₀ = 0.8) is reached, suggesting that thiosulfate is not the limiting substrate in these conditions (data not shown).

**Microscopy and X-ray Analyses**—*A. aeolicus* cells cultured with thiosulfate or elemental sulfur (harvested at the end of exponential phase) were subjected to optical and transmission electron microscopic observations. Micrographs show clearly the presence of black and white shiny refractive round particles associated with the cells grown with S⁰ only (Fig. 2, A and B). The same particles are also present when the cells grow with S⁰ and thiosulfate at the same time (data not shown). These particles look very similar to the “sulfur globules” already described and easily detectable by microscopy in many organisms (37, 38). Transmission electron microscopy on section preparations also revealed inclusions in the cytoplasm of cells coming from S⁰ cultures (Fig. 2, C–F). The thiosulfate samples lacked these inclusions. These observations indicate a different metabolism between the cells grown on the two media (S⁰ and S₂O₃⁻). To determine the granule composition, x-ray microanalysis (EDXS) was performed on unstained ultrathin sections from sulfur-grown cells, in different areas of the cell corresponding to inclusions (Fig. 3, granular zone 1) and to the rest of the cell (Fig. 3, nongranular zone 2, including cytoplasm and periplasm). It is clear that EDXS spectra from zones 1 and 2 are the same except for the presence of a peak for sulfur in the spectrum from the inclusions. The carbon, oxygen, chlo-
rine, and silicon peaks arise primary from the embedding Epon. A small copper peak is because of the TEM grid (we used a beryllium sample holder to limit the copper contribution). The osmium was associated with cell fixation and is more strongly detected in connection with the globules. Spectra indicated that the globules are composed of sulfur. The amount of sulfur detected is very low (after absorption correction, an overall sulfur/carbon ratio of around $5.10^{-4}$ was found), and no reliable estimation of the sulfur/carbon and sulfur/oxygen ratios in the granules could be obtained. Attempts to image the sulfur using energy-filtered electron microscopy or to determine the nature of the sulfur bonding by electron energy loss spectroscopy have not been successful because of the very small amount of sulfur available for the analysis.

This average low sulfur concentration also demonstrates that the brightest cytoplasmic areas are not homogeneous globules of several

FIGURE 2. Observation of cytoplasmic inclusions in *A. aeolicus* grown with elemental sulfur. A and B, optical micrographs of whole *A. aeolicus* cells grown with thiosulfate (A) or elemental sulfur (B) showing the characteristic shiny sulfur globules associated with the cells in the culture on S°. The bar represents 5 μm. C and D, transmission electron micrographs of *A. aeolicus* cultured with thiosulfate, lacking the inclusions. E and F, transmission electron micrographs of *A. aeolicus* sulfur-grown cells showing internal inclusions in the cytoplasm (some are indicated by arrows).

FIGURE 3. The internal inclusions present in the cytoplasm of *A. aeolicus* grown with elemental sulfur contain sulfur. A, STEM-HAADF image from an unstained ultrathin section of sulfur-grown *Aquifex* showing the presence of cytoplasmic inclusions. The scale bar represents 100 nm. Areas analyzed by EDXS are named zone 1 (corresponding to an inclusion) and zone 2 (corresponding to a portion of cytoplasm and periplasm). B, EDXS spectra from Epon; zone 1 and zone 2 (from A) indicate the presence of sulfur in zone 1 only.
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TABLE ONE
Hydrogenase and sulfur reductase activities of solubilized A. aeolicus membranes

| Preparation          | Hydrogenase | Sulfur reductase |
|----------------------|-------------|-----------------|
|                      | Specific activity | Total activity | Yield | Enrichment | Specific activity | Total activity | Yield | Enrichment | Total proteins |
| Membranes            | 0.06 units/mg | 150 units       | 100%   | 1 %        | 0.09 units/mg | 225 units       | 1 %   | 120 %      | 2500 mg       |
| Solubilized proteins | 0.15 units/mg | 130 units       | 87     | 2.5 %      | 0.25 units/mg | 217 units       | 96 %  | 110 %      | 867 mg        |
| DEAE (75 mM NaCl)    | 0.9 units/mg  | 108 units       | 72 %   | 15 %       | 1 units/mg    | 120 units       | 53 %  | 11 %       | 120 mg        |
| HTP (250 mM phosphate)| 3.5 units/mg  | 17.5 units      | 12     | 58 %       | 11 units/mg   | 55 units        | 24 %  | 122 %      | 5 mg          |
| MonoQ (flow-through)| 8.75 units/mg | 3.5 units       | 23     | 142 %      | 13.5 units/mg | 5.4 units       | 2.4%  | 150 %      | 0.4 mg        |
| MonoQ (350 mM NaCl)  | 17 units/mg   | 2 units         | 1.3    | 283 %      | 26 units/mg   | 3.1 units       | 1.4%  | 289 %      | 0.12 mg       |

