New Functional Sulfide Oxidase-Oxygen Reductase Supercomplex in the Membrane of the Hyperthermophilic Bacterium *Aquifex aeolicus*

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Aquifex aeolicus, a hyperthermophilic and microaerophilic bacterium, obtains energy for growth from inorganic compounds alone. It was previously proposed that one of the respiratory pathways in this organism consists of the electron transfer from hydrogen sulfide (H₂S) to molecular oxygen. H₂S is oxidized by the sulfide quinone reductase, a membrane-bound flavoenzyme, which reduces the quinone pool. We have purified and characterized a novel membrane-bound multi-enzyme supercomplex that brings together all the molecular components involved in this bioenergetic chain. Our results indicate that this purified structure consists of one dimeric bc₁ complex (complex III), one cytochrome c oxidase (complex IV), and one or two sulfide quinone reductases as well as traces of the monoheme cytochrome c₅₅₅ and quinone molecules. In addition, this work strongly suggests that the cytochrome c oxidase in the supercomplex is a ba₃-type enzyme. The supercomplex has a molecular mass of about 350 kDa and is enzymatically functional, reducing O₂ in the presence of the electron donor, H₂S. This is the first demonstration of the existence of such a respiratory carrying a sulfide oxidase-oxygen reductase activity. Moreover, the kinetic properties of the sulfide quinone reductase change slightly when integrated in the supercomplex, compared with the free enzyme. We previously purified a complete respirozyme involved in hydrogen oxidation and sulfur reduction from Aquifex aeolicus. Thus, two different bioenergetic pathways (sulfur reduction and sulfur oxidation) are organized in this bacterium as supramolecular structures in the membrane. A model for the energetic sulfur metabolism of Aquifex aeolicus is proposed.

Aerobic respiration in the mitochondria of eukaryotes involves electron transfer through membrane-embedded enzyme complexes that use NADH to reduce O₂ and generate a transmembrane proton gradient. The electron transport chain consists of the following four complexes: NADH and succinate dehydrogenases (complexes I and II), a bc₁ complex (complex III), and a cytochrome c oxidase (complex IV). In prokaryotes, however, a variety of electron acceptors and donors may be used by the same organism. The composition of bioenergetic chains can thus differ significantly from that of mitochondria. These prokaryotic pathways are far more complex than that of mitochondria and are thus flexible to adapt to environmental changes.

Aquifex aeolicus is a hyperthermophilic, chemolithoautotrophic, and hydrogen-oxidizing bacterium isolated from a hydrothermal system at 102 °C at Vulcano Island, Italy (1–3). It obtains energy for growth from an unusual electron transfer, from H₂ to O₂, but only in the presence of a source of sulfur (thiosulfate or elemental sulfur (S₀), which is intriguing (4). We have proposed the existence of unusual aerobic respiratory chains in this bacterium (electron transfer from H₂S to O₂ as well as from H₂ to O₂), at least when it is grown with S₀ (5, 6). We have also shown that it can reduce elemental sulfur with H₂, producing H₂S in these growth conditions (4). All three pathways might function simultaneously in the cell (5). Moreover, we have demonstrated that the reduction of elemental sulfur involves a multiprotein supercomplex carrying the sulfur reducing activity (4).

The idea that sequential enzymes within a metabolic pathway interact with each other to form highly organized enzymes complexes was formulated a long time ago for soluble enzymes (7). In mitochondria, much evidence suggests that the different respiratory complexes are assembled into supermolecular structures (supercomplexes) to perform their role as respirasomes. The composition, abundance, and stability vary between different organisms (8–10). Some macromolecular complexes containing respiratory or photosynthetic proteins have been found in bacteria and archaea. In most cases, they contain a bc complex and a cytochrome c oxidase or a reaction center (5, 11–16). A supramolecular assembly with NADH oxidase activity in the membranes of the bacterium Paracoccus denitrificans constitutes a complete respirasome and includes complexes I, III, and IV (17). Several roles for association between proteins of a respiratory pathway have been proposed in the literature, including substrate channeling, optimization of electron transfer, and protein stabilization (10).
New H₂S Oxidase-O₂ Reductase Respiratory Supercomplex

Previous studies on A. aeolicus membranes have revealed the possible existence of functionally relevant supercomplexes involved in pathways for H₂ oxidation and O₂ reduction and for H₂S oxidation and O₂ reduction (5). However, the precise protein composition of these complexes has not been established. Based on the probable interactions between certain of the respiratory components involved in O₂ reduction in A. aeolicus (5), we have begun a biochemical, functional, and physico-chemical characterization of the putative protein assemblies present in the membranes of this bacterium. Here, we describe the purification and characterization of an enzymatically functional and thermostable supercomplex containing all the protein components required for the electron transfer from H₂S to O₂. It includes the sulfide quinone reductase (Sqr), the bc₁ complex, and the cytochrome c oxidase enzymes, making this supercomplex the first of its kind to be recognized and analyzed. This work implies that A. aeolicus is the sole organism known to date that has two complementary energetic pathways (sulfur compounds reduction and oxidation) organized into supramolecular structures. Moreover, a modulation of properties of Sqr, depending on its environment, is suggested. A global view of the energetic sulfur pathway in A. aeolicus can be proposed on the basis of our results.

EXPERIMENTAL PROCEDURES

Growth of Bacteria

A. aeolicus VF5 was routinely grown at 85 °C in 2-liter bottles with 400 ml of medium containing only inorganic compounds, in the presence of elemental sulfur (7.5 g/liter) as described previously (4). Cells were harvested and stored at −80 °C.

Isolation of the A. aeolicus Supercomplex

Membranes (from 45 g of cells) were isolated as described previously (5). They were washed in 75 ml of 50 mM Tris-HCl, pH 7.6, 5% (v/v) glycerol, and 1.5 M NaBr for 30 min at room temperature with gentle shaking and ultracentrifuged at 45,000 rpm (Ti 45 rotor, Beckman) for 1 h at 4 °C to pellet them. They were resuspended in solubilization buffer (50 mM Tris-HCl, pH 7.6, 5% (v/v) glycerol, 750 mM aminocaproic acid) to give a protein concentration of 20 mg/ml and then solubilized with n-dodecyl β-D-maltoside (DDM) at 1% (w/v) final concentration. The solubilization was performed for 1 h, at 37 °C, with gentle shaking, and the solubilized membranes were ultracentrifuged as above. The supernatant was applied to a hydroxyapatite column (80 ml) equilibrated with 50 mM Tris-HCl, pH 7.6, 5% glycerol, 0.5% (w/v) aminocaproic acid, 0.02% (w/v) DDM (buffer A) at room temperature. The supercomplex, carrying cytochrome c oxidase activity, was followed during purification by detecting this activity directly in gel as described below. It was eluted from the first column with 300 mM potassium phosphate in buffer A. The eluate was concentrated ∼10-fold to give a volume of about 10 ml with concentrators (Vivaspun 100,000 molecular weight cutoff mem-

branes, Sartorius Stedim Biotech) and applied to a Superdex 200 gel filtration column (FPLC, 120 ml, GE Healthcare) equilibrated with buffer A at a flow rate of 0.6 ml/min. Fractions (1 ml) with cytochrome c oxidase activity were pooled and concentrated as described above. The fraction was then applied to a Q-Sepharose column (FPLC, 20 ml, Hiload, GE Healthcare) equilibrated with buffer A. Proteins were eluted by a gradient of 0–500 mM NaCl in buffer A at a flow rate of 1 ml/min. Fractions with cytochrome c oxidase activity (200 mM NaCl) were pooled, concentrated, and washed with buffer A as described above.

Isolation of A. aeolicus Sqr

Membranes were prepared as described above. Sqr was solubilized from membranes with 2% (w/v) DDM in 50 mM Tris-HCl, pH 7.6, 5% (v/v) glycerol, 750 mM aminocaproic acid (in the same conditions as for the supercomplex). The supernatant of ultracentrifugation as described above was applied on a DEAE-cellulose equilibrated with buffer A. Sqr was eluted at 150 mM NaCl in buffer A. The eluate, concentrated and washed in buffer A, was applied to a hydroxyapatite column in buffer A and eluted at 150 mM potassium phosphate in buffer A. The fraction was then applied to a Mono Q column (FPLC, 1 ml, Hiload, GE Healthcare) equilibrated with buffer A, at a flow rate of 0.5 ml/min. Fractions with Sqr activity (175 mM NaCl) were pooled, concentrated, and washed with buffer A as described above. This Sqr fraction is called Sqr₇ in the remainder of the paper.

Analytical Procedures

Electrophoresis—Proteins were separated by blue native gel electrophoresis apparatus (Bio-Rad) in all cases (5). After migration, gels were either stained with Coomassie Blue R-250, or enzymatic activities were revealed, or proteins were detected with antibodies, or c-type cytochromes were specifically revealed with 3,3′,5,5′-tetramethylbenzidine (19). Protein bands were cut out from gels and stored at −20 °C before analysis by mass spectrometry.

In-gel Enzyme Activities—Cytochrome c oxidase activity was revealed on the gels as described previously (5). Cytochrome bc₁ complex was detected at 37 °C as described previously (20). Sulfide quinone reductase activity was anaerobically revealed on the blue native gel, at high temperature, using sulfide as electron donor and quinone as electron acceptor. The assay mixture contained 50 mM BisTris, pH 7, 30 mM decyl-ubiquinone (DB), and 100 mM Na₂S. Anaerobic conditions were established by flushing the assay mixture with argon gas. Gels were incubated at 85 °C, and 1 mM 2,3,5-triphenyltetrazolium chloride was added to fix the red coloration of the bands corresponding to the Sqr.

Western Blotting—Proteins were subjected to native gels and transferred to nitrocellulose membranes (Electran) with the fast blot apparatus (Biometra) at 5 mA/cm² for 30 min. The blots were then processed and developed as described previously (21). Primary antibodies used were directed against components.
Sqr from *A. aeolicus* or against subunit 2 (CoxB) of cytochrome *c* oxidase from the bacterium *Acidithiobacillus ferrooxidans*, which we have shown here to cross-react with the terminal oxidase of *Aquifex*.

**Activity Measurements**

*Individual Enzyme Activity Assays*—All reactions were recorded on a Cary 50 Bio (Varian) spectrophotometer equipped with a Peltier-type temperature control system (single cell peltier accessory, Varian) using the purified supercomplex or the Sqr<sub>free</sub> prepared as detailed above. All activities were measured at least three times to ensure reproducibility.

Sulfide quinone reductase activity was determined as described previously (5) using sodium sulfide as the electron donor and DB as the electron acceptor. This activity has been determined at various temperatures. When used, antimycin A was added at a final concentration of 50 μM. One enzyme unit is defined as the amount of Sqr that catalyzes the reduction of 1 μmol of DB/min.

*bc<sub>1</sub>* complex activity was measured anaerobically at 289–300 nm, at 40 °C, after the reoxidation of reduced decyl-ubiquinone (DBH<sub>2</sub>) using horse heart cytochrome *c* as electron acceptor. DB was reduced with NaBH<sub>4</sub>, and a freshly prepared solution was used. The reaction mixture contained 50 mM Tris-HCl, pH 7, 50 μM ferric cytochrome *c*, 50 μM DBH<sub>2</sub>, and 0.6 mM KCN final concentration. When used, antimycin A was added at a final concentration of 0.2 mM. One enzyme unit is defined as the amount of *bc<sub>1</sub>* complex that catalyzes the oxidation of 1 μmol of DBH<sub>2</sub>/min. The *bc<sub>1</sub>* complex was also assayed, at 550 nm, after reduction of the ferric cytochrome *c*.

Cytochrome *c* oxidase activity was measured at 550 nm at 40 °C, by oxidation of horse heart ferrous cytochrome *c* as described previously (5). When used, KCN was added at a final concentration of 0.6 mM. One enzyme unit is defined as the amount of cytochrome *c* oxidase that catalyzes the oxidation of 1 μmol of cytochrome *c*/min.

The stability of the Sqr was measured following the enzyme activity after preincubation of the Sqr<sub>free</sub> and the supercomplex at 80 °C. The activity was measured at 25 °C in the spectrophotometer, as described above.