TABLE TWO
Yields and enrichments of the hydrogenase and SR activities

| Preparation          | Hydrogenase | Sulfur reductase |
|----------------------|-------------|-----------------|
|                      | Specific activity | Total activity | Yield | Enrichment | Specific activity | Total activity | Yield | Enrichment | Total proteins |
| Membranes            | 0.06 units/mg | 150 units       | 100%   | 1 %        | 0.09 units/mg | 225 units       | 1 %   | 120 %      | 2500 mg       |
| Solubilized proteins | 0.15 units/mg | 130 units       | 87     | 2.5 %      | 0.25 units/mg | 217 units       | 96 %  | 110 %      | 867 mg        |
| DEAE (75 mM NaCl)    | 0.9 units/mg  | 108 units       | 72 %   | 15 %       | 1 units/mg    | 120 units       | 53 %  | 11 %       | 120 mg        |
| HTP (250 mM phosphate)| 3.5 units/mg  | 17.5 units      | 12     | 58 %       | 11 units/mg   | 55 units        | 24 %  | 122 %      | 5 mg          |
| MonoQ (flow-through)| 8.75 units/mg | 3.5 units       | 23     | 142 %      | 13.5 units/mg | 5.4 units       | 2.4%  | 150 %      | 0.4 mg        |
| MonoQ (350 mM NaCl)  | 17 units/mg   | 2 units         | 1.3    | 283 %      | 26 units/mg   | 3.1 units       | 1.4%  | 289 %      | 0.12 mg       |

hundreds of nanometers consisting of either sulfur- or thiosulfate-related compounds. Sulfur molecules or unrevealed nanometer scaled aggregates may be strongly diluted in this sulfur-rich area. Evidence for Sulfur Reducing Activity in the Aquifex Membrane—As A. aeolicus can produce hydrogen sulfide, we have searched for a sulfur reductase activity in the cells. SR activity was routinely measured under a hydrogen atmosphere (used as electron donor) in an assay mixture containing sulfur powder (used as electron acceptor). The measure of H2S produced was representative of the catalysis. After centrifugation of the cell extract, the sulfur reductase activity was found to be associated with the membrane fraction, whereas soluble fraction was inactive. In the intact membrane fraction, the hydrogenase specific activity was 0.06 units/mg, and the sulfur reductase specific activity was 0.09 units/mg. Various conditions have been used to solubilize the SR enzyme activity. Membranes were treated with different detergents and “protective reagents” (to prevent dissociation) to determine the optimal conditions for solubilization and stabilization of the sulfur reducing activity (TABLE ONE). Whatever the detergent used (n-dodecyl β-D-maltoside and sodium deoxycholate) and regardless of the presence of glycerol, phosphatidylcholine, or ACA as protectors, the specific activity obtained for hydrogenase activity was in a same order range (TABLE ONE). SR activity was recovered only when membranes were solubilized by sodium deoxycholate in the presence of ACA and glycerol. We can conclude that the enzymes required for the electron transport from hydrogen to S- are present in A. aeolicus membranes.

We have obtained evidence for the presence of a sulfur reducing activity in the solubilized membranes of A. aeolicus grown with S- or with S2O3. However, extracts of cells grown with elemental sulfur showed a specific sulfur reducing activity 10 times higher than the specific activity measured with cells grown with thiosulfate (TABLE ONE). In contrast, no major difference between the two media was observed in hydrogenase activity. We thus decided to use cells cultured on H2/S- medium to work on the sulfur reducing activity.

Purification of a Membrane-bound Sulfur-reducing Complex—A complex transferring electrons from hydrogen to sulfur was purified by DEAE, hydroxylapatite, and MonoQ ion exchange chromatography in the presence of sodium deoxycholate, ACA, and glycerol (TABLE TWO). Various fractions had hydrogenase-SR activity or hydrogenase activity alone. The heterogeneity could be explained by the presence of two membrane-bound hydrogenases and the loss of one or more proteins without catalytic activity but that modulate the hydrogenase-SR activity or stability. Only the fractions with higher activity (hydrogenase-SR and hydrogenase) were combined to give the two final preparations. This, however, resulted in a low yield. The sulfur reductase activity was purified 290-fold compared with the cell-free extract, with an overall yield of 1.5% (TABLE TWO).

Native gel electrophoresis on the purified fraction showed one major band associated with hydrogenase activity, indicating a homogeneous complex. Loading of this fraction on a Superose 6 gel filtration column gave a complex with a molecular mass around 600 kDa but with less activity (Fig. 4). We have tried to purify SR alone without any success. Although hydrogenase I could be purified alone (the large and the small subunit), or with cytochrome b1, or associated with cytochrome bc1 complex, SR by itself could not be purified.