*Measurement of Oxygen Uptake*—Respiratory activities using sulfide as electron donor were determined with a Clark-type oxygen electrode (Rank Brothers oxygraph). Oxygen consumption was measured in a 3-ml reaction system at 60 °C in 50 mM Tris-HCl, pH 7, with 3–15 μg of supercomplex prepared as detailed above or solubilized membranes, supplemented (when specified) with 4 μM DB and 1 μM horse heart cytochrome *c*. Sulfide oxidase activity was started by the addition of 100 μM Na<sub>2</sub>S. When used, KCN and antimycin A were added at final concentrations of 0.6 and 0.2 mM respectively. The oxygen uptake rate was negligible in the absence of sample. All reactions (enzymatic and nonenzymatic) were measured at least in triplicate. To avoid damage to the apparatus, oxygen uptake was not determined at higher temperatures. One enzyme unit corresponds to the amount of enzyme catalyzing the consumption of 1 nmol of O<sub>2</sub>/min.

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**Spectroscopy**

*UV-visible Spectroscopy*—Spectra were recorded on a Cary 50 Bio (Varian) spectrophotometer at room temperature. Heme contents were calculated from the reduced spectra using the following adsorption coefficients, Δε<sub>552–540</sub> = 19 mm<sup>-1</sup>-cm<sup>-1</sup> for heme B and Δε<sub>560–571</sub> = 18 mm<sup>-1</sup>-cm<sup>-1</sup> for heme C (21).

*Electron Paramagnetic Resonance (EPR)*—Spectra were recorded at liquid helium temperatures with a Bruker ESP 300E X-band spectrometer equipped with an Oxford Instruments cryostat and temperature control system. Spectra were recorded on untreated (“as prepared”) and reduced *A. aeolicus* supercomplex. For that purpose, freshly prepared solution of 1 mM sodium dithionite in 20 mM Tris-HCl, pH 7.6, was added to the sample to obtain a 5 mM final concentration of the oxidant. The sample was frozen in liquid nitrogen.

*Fluorescence Spectroscopy*—The presence of FAD was determined using a Fluoromax-3 spectrofluorometer (Horiba Jobin Yvon). Emission spectra were obtained using an excitation wavelength of 375 nm with a slit of 5 nm. The sample (purified supercomplex) was diluted in 50 mM Tris-HCl, pH 7.6, 0.02% DDM at a concentration of 0.5 mg/ml.

**Mass Spectrometry**

Tryptic digestion of excised gel plugs, ion trap, and MALDI-TOF mass spectrometry protein identification were performed as described previously (5).

**Analytical Ultracentrifugation**

Sedimentation velocity experiments were performed using a Beckman XL-I analytical ultracentrifuge and an AN-60 Ti rotor (Beckman Coulter). The purified supercomplex at 3.5 mg/ml was dialyzed against buffer A at 4 °C and diluted in the same buffer for sedimentation velocity experiments, which were carried out at 1.75, 0.35, and 0.10 mg/ml. A volume of about 100 μl, for the most concentrated sample, or 400 μl was loaded into 0.3- or 1.2-cm optical path two-channel cells, with buffer A but without DDM in the reference channel. Scans were recorded at 12 °C and 42,000 rpm every 15 min, overnight, at 278 and 413 nm, and by interference.

The solvent density, ρ = 1.017 g/ml, and viscosity, η = 1.58 centipoise at 12 °C, were estimated from measurements with the density meter DMA 5000 and viscosity meter AMVn (Anton PAAR) at 20 °C and extrapolation to 12 °C on the basis of the ratio of the values for water. We estimate the partial specific volume of the polypeptide chains with the Sednterp software (free and available on line) and considered the mean value, υ<sub>p</sub> = 0.75 ml/g at 12 °C and υ<sub>DDM</sub> = 0.82 ml/g for DDM (22). The sedimentation profiles were analyzed with the size distribution analysis function (23) of the program Sedfit (free and available on line).

The value of the sedimentation coefficient depends on the complex shape (described by the hydrodynamic radius *R*<sub>d</sub>), partial specific volume (inverse of the particle density), *v*, and molar mass, *M*, according to the Svedberg Equation 1,

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S = M(1 - \rho v)/(N_A 6\pi\eta R_d)
\]  

(Eq. 1)
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The term $M(1 - \rho \tilde{v})$ for the supercomplex, solubilized in DDM and in a solvent containing glycerol, with a density significantly different from water, depends on supercomplex protein molar mass, $M_p$, detergent binding $B_{Det}$ (g/g), and hydration $B_w$ (g/g), with $\tilde{v}_p$, $\tilde{v}_{Det}$ and $\tilde{v}_w$ the corresponding partial specific volumes (24, 25) as shown in Equation 2,

$$M(1 - \rho \tilde{v}) = M_p((1 + B_{Det} + B_w) - (1 - \rho \tilde{v}_p) + B_{Det}(1 - \rho \tilde{v}_{Det}) + B_w(1 - \rho \tilde{v}_w)) \quad (Eq. 2)$$

We took a typical value of $B_w$ to be 0.3 g/g. The frictional ratio $f_{fmin}$ relates $R_s$ to the minimum radius from the anhydrous volume, $f_{fmin} = 1.25$ for a globular compact macromolecule. From the amino acid composition, we calculate that the putative supercomplex of 310 kDa, including one dimeric $bc_1$ complex, one cytochrome $c$ oxidase, and one Sqr, has an extinction coefficient at 280 nm $\epsilon_{280} = 1.63 \text{ cm}^{-1} \text{mg}^{-1} \text{ml}^{-1}$. For relating the fringe displacement, $J$, measured using interference optics, to concentration, we assumed refractive index increments of 0.187 and 0.143 ml/g for protein and DDM.

**RESULTS**

**Purification of a Supercomplex Containing a Cytochrome c Oxidase**

Based on previous studies, we decided to purify a superstructure that reduces O₂, starting from cells cultured in H₂/S²/O₂ medium. The fractions displaying cytochrome c oxidase activity on blue native gel were combined, to give the final preparation of the purified complex. A supercomplex with a functional cytochrome c oxidase was then purified as described under “Experimental Procedures.”

Blue native gel electrophoresis of the purified fraction shows one major band of molecular mass around 350 kDa stained with Coomassie Blue (Fig. 1A), associated with cytochrome c oxidase activity (Fig. 1B), indicating that the complex is homogeneous. Two other very faint bands could be detected in some preparations (around 50 and 100 kDa), but these were not associated with the cytochrome c oxidase activity.

**Identification of the Supercomplex Components**

**Mass Spectrometry**—To precisely define the composition of the purified supercomplex, protein bands were directly cut out from blue native gels, digested with trypsin, and analyzed by ion trap mass spectrometry. The analysis of the 350-kDa band (Fig. 1A) confirms that the purified supercomplex contains a cytochrome c oxidase with identification of the two subunits Cox A₂ and Cox B₂ (Table 1). This enzyme, which we have named cytochrome c oxidase II, is the same as the one we previously detected in *Aquifex* membranes as part of potential supercomplexes (5). In addition, the fully assembled $bc_1$ complex, containing the three subunits cytochrome $c_1$

![FIGURE 1. Migration of the purified supercomplex on blue native gel and in-gel detection of supercomplex components. 25 µg of supercomplex were loaded on 4–12% gels in all cases. A, detection by Coomassie Blue staining. B, detection of cytochrome c oxidase activity by in-gel assay. C, detection of $bc_1$ complex. D, detection of Sqr activity. Molecular mass markers are indicated in kDa.

**TABLE 1**

| Protein name | NCBI entry | Gene | Coverage | Mass |
|--------------|------------|------|----------|------|
| Cytochrome c | 2982798    | cyc  | 36.0     | 27.6 |
| Resiske-1 iron-sulfur protein | 2982799 | petA | 18.2     | 19.4 |
| Cytochrome c oxidase subunit II | 2984392 | coxB2 | 23.3     | 9.5  |
| Sulfide quinone reductase | 2984385 | sgr  | 18.4     | 47.4 |
| Hypothetical protein qg_814 | 2983372 | qg_814 | 9.7      | 32.5 |
| Cytochrome b | 2982800    | petB | 2.7      | 46.9 |
| Cytochrome c oxidase subunit 1 | 2984382 | coxA2 | 2.2      | 66.5 |

* Protein name is name as given in the NCBI database.
* NCBI entry is the accession number.
* Coverage indicates protein sequence coverage by the matching peptides (in %).
* Peptides indicate the number of different peptides matching the protein sequence.
* Mass indicates the theoretical molecular mass in kDa of the identified protein calculated from the amino acid sequence without possible processing or modifications.
New $\text{H}_2\text{~S}$ Oxidase-$\text{O}_2$ Reductase Respiratory Supercomplex

The subunits of the supercomplex were visualized by gel electrophoreses on an SDS-PAGE (Fig. 2A). The presence of seven major protein bands was revealed by Coomassie Blue staining. Analysis by MALDI-TOF mass spectrometry of all of them confirmed the composition of the supercomplex (Fig. 2B). Three of these proteins (cytochromes $c_1$ and $b$ and Rieske protein) are components of the $b_2c_1$ complex. Sqr was detected, as well as the small subunit of the cytochrome $c$ oxidase (Cox $b_3$). Cox $A_2$ was absent from the gel, probably due to its high hydrophobicity (12 predicted transmembrane segments (5)). This result confirms the results obtained by ion trap mass spectrometry. Moreover, staining of cytochromes $c$ with 3,3′,5,5′-tetramethylbenzidine in SDS gel revealed the presence of one band only with a molecular mass of about 25 kDa, corresponding to cytochrome $c_1$ (data not shown).

**Western Blotting**—We used antibodies for detection of the components after separation of the supercomplex on a native PAGE, which confirms the presence of the Sqr and the cytochrome $c$ oxidase in the same band (Fig. 2C). Sqr was also detected with antibodies, after migration on a blue native gel as well as on a denaturing gel (data not shown).

All the results indicate that the 350-kDa purified supercomplex contains three membrane-attached constituents, the Sqr, the $b_2c_1$ complex, and the cytochrome $c$ oxidase II. This is consistent with the proposition of the existence of an $\text{H}_2\text{~S}$ oxidation/$\text{O}_2$ reduction pathway in *A. aeolicus* (5, 26).

**Homogeneity of the Supercomplex**

The homogeneity and association state of the purified supercomplex were investigated by analytical ultracentrifugation. Fig. 3 shows the experimental and fitted sedimentation velocity profiles obtained at 413 nm for the supercomplex at 1.75 mg/ml (Fig. 3, A and B), the superposition for this sample of the distributions, $c(s)$, of sedimentation coefficients obtained at 278 and 413 nm and by interference (Fig. 3D), and the superposition of the $c(s)$ at 413 nm for the supercomplex at 1.75, 0.35, and 0.10 mg/ml (Fig. 3C). The three samples show qualitatively the same behavior, with limited nonideal effects, leading to smaller $s$ values at larger protein concentration. The proportion of the largest major species increases slightly with increased concentration (from 67% at 0.10 mg/ml to 75% at 1.75 mg/ml of the total absorbance at 278 nm). This may indicate slow association–dissociation equilibrium but is not easily distinguishable from sample heterogeneity. The largest main species sediments at $s = 6.8$ S ($s_{20,w} = 11.4$ S), corresponding to the protein-detergent supercomplex. The $s$ value allows an estimation of the protein part within the complex, considering an amount of bound DDM/g of protein ($B_{	ext{prot}}$) in the range 0–1 g/g, for frictional ratios of 1.25 to 1.5 (i.e. for a globular to a moderately extended shape), of 240 ± 50 to 320 ± 70 kDa (complexes of 340 to 450 ± 50 kDa). The number of fringes per absorbance unit at 278 nm is 2.5. It corresponds, considering $\xi_{280} = 1.63$ cm$^{-1}$ (mg/ml)$^{-1}$ for the putative supercomplex and $\xi_{prot} = 0$ for the detergent, to $B_{	ext{Det}} = 0.6$ g/g. This species has rather a large absorbance at 413 nm, in agreement with the presence of cytochromes in the supercomplex ($A_{413}/A_{278} = 0.9$). Two ill-resolved minor species are identified, from the superposition of the $c(s)$ at