Identification of the Complex Components—The constituents of the sulfur-reducing complex were identified first performed by gel electrophoresis separation and mass spectrometry analysis. The fraction obtained after purification was loaded onto a 10% denaturing gel, which was silver-stained as described under “Experimental Procedures” (Fig. 4B). Ten bands were cut out, the proteins digested by trypsin, and the peptides analyzed with a MALDI-TOF spectrometer, and nine proteins were identified. The majority of these proteins is known to be generally involved in electron transport chains or in energy conservation.

Membrane-bound hydrogenase I and hydrogenase II (small and large subunits) are present in the transport electron chain (22). Another protein with a molecular mass of approximately 110 kDa has been identified.
as the product of \textit{aq.1234} predicted to encode the \textit{Me}_{2}SO reductase chain \textit{A} (DmsA). \textit{aq.1234} is part of a putative operon containing two other genes \textit{aq.1232} (predicted as \textit{dmsB1}) and \textit{aq.1231} (predicted as \textit{dmsC}) coding for a small hydrophilic subunit and a hydrophobic membrane anchor, respectively. As no \textit{Me}_{2}SO reductase described to date indicated hydrogenase I and hydrogenase II.

Ten bands, indicated by arrows, were analyzed by MS (dotted lines), the mass spectrosopy data are given as supplemental material and/or N-terminal sequencing after transference. The cytochrome \textit{c}, and the cytochrome \textit{b} from the \textit{bc} complex are also part of the SR complex (28). The Rieske protein has not been identified in this experiment.

Sequence determination realized after transfer confirms the presence of hydrogenase I and hydrogenase II in the complex. The N-terminal sequence of SreB subunit (MPQYALV . . . ) corresponding to the deduced amino acid sequence from \textit{sreB} gene is also identified. Apparently, some of the bands on the gel did not originate from individual subunits but were formed by aggregates or more likely by sample buffer-resistant subcomplexes. Thus, the SreB protein sequence is found associated with one hydrogenase large subunit (N-terminal sequences of hydrogenase I and II large subunits are identical up to 20 residues). We did not obtain the N-terminal sequence of the SreA subunit, but we detected it with antibodies against DmsA from \textit{E. coli} by Western blotting experiments (data not shown).

The cytochromes \textit{b} and \textit{b}^{III}, electron partners of hydrogenase I and II, respectively, were not detected by mass spectrometry. However, as we can detect an electron transfer from hydrogenase to SR, these cytochromes must be present. In order to test the presence of cytochromes \textit{b}, we have performed low temperature absorption spectra of the hydrogenases-SR complex and the hydrogenase-\textit{bc} complex (Fig. 5). In the hydrogenase I-\textit{bc} complex sample, in the \textit{a} region, one peak is present at 557.2 nm with two shoulders at 555 and 561, and a second one is present at 548.5 nm. The first corresponds more likely to cytochrome \textit{b} from the \textit{bc} complex described by Schütz \textit{et al.} (28) and to cytochrome \textit{b}^{III} from the hydrogenase I complex (22). The second peak, with a more positive redox potential, corresponds to cytochrome \textit{c}.

Low temperature spectra of the SR complex show a new peak at 554 nm, which could correspond to cytochrome \textit{b}^{III} from the hydrogenase II complex. This peak appears only after reduction by dithionite sodium demonstrating the low redox potential of this cytochrome. The bands characteristic of the molybdenum cofactor are not detectable because of the interference of \textit{a} bands from cytochromes \textit{b}.

\textbf{Molybdenum Cofactor Analysis—} The use of a molybdopterin cofactor to catalyze the sulfur reduction is intriguing because this cofactor normally catalyzes oxygen atom transfer, hydrogen atom transfer, or a combination of the two (39, 40). The SR enzymes form a specific group distinct from other molybdopterin-guanine dinucleotide-dependent enzymes in the \textit{Me}_{2}SO reductase family (41).

ICP-MS metal analysis revealed the presence of iron, sulfur, nickel, and molybdenum in enriched sulfur reductase fractions only (data not shown). The molybdenum cofactor in the sulfur reductase complex was quantified by fluorescence, and we used purified TMAO reductase (TorA), a well characterized molybdenum cofactor-containing enzyme of \textit{E. coli}, as a standard. The fluorescence excitation and emission spec-
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tra of the cofactor released from the sulfur-reducing complex and from TorA are shown in Fig. 6. Spectra for TorA exhibit maxima at 370 and 450 nm for excitation and emission, respectively, similar to those usually described for the molybdenum cofactor (42). For the cofactor isolated from the sulfur reductase complex, we can observe very similar spectra with a shift toward lower wavelengths for the excitation maximum, detected at 325 nm. Comparison of the fluorescence intensity with the control TorA allowed us to calculate that \( \frac{1}{2.2} \mu M \) of cofactor is present in \( 2.2 \mu M \) of SR complex.