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**FIGURE 2. Protein composition of the purified supercomplex.** *A,* separation of the subunits of the supercomplex (25 µg) on a 4–20% SDS gel and staining with Coomassie Blue. Arrowheads indicate subunits identified by mass spectrometry (B). The upper band (>100 kDa), not marked with an arrowhead, was not identified. *B,* identification of subunits by MALDI-TOF mass spectrometry. *C,* migration of the supercomplex (30 µg) on a 5–15% Tris-glycine native gel and detection with Coomassie Blue (lane 1). *A. ferrooxidans* Cox $B$ antibodies (lane 2) for detecting the *Aquifex* cytochrome $c$ oxidase; and *A. aeolicus* Sqr antibodies (lane 3).
different concentrations with the various optics, at $s = 3.8$ and $5.05 \pm 0.5$ S ($s_{20,w} = 6.4$ and $8.4 \pm 0.8$ S), accounting for 10 and 5%, respectively, of the signal at 278 nm, with a low signal at 413 nm. The $s_{20,w} = 6.4$ S species absorbs less at 413 nm compared with that of 8.4 S ($A_{413}/A_{278} = 0.2$ and 1.1, respectively). These $s_{20,w}$ values correspond to globular proteins of about 120 and 180 kDa, respectively. The species at 120 kDa might correspond to a dimer of Sqr (visualized on blue native gel in some supercomplex preparations, see below and Fig. 1D) for which $A_{413}/A_{278}$ between 0.1 and 0.2 is expected. The species at 180 kDa, not detected by blue native gel (Fig. 1), may correspond to a small amount of a dissociated species of the supercomplex. In addition, a contribution at $s = 2.5$ S ($s_{20,w} = 4.3$ S assuming hydrated micelles of DDM) is characterized by a large interference signal with a number of fringes per absorbance unit at 278 nm between 34 for the most concentrated samples and 54 for the most diluted one. It corresponds reasonably well to the detergent micelles, with possibly dissolved absorbing impurities (22).

When the purified supercomplex was subjected to a size exclusion chromatography with light scattering detection, the elution profile showed two resolved peaks (data not shown).

The first is characterized by $A_{413}/A_{280} = 0.95$ and corresponds to the supercomplex with a molecular mass of 375 ± 50 kDa. Combining the $A_{280}$ and the refractive index signals allows estimation of an amount of bound DDM of 0.5 g/g (based on the extinction coefficient of the putative supercomplex) and, with the light scattering signal, a protein molecular mass of 235 kDa. The second peak corresponds at least in part to the detergent micelles with a low absorbance and a molecular mass of about 75 kDa. This result is consistent with the sedimentation velocity and blue native gels experiments and confirms that the purified complex is compact.

**Spectroscopic Analysis**

**Optical Spectroscopy**—In agreement with supercomplex composition, UV-visible absorption spectra of the purified 350-kDa complex, recorded at ambient temperature, showed the presence of cytochrome $c$, with peaks at 424 and 554 nm, cytochrome $b$, with peaks at 424 and 561 nm, and cytochrome $c$ oxidase, with peaks at 448 and around 600 nm in the reduced state (Fig. 4A). These signals arise from the dimeric cytochrome $b$ and monohemes cytochrome $c_i$ from the $b_{c_1}$ complex and the cytochrome $c$ oxidase II identified above (27, 28). Addition of sodium ascorbate to the "as prepared" sample leads to reduction of about 40% of total hemes C and 30% of

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**FIGURE 3.** Sedimentation velocity experiments of *A. aeolicus* purified supercomplex. A, superimposition, for the supercomplex at 1.75 mg/ml in buffer A, of selected experimental sedimentation profiles obtained at 413 nm during 6 h at 42,000 rpm, at 12 °C, in 3-mm optical path length cell and of their modeled profiles with the $c(s)$ analysis. B, corresponding residuals. C, superposition of the $c(s)$ analysis for the supercomplex at 1.75, 0.35, and 0.10 mg/ml followed at 413 nm. D, result of the $c(s)$ analysis for the supercomplex at 0.35 mg/ml followed at 278 and 413 nm and using interference optics. $s_{20,w}$ values were calculated using $\gamma = 0.75$ ml/g, corresponding to the protein part. The errors in $s_{20,w}$ related to the choice of $s_{20,w}$ are less than 3%.

**FIGURE 4.** Spectral properties of the supercomplex. A, optical spectra. Proteins (3.5 mg/ml in buffer A) were analyzed as prepared (dotted line), reduced by sodium ascorbate (dashed line), and reduced by sodium dithionite (solid line). The inset shows the difference spectrum (dithionite-reduced minus as prepared). B and C, EPR spectra. The purified supercomplex (3.5 mg/ml in buffer A) was analyzed without any addition (as prep, solid line) or reduced with 5 mM sodium dithionite (dithionite-reduced, red, dashed line), in the region of the low field component of the low spin ferric heme resonances (B) and in the region of the Rieske iron-sulfur cluster (C). Instrument settings were as following: temperature, 15 K; microwave frequency, 9.406 GHz; microwave power, 6.4 milliwatts; modulation amplitude, 3 millitesla. Nine scans were averaged for each spectrum.
new H2S oxidase–O2 reductase respiratory supercomplex

These experiments demonstrate that the bc1 complex, the ba3 cytochrome c oxidase, and the Sqr in the supercomplex possess their redox cofactors suggesting a native and active state for all of them. We propose a stoichiometry of the supercomplex components on the basis of the spectroscopic analyses. The relative amount of hemes C and B in the supercomplex determined by optical spectroscopy suggests that it contains one cytochrome c oxidase for one homodimeric bc1 complex. The semi-empirical formula developed by de Vries and Albracht (41) allows calculation of the concentration of low spin hemes by EPR and thus determination that the two hemes at gz = 3.00 and gz = 3.71 are present in an approximate 1:2 stoichiometry, confirming the stoichiometry of one oxidase for one dimeric bc1 complex in the supercomplex.

Functional Role of the Supercomplex

To determine whether the three enzyme species associated in the supercomplex (bc1 complex, cytochrome c oxidase, and Sqr) are active, activities were determined in-gel and quantified by spectrophotometry.

Detection on Blue Native Gels—In addition to the cytochrome c oxidase activity, the bc1 complex was revealed at 350 kDa (Fig. 1C). To demonstrate the presence of active Sqr in the native gel, we developed the assay for specific detection of Sqr activity, as described under “Experimental Procedures.” Sqr activity was detected at 350 kDa, corresponding to the supercomplex, but also in two bands at about 50 and 100 kDa on the blue native gel with a faint intensity (Fig. 1D). They correspond to the two faint bands sometimes seen on the Coomassie Blue-stained gel and could also correspond to the minor species identified by analytical ultracentrifugation (at least for the 100-kDa species). When these two bands were cut out from the gel and analyzed by ion trap mass spectrometry, only the Sqr was identified (data not shown). These bands probably result from an equilibrium dissociation of the complex rather than an artifact of purification. Taken with the knowledge that Aquifex Sqr is a protein of 47 kDa, this experiment shows that it may exist in monomeric as well as in dimeric forms and that both are catalytically active on blue native gel. The fact that only Sqr appears to dissociate from the supercomplex in these conditions suggests that this component is associated less tightly than the rest.

Individual Enzyme Activities Measurements—Specific activities of the three enzymes in the supercomplex were determined spectrophotometrically at 40 °C (Table 2). Sqr, bc1 complex, and cytochrome c oxidase are active at this temperature. Sqr and bc1 complex activities are significantly inhibited...
New $\text{H}_2\text{S}$ Oxidase-$\text{O}_2$ Reductase Respiratory Supercomplex

![Graph](image)

**FIGURE 5. $\text{H}_2\text{S}$ oxidase-$\text{O}_2$ reductase activity of the supercomplex.** Oxygen consumption was measured at 60 °C using Na$_2$S as electron donor. The reactions were made in the presence (+) or absence (−) of horse heart cytochrome c (cyt) at 1 µM and quinone (DB) at 4 µM. Rates were corrected for the nonenzymatic reactions. The 100% activity corresponds to 3230 ± 610 units/mg.

by antimycin A, a quinone analog. Cyanide, an inhibitor of cytochrome c oxidases, inhibits the terminal enzyme demonstrating the specificity of the measured reactions (Table 2).

Specific activities of the $bc_1$ complex and cytochrome c oxidase are in the same range as those described in the literature (17), despite the fact that they were determined at a temperature far from the optimum (80 °C) and that the enzymes were not isolated but in association with proteins inside the supercomplex. Interestingly, the specific activity of the Sqr embedded in the supercomplex is very high compared with the specific activities of other purified Sqr described previously, except for the one of *A. ferrooxidans* (26, 42–44). These results show that the three enzymes are functional in the purified protein association.

**Electron Transfer from Sulfide to Oxygen**—To investigate whether the supercomplex is able to oxidize $\text{H}_2\text{S}$ (via Sqr) and reduce $\text{O}_2$ (via the cytochrome c oxidase), we measured $\text{O}_2$ consumption activities with a Clark-type electrode using Na$_2$S as electron donor at 60 °C. The $\text{O}_2$ was consumed by the supercomplex without any addition in the reaction medium (Fig. 5, −cyt −DB condition). Such an activity has also been detected with solubilized membranes (data not shown). The activity measurement was proportional to the concentration of the supercomplex in the reaction medium. This suggests that no dissociation or aggregation process of the enzymatic species occurs during the catalysis. $\text{O}_2$ consumption was inhibited by cyanide (100% inhibition) and antimycin A (68% inhibition), demonstrating that $\text{O}_2$ is reduced by the oxidase, and the electrons are provided by the Sqr and by the $bc_1$ complex. Using quinol (DBH$_2$) as substrate, a quinol oxidase-oxygen reductase activity was detected in the same conditions not only with the isolated supercomplex but also with the membranes (data not shown).

It follows that the purified protein superstructure transfers electrons from $\text{H}_2\text{S}$ to $\text{O}_2$ and that it is an active sulfide oxidase-oxygen reductase supercomplex. Quinone molecules are apparently contained in this supercomplex, and also cytochrome c$_{555}$ (shown by EPR), both being electronic shuttles between the enzymes. This new protein assembly can be considered as a true respirasome.

Addition of exogenous quinone (DB) does not enhance the activity, suggesting that the amount of endogenous bound quinones in the isolated supercomplex allows a maximal electron transfer rate in our conditions (Fig. 5, −cyt + DB condition) (Sqr and $bc_1$ complex are active with DB, although the physiological quinone is a demethylmenaquinone (28, 45)). On the other hand, a 2-fold higher activity could be obtained by addition of 1 µM of horse cytochrome c in the medium (Fig. 5, + cyt −DB and + cyt + DB conditions). In these conditions, an inhibition of the electron transfer from sulfide to oxygen of 71 and 100% is measured with antimycin A and KCN, respectively.

**Functional Consequences of Association into a Supercomplex on the Sqr**

To understand the impact on the Sqr enzyme of the physical association with its physiological partners ($bc_1$ complex and cytochrome c oxidase), we studied the isolated Sqr enzyme purified from the membranes (Sqr$_{free}$). Its enzymatic and biochemical characteristics are essentially the same as those reported while this manuscript was under consideration (46), except for the value of the specific activity, which was lower than what we found. The N-terminal sequence confirms that the N-terminal part of the enzyme is not cleaved for translocation across the membrane, despite the fact that it is proposed to be periplasmic (data not shown). The signal required for translocation may be at the C-terminal part of the protein as in the Sqr of *Rhodobacter capsulatus* (47).

In addition to the pure Sqr$_{free}$, we could also obtain a pure Sqr fraction released from the supercomplex (called Sqr$_{released}$). In contrast to what we and others (46) obtained for Sqr$_{free}$, migration on blue native gel shows beyond a doubt the absence of the trimeric state for Sqr$_{released}$ (Fig. 6). The nature of the protein in each band has been confirmed by ion trap mass spectrometry and Western blotting (data not shown). This suggests that the oligomeric state of the Sqr is different according to whether it is free or embedded in the supercomplex.