Genetic Organization and Sequence Analysis—The gene cluster encoding the SR consisted of three open reading frames, sreA, -B, and -C (aq_1234, aq_1232, and aq_1231, respectively). The deduced amino acid sequences from the three SR proteins are similar to those of the molybdoprotein family, which includes the Me_2SO reductases, formate dehydrogenases, nitrate reductases, and sulfur reductases (43, 44). In this predicted operon, there were no genes encoding a specific chaperone like TorD from E. coli or SreE from A. ambivalens (18, 45). By using the amino acid sequence of the Aquifex Sre chain A for a BLAST search,
we found homologous molybdoproteins, including a typical selenocysteine-containing anaerobic dehydrogenase from *Thiobacillus denitrificans* ATCC 25259 (48.9% identity) and a protein annotated Me$_2$SO reductase chain A from *Mesorhizobium loti* (48.5% identity), whose cellular function is still unknown. 20 and 18% of identity have been found with the catalytic subunit PsrA of the polysulfide reductase from *W. succinogenes* and with SreA of the sulfur reductase from *A. ambivalens*, respectively. Unlike PsrA and SreA, the N terminus of Aquifex SreA, like those from the proteins most similar to it (from *T. denitrificans, M. loti, Magnetospirillum magnetotacticum, Rubrivivax gelatinosus, Magneto- coccus*, and *Burkholderia*), does not contain either a typical twin arginine motif or a signal peptide suggesting that the proteins are located in the cytoplasm (46). Molybdoenzymes of the Me$_2$SO reductase family are organized in four functional groups according to their sequence homology and catalytic specificity. One of these groups is made up of thiosulfate, tetrathionate, polysulfide, and sulfur reductases. Sequence comparison between the members of this group showed that a cysteine residue is conserved and could coordinate the molybdenum atom (41, 47). This conserved cysteine is also found in SreA from *A. ambivalens* (Cys-176) (Fig. 7). However, this sequence region is significantly different from other enzymes belonging to this group. As found in several other molybdoenzymes, a cysteine-rich motif is present in SreA. However, this Fe/S cluster binding site is different (Cys-176) from those described in other molybdoproteins belonging to class I (48). One additional cysteine residue (Cys-56) is present compared with the classical motif, and the fourth cysteine is not present.

The amino acid sequence derived from the sreB nucleotide sequence is predicted to carry four [4Fe-4S] clusters and is very similar to the FeS proteins from *M. magnetotacticum, Magnetococcus, Burkholderia, R. gelatinosus,* and *T. denitrificans* (51–58% identity). The identity with chain B from the sulfur reductases from *W. succinogenes* and *A. ambivalens* (PsrB and SreB) and the Me$_2$SO reductase from *E. coli* (DmsB) is around 27–29%.

The gene *ag_1231* encodes a hydrophobic putative membrane protein of 39 kDa. Analysis of the amino acid sequence by several algorithms suggested that the protein has eight transmembrane helices. *Aquifex* SR chain C is similar to a hydrophobic protein annotated Me$_2$SO reductase anchor subunit of *T. denitrificans* (28% identity). The percentage of identity with the anchor subunit of the enzymes from *W. succinogenes, A. ambivalens,* and *E. coli* (DmsC) is 17, 14, and 19%, respectively. SreC very likely provides an anchor for both SreAB and the quinone binding/oxidation site. The residues His-65 and Glu-87, which have been demonstrated as involved in quinone fixation in DmsC variants from *E. coli,* are conserved in SreC from *A. aeolicus* (49). In *E. coli* DmsC, these residues are located at the end of helix two and close to the periplasmic side of the cell membrane. Topology prediction for SreC from *A. aeolicus* proposes that the N and C termini are located on the cytoplasmic side of the cell membrane, and the potential quinone-binding site is also located close to the cytoplasmic side of the membrane. This orientation is probably in line with the cytoplasmic location of the sulfur reductase catalytic subunit, which is different from the periplasmic location of Me$_2$SO reductase (subunit A) from *E. coli*.

**Catalytic Properties**—Hydrogenase catalyzes quinone reduction by hydrogen in various systems (50). In *A. ambivalens*, the sulfur reducing activity was lost in solubilized membrane, and activity was restored by adding quinone or cytochrome c (18). If no modification of sulfur reducing activity was observed in the presence of the soluble or membrane-bound cytochrome $c_{555}$ from *A. aeolicus,* addition of quinone in the reactional medium increased the sulfur reducing activity. As the quinone pool of *Aquifex* was determined to be naphthoquinone (28), we have used 1,4-hydroxynaphthoquinone ($E^0 = −145$ mV). The addition of NADPH increases the sulfur reducing activity, but in our experimental conditions (80°C, 20 μM NADPH) NADPH alone cannot be used as an electron donor (TABLE THREE). Spectroscopic measurement of NADPH cannot be detected because of the presence of the sulfur. No effect of NADH was detected. The hydrogenase activity of the isolated