The thermal stability (half-life determined at 80 °C) and the kinetic parameters of the Sqr have been compared in both states (Sqr$_{free}$ versus associated with their redox partners within the supercomplex), as reported in Table 3; the properties of this enzyme are not significantly modified, except for the Michaelis constant ($K_m$) for the quinone and the catalytic efficiency. The data suggest a higher affinity for this substrate when the Sqr is integrated in the multienzymatic complex.

**DISCUSSION**

**Stable Association of All Components Involved in $\text{H}_2\text{S}$ Oxidation-$\text{O}_2$ Reduction Pathway**—We have described a new functional respiratory supercomplex containing the Sqr enzyme, the $bc_1$ complex, and a $ba$_{3} cytochrome c oxidase, which are properly arranged into a functional structure. Thus,
it contains all the molecular components required for the
electron transfer from H_2S as electron donor to O_2 as an ac-
ceptor. It has a sulfide oxidase-oxygen reductase activity and
is thus a complete respirasome. To our knowledge, this is the
first time that Sqr is found as part of a complex.

In a variety of phototrophic and chemolithoautotrophic
bacteria, Sqr feeds electrons into the photosynthetic or respi-
ratory electron transfer chain via a bc-type complex (43). The
implication of Sqr in the oxygen reduction pathway in A. ae-
olicus was proposed a few years ago by Nübel et al. (26) and
recently by our group (5). However, this work is the first evi-
dence for the association of all enzymes involved in this path-
way into a membrane-bound supercomplex. We have dem-
onstrated that it is very stable, as it is not destabilized by
relatively harsh solubilization conditions and three purifica-
tion steps, unlike other mitochondrial or bacterial respiratory
supercomplexes that are stabilized by relatively weak interac-
tions (15, 48). This superstructure could not have been pre-
dicted from the organization of the genes in the genome.

**Organization of the Supercomplex**—This new multienzyme
protein assembly is shown in the scheme in Fig. 7 with the bc_1
complex interacting with the cytochrome c oxidase, as we
have previously shown that they both associate in the mem-

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**FIGURE 6. Migration of the purified Sqr on blue native gel.** 4–15% gels
were used, and 10 μg of Sqr were loaded in all cases and detected by Coo-
massie Blue staining. **A.** Sqr_reconst. **B.** Sqr_free. Protein bands are
indicated by *arrowheads* and correspond to the monomer (58 kDa), dimer (106 kDa), tri-
mer (175 kDa), and an higher oligomeric state (240 kDa) of the Sqr. Molecular
mass markers are indicated in kDa.

**FIGURE 7. Model of the A. aeolicus energetic sulfur metabolism.** The H_2/S
and H_2S/O_2 respiratory pathways are structurally organized in supercom-
plexes. Cytochrome oxidase, bc_3, cytochrome c oxidase (composed of Cox A_2 and Cox B_2 subunits); bc, monomeric bc, complex (composed of cytochrome
b, cytochrome c_1, and the Rieske protein); c, monoheme cytochrome c_552; Sqr, sulfide quinone reductase; SOR, sulfur oxyge
nase reductase; S(S), elemental sulfur (the true substrate and the number of sulfur atoms in the molecule in vivo are not known); S_2O_3, thiosulfate; SO_3^-, sulfite; H_2S, hydrogen sulfide. Evidence for cytoplasmic sulfur globules was reported previously (4). The membrane-bound protein symbolized by *dotted lines* is a putative H_2S transporter.

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**TABLE 3**

Comparison of the properties of the isolated Sqr and the Sqr integrated in the supercomplex

| State of the Sqr | Isolated (Sqr_free, this study) | Integrated in the supercomplex (this study) | Isolated (from Ref. 46) |
|-----------------|-------------------------------|---------------------------------------------|------------------------|
| Oligomeric state on blue native gel | 4 states at 58, 106, 175 and 240 kDa | 2 states at 58 and 106 kDa | 4 states at 50, 90, 150, 280 kDa |
| t_1/2 at 80°C | 18 h | 15 h | 32 h |
| k_ cat (s^-1 μM^-1) | 75^b | 60^d | 59^c |
| K_m (μM) | 1.66 ± 0.68 μM | 0.71 ± 0.4 μM | 0.71 ± 0.4 μM |
| k_ cat/K_m | 45.2 s^-1μM^-1 | 84.5 s^-1μM^-1 | 2.16 ± 0.19 μM^-1 |

^a Kinetic parameters were calculated using the monomeric concentration of isolated Sqr and on the basis of one monomeric Sqr in the supercomplex.

^b Data were determined at 25°C.

^c Data were determined at 40°C.

^d Temperature was not specified.
brane of *A. aeolicus* (5). Because Sqr is considered to be an integral monotypic membrane protein (44, 49) and uses quinone as substrate, we propose that the Sqr interacts with the $bc_1$ complex inside the supercomplex, which includes one dimeric $bc_1$ complex and one cytochrome $c$ oxidase complex (Fig. 7). This is consistent with the fact that dimers of $bc_1$ complexes constitute the minimal structural and functional unit (50, 51). Supercomplexes containing a dimeric $bc_1$ complex and one or two copies of cytochrome $c$ oxidase have been purified from yeast mitochondria. Structural models showed that the terminal enzyme is peripherally located in these supercomplexes and that one copy of this enzyme is lacking in a substantial number of particles (52).

The *A. aeolicus bc$_1$* complex is typical (28), but the isolated Sqr enzyme, characterized very recently, has been proposed to be a trimer (46, 49), which differs from other organisms. We detected this trimer on blue native gel with Sqr$_{free}$. We have shown that the Sqr$_{released}$ appears to be a monomer as well as a homodimer on the blue native gel and that this Sqr fraction seems to be less stable than Sqr$_{free}$ or Sqr inserted in the supercomplex (data not shown). Moreover, we have shown that Sqr is active in the oligomeric as well as in the monomeric state. The precise stoichiometry of the Sqr in the supercomplex remains unknown. However, the experimental molecular mass of the purified superstructure (around 350 kDa) and the proposed stoichiometry of components based on spectroscopy exclude the possibility of a trimeric state of the Sqr in the supercomplex. We propose that Sqr in the supercomplex is a monomer or a dimer. Sqr from *A. aeolicus*, whether in the supercomplex or free, is much more active than purified Sqr from other organisms, even though the specific activity may well be substantially underestimated, because it was determined at 25 and 40 °C, much lower temperatures than those optimal for growth. These data call for further investigation.

**Presence of Electronic Intermediates in the Supercomplex—**The supramolecular assembly contains one of the two cytochromes $c_{555}$ known in *A. aeolicus*, which is probably the membrane-tethered one (called $c_{555}$m) (39, 53). This cytochrome is present in substoichiometric amounts, as was also the case for the purified NADH oxidase respirasome from *P. denitrificans* (0.4 cytochrome $c_{552}$/1 cytochrome $c$ oxidase) (17). In *A. aeolicus* membranes, however, the $g_s$ peak corresponding to this cytochrome in EPR spectrum has a very high intensity compared with that of the cytochrome $c$ oxidase. This suggests that this electron carrier could be present in the supercomplex in a large amount and that part of it could even be free in membranes (data not shown). This would indicate that cytochrome $c_{555}$ is partially lost during supercomplex purification steps as the $c_{552}$ from *P. denitrificans* (40–80% loss). Such a loss would be consistent with the idea that this cytochrome is a “shuttle” between the two partners ($bc_1$ complex and cytochrome $c$ oxidase) and might thus be loosely bound to be able to interact with both of them. As the sulfide oxidase-oxygen reductase activity is significantly enhanced by the addition of horse heart cytochrome $c$, the electron transport between $bc_1$ complex and cytochrome $c$ oxidase was impeded by loss of cytochrome $c_{555}$. This indicates that the $bc_1$ complex and the cytochrome $c$ oxidase can tightly interact in the absence of this electron carrier. In addition, quinones are retained in the supercomplex after isolation, as with the NADH oxidase purified from *P. denitrificans*, where the ubiquinone is specifically enriched (17). We propose that electrons delivered from Sqr into the quinone pool pass through the cytochrome $bc_1$ complex and finally reduce the cytochrome $c$ oxidase via the cytochrome $c$, as shown in the scheme of Fig. 7.

**Supramolecular Organization of the Respiratory Chains in *A. aeolicus*: Functional Significance—**The sulfide oxidase/oxygen reductase supercomplex is the second respirasome that we have characterized from *A. aeolicus*. A few years ago, we demonstrated that enzymes that couple periplasmic oxidation of hydrogen to cytoplasmic sulfur reduction form a membrane-bound supercomplex containing a hydrogenase and a sulfur reductase connected via quinones (4). It thus appears that the two respiratory pathways of *Aquifex*, sulfur reduction and sulfur oxidation, which are probably complementary or antagonistic in vivo, are organized into supramolecular structures (Fig. 7), which is a unique feature among prokaryotic as well as eukaryotic organisms. Indeed, all the supercomplexes identified in the mitochondrial membranes belong to the same energetic pathway.

Several studies of mitochondrial as well as bacterial supercomplexes have highlighted that the isolated respiratory complexes could be structurally stabilized when associated with their physiological partners (17, 54). Another main functional advantage that may originate from the interaction of consecutive enzymes is substrate channeling. The enzymes embedded in multiprotein complexes can also have different kinetic behavior compared with free enzymes (55), particularly when the subunit composition is different. Our present results suggest that interaction of the H$_2$S/O$_2$ pathway components into a supercomplex modifies the oligomerization state of the Sqr but does not stabilize the Sqr. However, the kinetic behavior of the Sqr is slightly altered, as the catalytic efficiency ($k_{cat}/K_m$) of this enzyme is increased when it is present in the supercomplex. The fact that the solubilized supercomplex is stable with time (there appears to be no dissociation) suggests that the Sqr$_{free}$ that we find could be part of a second pool of enzyme in the membrane that is not associated. The observation that the Sqr$_{released}$ appears to have a different quaternary structure from the Sqr$_{free}$ (Fig. 6) supports the idea of the existence in vivo of two pools, and these two pools possibly have different physiological roles. The fact that the kinetic parameters of Sqr inserted in the supercomplex and Sqr$_{free}$ appear to be different is compatible with this possibility.

**Energetic Sulfur Metabolism in *A. aeolicus*:—** *A. aeolicus* requires a sulfur compound for growth, in addition to molecular hydrogen and molecular oxygen, which is puzzling. Why are these three components? Genes encoding enzymes involved in the oxidation as well as in the reduction of sulfur compounds are present in its genome. We have previously characterized several enzymes involved in sulfur compound utilization (Fig. 7) as follows: a cytoplasmic sulfur oxygenase reductase catalyzing the disproportionation of elemental sulfur in the presence of O$_2$ (56), the membrane-bound sulfur
reductase (4), and the Sqr (this work and see Refs. 26, 46, 49).

It is known that A. aeolicus can grow in the presence of elemental sulfur or thiosulfate if H₂ and O₂ are also present (4). We have also found that A. aeolicus can grow in the presence of sodium sulfide, but as in the previous case, H₂ and O₂ are required (data not shown). No growth was observed with H₂S and O₂ only, which raises some questions. This suggests that this bacterium can use oxidized or reduced sulfur compounds to grow and has a very versatile metabolism.

Brito et al. (42) have proposed that in Acidaminus ambivalens, an acidophilic and hyperthermophilic archaea, Sqr oxidizes sulfide produced by sulfur oxygenase reductase and thus participates into an energetic spiral allowing the maximum of energy to be obtained from sulfur compounds. We propose that an analogous process occurs in Aquifex with an energetic coupling between the sulfur reduction and oxidation pathways. The sulfur that might be stored in cytoplasmic inclusions by the cell (4) could be used by the sulfur reductase and the sulfur reductase producing H₂S (Fig. 7). H₂S would in turn be used by Sqr to reduce oxygen and produce ATP. H₂S transport across the membrane could occur by simple diffusion without facilitation by membrane channels as recently demonstrated (57). We have characterized most of the enzymes involved in the intricate sulfur metabolism in A. aeolicus, and we will now concentrate our efforts in understanding how the various energetic pathways are connected, as a way to gain insight of why A. aeolicus requires H₂, O₂, and a source of sulfur for growth.

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