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**TABLE THREE**

**Catalytic properties of the sulfur-reducing complex**

The reaction catalyzed by the complex is $H_2 + S_2\text{O}_3^{-} + S_2\text{O}_4^{-} + H^+$ → $S_2\text{O}_3^{-} + H_2$. Various electron donors (H$_2$, NADPH, or NADH) have been tested. Depending on the type of activity assayed (hydrogenase or sulfur reductase), S’ or MV have been used as an electron acceptor. Carbon monoxide (CO) is a specific inhibitor of hydrogenases. The reactions have been made in the presence or absence of NADPH. The following abbreviations are used: MV$_{ov}$ oxidized methyl viologen; MV$_{red}$, reduced methyl viologen. One unit of SR activity corresponds to the uptake of 1 μmol of H$_2$/min, and 1 unit of hydrogenase activity corresponds to the uptake of 1 μmol of H$_2$/min.

| Gas phase | Electron donor | Electron acceptor | Reaction | NADPH | Specific activity |
|-----------|---------------|-------------------|----------|-------|------------------|
|           |               |                   |          |       | units/mg          |
| H$_2$/Ar  | H$_2$         | S'                | S':H$_2$S| −     | 0.3 ± 0.1        |
| H$_2$/Ar  | H$_2$         | S'/Ar             | S':H$_2$S| +     | 1 ± 0.1          |
| H$_2$/Ar  | H$_2$         | MV                | H$_2$2H$^+$| −   | 0.3 ± 0.03       |
| H$_2$/Ar  | H$_2$         | MV                | MV$_{ov}$MV$_{red}$ | + | 0.3 ± 0.02 |
| H$_2$/Ar + CO | H$_2$  | S'                | S':H$_2$S| +     | 0.1 ± 0.02       |
| H$_2$/Ar + CO | H$_2$  | MV                | MV$_{ov}$MV$_{red}$ | + | 0.1 ± 0.02       |
| Argon     | NADPH         | S'                | S':H$_2$S| −     | NADPHCNADP       |
| Argon     | NADPH         | MV                | MV$_{ov}$MV$_{red}$ | + | NADPHCNADP       |
| Argon     | NADPH         | S'                | S':H$_2$S| +     | NADPHCNADP       |
| Argon     | NADPH         | MV                | MV$_{ov}$MV$_{red}$ | + | NADPHCNADP       |
Sulfur-reducing Complex from the Hyperthermophile *Aquifex*

**TABLE FOUR**

| Substrate specificity of the *Aquifex* sulfur reductase enzyme |
|---------------------------------------------------------------|
| The reaction of the molybdenum sulfur reductase has been assayed with different substrates: S\(^{\circ}\), tetrahionate (S\(_{4}\)O\(_{6}\)), thiosulfate (S\(_{2}\)O\(_{3}\)), TMAO, and Me\(_{2}\)SO. The reaction is made in the absence of hydrogen and the presence of carbon monoxide (CO) so that hydrogenase is not active and in the presence of NADPH. Reduced benzyl viologen is directly used as the electron donor at the catalytic site of the SR. 100% of activity corresponds to the maximal activity obtained in micromoles of BV oxidized per min/mg of protein. One unit of SR activity corresponds to the uptake of 1 \(\mu\)mol of H\(_{2}\)/min. DMS is dimethyl sulfate; TMA is trimethylamine. |
| Electron acceptor | Reaction | NADPH | Activity % |
|-------------------|----------|-------|------------|
| S\(^{\circ}\) | S\(^{\circ}\):H\(_{2}\)S | − | 35 |
| S\(^{\circ}\) | S\(^{\circ}\):H\(_{2}\)S | + | 75 |
| S\(_{2}\)O\(_{6}\) | S\(_{2}\)O\(_{6}\):H\(_{2}\)S | − | 75 |
| S\(_{2}\)O\(_{4}\) | S\(_{2}\)O\(_{4}\):H\(_{2}\)S | + | 100 |
| S\(_{2}\)O \(_{3}\) | S\(_{2}\)O\(_{3}\):H\(_{2}\)S | − | 0 |
| S\(_{2}\)O \(_{2}\) | S\(_{2}\)O\(_{2}\):H\(_{2}\)S | + | 0 |
| TMAO | TMAO:TMA | − | 18 |
| TMAO | TMAO:TMA | + | 18 |
| Me\(_{2}\)SO | Me\(_{2}\)SO:DMS | − | 0 |
| Me\(_{2}\)SO | Me\(_{2}\)SO:DMS | + | 0 |

Sulfur reductase complex was also measurable with hydrogen as electron donor and MV as electron acceptor. When CO, which is a specific inhibitor of hydrogenases, was added in the reaction medium and hydrogen was used as electron donor, no hydrogenase activity was detected with MV as electron acceptor, and no sulfur reduction activity was detected using S\(^{\circ}\) as electron acceptor (even in presence of NADPH), indicating that hydrogenase has to be active to have a sulfur reductase activity in these conditions. The bi-directional and oxygen-stable hydrogenase I, purified separately, did not have sulfur reductase or sulfhydrogenase activity with H\(_{2}\) or NADPH as electron donor. We can conclude that an active hydrogenase-sulfur reductase supercomplex exists, which transfer electrons from hydrogen to sulfur. The effect of temperature on SR activity was assayed with H\(_{2}\) as electron donor and sulfur as electron acceptor as follows: 100% activity was found at 80 °C, 75% at 50 °C, and only 8% at 25 °C. In contrast, no activity was detectable below 40 °C with Sre from *A. ambivalens* (18).

Some substrates have been tested with hydrogen as electron donor, and H\(_{2}\)S production was measured. In these conditions, we found that elemental sulfur and polysulfide are reduced, both with activities of about 85% that for tetrahionate reduction. No activity was measured with thiosulfate as electron acceptor. Similar experiments were done using reduced BV as electron donor (in the presence of quinine and NADPH). Oxidation of this molecule was followed spectrophotometrically, and we have slipped the hydrogen production or consumption activity from the complex by using CO under argon atmosphere (TABLE FOUR). The sulfur reductase complex does not catalyze Me\(_{2}\)SO reduction, but it has an activity with TMAO. As when hydrogen is the electron donor, only tetrahionate, polysulfide, and sulfur can be used as electron acceptors.

In summary these data suggest that there is an energetic coupling between hydrogen oxidation by the hydrogenase and sulfur reduction, as in *A. ambivalens* and *W. succinogenes* (8, 16, 18, 51). Two separate complexes associated in a stable and functional supercomplex are present in *Aquifex* and *Acidianus* membranes. The electron transfer between the hydrogenase complex and the SR complex is mediated by quinones, and unknown proteins are probably involved in the stability or the functionality of this molecular edifice.

**DISCUSSION**

Existence of a Supercomplex Involved in Sulfur Respiration in *A. aeolicus* Membranes—We have purified a respirasome coupling hydrogen oxidation and sulfur reduction in the presence of quinone. By a proteomic approach, we have demonstrated that this complex of 600 kDa includes hydrogenase I complex, the molybdoenzyme annotated Me\(_{2}\)SO reductase in *Aquifex* genome and renamed Sre, hydrogenase I complex, cytochrome bc\(_{1}\) complex, and other proteins of unknown function. The supercomplex involved in hydrogen-sulfur pathway is thus purified in association with a complex containing enzymes involved in the hydrogen-oxygen pathway (hydrogenase I complex and bc complex) (22, 28, 52). The occurrence in vivo of specific interactions among the respiratory complexes has been long suspected because they can be isolated in stoichiometric supramolecular assemblies that are stable and functionally active. The existence of respirasome-like supercomplexes has been described in yeast, mammals, and plants, as well as a variety of bacterial respiratory chains (53). The term “respirasome” was suggested because these supercomplexes can autonomously carry out respiration in the presence of ubiquinone and cytochrome c (54).

To our knowledge, the only complex from *A. aeolicus* characterized to date is complex I (55). Studies by electron microscopy and single particle analysis of this complex have shown a better preservation of the structure of its peripheral arm and may indicate that complex I is more stable than that from the other species (27). This unusual stability is probably an adaptation to high temperatures and can explain the presence of a supercomplex in this bacterium. A variety of theories have been advanced to explain the enhanced stability of proteins from thermophilic or hyperthermophilic organisms in terms of their amino acid composition, hydrophobic packing in protein cores, and oligomerization (56). Recently, it has been proposed on the basis of neutron scattering experiments on various bacteria that hyperthermophilic proteins may achieve hyperthermostability by resilience rather than rigidity. Association between complexes involved in the same bioenergetic pathway not only enhances the global flux but also increases the resilience of the system and thus the stability at high temperatures (57–59). As in the complete respirasome comprising complexes I, III, and IV isolated from *Paracoccus denitrificans*, we can also conclude that structural stabilization of a labile membrane protein complex is a major function of supercomplex formation (60).

Moreover, in addition to structural stabilization of labile membrane proteins, supercomplex formation seems to have a major function in substrate channeling. In *Aquifex*, because of the diversity of the putative energetic substrates (19, 21), the association of various complexes organized in supercomplexes is a possible level of regulation of electron partitioning (54).
Sulfur Reductase of Aquifex—According to amino acid sequences, the SR subunits belong to the Me$_2$SO reductase family of molybdo-oxidoreductases. Over the last few years, it has been established that enzymes of Me$_2$SO reductase family are of great importance in the electron transfer chains and also in a variety of metabolic reactions. Novel additions to this family include arsenate reductase, dimethyl sulfide dehydrogenase, and sulfur reductase (48). A recent phylogenetic analysis of the sulfur/polysulfide/thiosulfate/tetrathionate reductase amino acid sequences revealed that these proteins belong to the Me$_2$SO reductase family (18, 41, 47).

Like all members of the Me$_2$SO reductase family, SR from A. aeolicus probably possesses a bismolybdopterin-guanine dinucleotide molybdenum cofactor as all the genes implicated in its synthesis are present in the genome. In Me$_2$SO reductases, a serine residue of the protein has been identified as the ligand of molybdenum ion, whereas the corresponding ligand is a cysteine or an aspartic acid in nitrate reductases and a selenocysteine in formate dehydrogenases (44, 61). The molybdenum cofactor at the Aquifex active site is probably coordinated by a cysteine residue as in other SR. However, the presence of a serine adjacent to this cysteine in Aquifex protein and its homologues (Aquifex SreA-like proteins, Fig. 7) raises the question of the molybdenum coordination for this group of proteins. All molybdoproteins showing the higher degree of identity with SreA from Aquifex have cysteine (or selenocysteine) as putative molybdenum ligand and the atypical Fe/S cluster binding site. None of these proteins have been purified, and their cellular functions are still unknown. However, no Me$_2$SO and nitrate reductase activities have been found in membranes from Thiobacillus to date, and an H$_2$/sulfur metabolism has been described in this bacterium (62).

We have shown that A. aeolicus can grow with elemental sulfur and contains a sulfur reductase. The sulfur reducing activity (electron transfer from hydrogen to sulfur) is more important when the cells grow with sulfur than with thiosulfate. Various electron acceptors of the SR have been tested in vitro, and the results show that the enzyme seems to be specific for compounds that contain more than two sulfur atoms (tetrathionate, S$^2_4$, and polysulfide). During growth, the true substrate used by the bacteria is not known because we do not know the stability and solubility of elemental sulfur at 85 °C and whether this compound is modified by the cells before its use by the SR. When sulfur is reduced in the cytoplasm, like in Pyrococcus, it has been speculated that elemental sulfur diffuses across the cytoplasmic membrane and is readily converted to polysulfide in aqueous solutions of sulfide, a product of sulfur respiration (8). Moreover, the predominant species of polysulfide (number of sulfur atoms in the molecule) at this temperature is unknown. Me$_2$SO is not reduced by this enzyme confirming that it belongs to a distinct group, including sulfur/polysulfide/tetrathionate/thiosulfate reductases. This result could be explained by the absence of a tyrosine residue near the active site of Sre from Aquifex, which is important for the ability to utilize sulfur oxides in Me$_2$SO reductases and biotin sulfioxide reductases (63).

NADPH increases sulfur reducing activity by a factor 3–10 depending on the enzyme preparation and does not have any effect on hydrogenase activity. Because of the cytoplasmic localization of Sre from Aquifex, we can suppose a direct interaction between NADPH and the enzyme. This type of interaction has been already described in the thermophilic bacteria strain TI-1 (12), where NADH was proposed to be the electron donor of the SR. However, this enzyme seems to be different from SR from A. aeolicus as it is homodimeric, soluble, not described to contain molybdobium, and not linked with the energetic metabolism of the bacteria. To our knowledge, the only molybdenzyme member of the Me$_2$SO reductase family that presents a direct interaction with NADPH is the biotin sulfoxide reductase from Rhodobacter sphaeroides (64). The potential NADPH-binding motif described in the biotin sulfoxide reductase (Ala/Gly-Xaa-Gly-Xaa-Xaa-Ala/Gly) was present in SreA from A. aeolicus ($^{67}$AKGSSG$^{92}$). The exact role of the NADPH is not clear. It could regulate the enzyme activity or be directly involved in electron transfer. We have demonstrated that a quinone is implicated in the energetic coupling between hydrogen oxidation and sulfur reduction. In P. abyssi, a possible role of cytochrome $c$, not evidenced in A. aeolicus electron transfer, has been proposed (17). The periplasmic cytochromes $c_{555}$ and $c_{553}$ are not the electron-transferring elements between hydrogenase II and SR. This result excludes the exiting of electrons from the membrane during the transfer from one enzyme to another.

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3 V. Bonnefoy, LCB Marseilles, personal communication.
another enzyme. This is also in accordance with the possible role of these cytochromes in the electron transfer chain from the bc1 complex to cytochrome oxidase (22, 28, 52) and with the cytoplasmic localization of sulfur reduction.

**Cytoplasmic Localization of Sulfur Reduction**—The electron transfer from hydrogenase to SR is not unique to *A. aeolicus*. This pathway has been studied in *Wolinella*, but the formation of hydrogenase-SR supercomplex has been described in only two hyperthermophilic Archaea (*Acidianus* and *Pyrococcus*). The chemolithoautotrophic H2 oxidation with S- as electron acceptor requires at least two enzymes in a very short electron transport chain as follows: a hydrogenase and a sulfur reductase or polysulfide reductase (Par), both membrane-bound. In these organisms, the sulfur or polysulfide reduction occurs in the periplasm as shown by the orientation of the reductase toward the outside of the cytoplasmic membrane. Molybdenum acquisition is a cytoplasmic event, and all periplasmic molybdenoenzymes contain the sequence motif CysX(Cys)2Cys addressing the protein toward the periplasmic space via the twin arginine translocation systems (46). This motif is absent in SreA from *Aquifex* suggesting a cytoplasmic location of the sulfur reduction. This analysis is in line with the microscopy results showing sulfur granules in the cytoplasm.

Sulfur globules have been studied for years and are present in metabolically different sulfur-accumulating bacteria, including many sulfide-oxidizing organisms. The chemical nature of sulfur in the globules is still a much debated question (65, 66), and it seems that three different forms are found, depending on the metabolic group of the bacteria tested: cyclo-octasulfur (S8), polythionates, and sulfur chains. The sites of deposition of sulfur granules vary between bacteria, and may be extra- or intracellular (cytoplasmic or periplasmic). Our EDXS analysis of the cytoplasmic *A. aeolicus* granules showed that they contain sulfur. In contrast to the chemotrophic sulfur-oxidizing bacteria where sulfur granules are enclosed by a protein envelope constituted by Sgp proteins (67), we did not find proteins in the *Aquifex* genome, with significant similarities to the Sgp proteins, suggesting that if sulfur inclusions are surrounded by a protein layer in this bacterium, these proteins are different from those of the Chromatiaceae. Little is known about the function and the formation of the sulfur globules. For the bacteria *Thioploca* and *Thiornimargarita*, it is proposed that stored sulfur is used when food is in short supply (37). In bacteria like *Rhodobacter capsulatus* and the Chromatiaceae, the extracytoplasmic localization of the sulfur globules is consistent with the localization of the enzymes involved in sulfur production such as sulfide-quinone reductase or sulfate-oxidizing flavocytochrome c (26, 68, 69). In *A. aeolicus*, sulfur might be stored and used in the cytoplasm by the cytoplasmic sulfur reductase or other sulfur metabolism enzymes like the soluble sulfur oxygenase reductase. A possible role of sulfide-quinone reductase in the sulfur production cannot be ruled out, and the periplasmic location of *Aquifex* sulfide-quinone reductase needs to be confirmed experimentally because it has been proposed recently that the sulfide-quinone reductase from *Acidithiobacillus ferrooxidans* is cytoplasmic (70).

**Mechanism of Sulfur Respiration in A. aeolicus**—In *A. aeolicus*, we propose that the electron transport chain is composed of at least two enzymatic complexes most likely connected via quinones (Fig. 8). As we have purified hydrogenase I in complex with the cytochrome bc1 complex, we therefore propose that, in our multiprotein complex, the electrons used for S reduction by the sulfur reductase arise from the hydrogend cleavage by hydrogenase II. The order of genes and the nature of the cytochrome b (cyt b3) of this enzyme are unusual and have been found in very few microorganisms (22, 47, 18). The genes for the small and the large subunits of hydrogenase II are separated by two genes coding for the integral membrane cytochrome b11 and an iron-sulfur protein (annotated HdrlD, aq_961). Cytochrome b13 (lsp1 in *Acidianus*) has an arrangement of hemes different from that in cytochrome b1 (cytochrome anchoring hydrogenase I to the membrane) (71). The *A. ambivalens* hydrogenase present in the sulfur-reducing complex has the same operon arrangement as *Aquifex* hydrogenase II. It is envisaged that quinone is reduced by electrons from the cytochrome b3 subunit of hydrogenase II as in *Acidianus* and *Wolinella*. The electrons are then transferred to SreC via a possible quinone site. SreB is thought to serve as mediator of electron transfer between SreC and the catalytic subunit of SR. From the results of this study, *A. aeolicus* hydrogenase and SR both possess subunits similar in structure and properties to their homologues in *Acidianus* and *Wolinella*.

The proteins constituting the supercomplex are located in the periplasmic space (hydrogenase I and II, bc complex), in the membrane (cytochromes b, subunit C of Sre), and in the cytoplasmic space (molybdenoenzyme). These localizations allow the energetic coupling from hydrogen oxidation in the periplasm to sulfur reduction in the cytoplasm. To our knowledge, the only similar respiratory chain characterized to date showing the same electronic transfer from the periplasm to the cytoplasm is the respiratory chain from hydrogen to CoM-S-S-CoB involving a periplasmic membrane-bound hydrogenase and a cytoplasmic membrane-bound heterodisulfite reductase in some methanogenic bacteria (8, 72).

In conclusion, the results of our investigation are compatible with the existence of functionally relevant association between hydrogenase and SR, involved in the sulfur metabolism of *A. aeolicus*.

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