A New Iron-oxidizing/O₂-reducing Supercomplex Spanning Both Inner and Outer Membranes, Isolated from the Extreme Acidophile Acidithiobacillus ferrooxidans

Cindy Castelle, Marianne Guiral, Guillaume Malarte, Fouzia Ledgham, Gisèle Leroy, Myriam Brugna, and Marie-Thérèse Giudici-Orticoni

From the Laboratoire de Bioénergétique et Ingénierie des Protéines, IBSM-CNRS, Marseille Cedex 20, France

The iron respiratory chain of the acidophilic bacterium Acidithiobacillus ferrooxidans involves various metalloenzymes. Here we demonstrate that the oxygen reduction pathway from ferrous iron (named downhill pathway) is organized as a supercomplex constituted of proteins located in the outer and inner membranes as well as in the periplasm. For the first time, the outer membrane-bound cytochrome c Cyc2 was purified, and we showed that it is responsible for iron oxidation and determined that its redox potential is the highest measured to date for a cytochrome c. The organization of metalloproteins inside the supramolecular structure was specified by protein-protein interaction experiments. The isolated complex spanning the two membranes had iron oxidase as well as oxygen reductase activities, indicating functional electron transfer between the first iron electron acceptor, Cyc2, and the Cu₄ center of cytochrome c oxidase aa₃. This is the first characterization of a respirosome from an acidophilic bacterium. In Acidithiobacillus ferrooxidans, O₂ reduction from ferrous iron must be coupled to the energy-consuming reduction of NAD⁺ (P) from ferrous iron (uphill pathway) required for CO₂ fixation and other anabolic processes. Besides the proteins involved in the O₂ reduction, there were additional proteins in the supercomplex, involved in uphill pathway (bc complex and cytochrome Cyc₄₂), suggesting a possible physical link between these two pathways.

Oxidation of ferrous iron (Fe(II))² by microbes is an important component of the iron geochemical cycle (1). In modern environments, bacteria that can oxidize Fe(II) are ubiquitous, inhabiting and affecting a wide variety of environments where Fe(II) is present. One of these bacteria is Acidithiobacillus ferrooxidans, a Gram-negative strictly acidophilic bacterium that has gained considerable interest because of its use in microbial leaching, and its emerging role as a model for studying organism life in an extreme acidic environment. It is an obligate chemolithotrope, and the sole energy-producing process that it uses for growth and cell maintenance involves oxidation of reduced sulfur compounds and/or Fe(II) under acidic conditions, using O₂ as oxidant. Electrons from Fe(II) can either be transported along a “downhill” or a “uphill” electron pathway, toward a higher (O₂/H₂O) or lower (NAD(P)⁺/NAD(P)H) redox couple (2–4). The respiratory chain of Acidithiobacillus ferrooxidans has been analyzed in detail in recent years. The downhill and uphill pathways, involving proteins encoded by the rus and petl operons, conclude with reduction of the final target by a cytochrome c oxidase (cyt c oxidase) and bc₁ complex, respectively (4, 5). However, despite substantial efforts, the pathway between Fe(II) and O₂ has not yet been completely elucidated, and new strategies need to be developed to understand the energetics. Nevertheless, there is general agreement that Fe(II) is oxidized on the cell surface at the outer membrane and that cyt c oxidase alone does not catalyze this reaction (6–10). The organization of rus operon, the subcellular localization of the proteins, and studies of protein-protein interactions all suggest that electron transfer occurs through the high molecular weight c-type cytochrome Cyc2 located in the external membrane, the periplasmic blue copper protein rusticyanin (RcY), the periplasmic diheme cytochrome c Cyc1, and the aa₃ cyt c oxidase. In addition to genes encoding the bc₁ complex, a gene coding for a diheme cytochrome Cyc₄₂ is also present in the petl operon, and it has been recently proposed (7) that the bc₁ complex receives electrons from this cytochrome and transfers them to the quinol pool. Electrons would then be transferred to the complex I that is supposed to function also in the reverse direction to produce NADP(H) (3). Moreover, it has been proposed (7) that the branching point between these two pathways is at the level of RcY.

This raised questions about the electron transfer pathways from Fe(II) to O₂ and to NAD(P). In Acidithiobacillus ferrooxidans, no efficient genetic systems are available to disrupt genes by a procedure such as homologous recombination. We have used various approaches to probe the physical and energetic coupling between all the proposed components of the downhill oxidizing chain, and we demonstrate a supramolecular organization of the downhill pathway going from the external membrane to the inner one.

**EXPERIMENTAL PROCEDURES**

Preparation and Solubilization of the Membranes

Bacteria were grown according to Ref. 6. Cells (30 g) were resuspended in 100 ml of 50 mM BisTris, pH 7, supplemented...
New Two Membrane-spanning Respirasome from an Acidophile

with 10 µg/ml DNase I (from bovine pancreas; Roche Applied Science) and protease inhibitors (mixture tablets, Complete, Roche Applied Science). They were broken at 18–20 kpsi in a French press cell. Membranes were pelleted by centrifugation at 145,000 × g for 1 h at 4 °C, then resuspended at a protein concentration of 12 mg/ml, and solubilized in 50 mM BisTris, pH 7, containing 5% glycerol, 750 mM ammonium thioglycolate, and dodecyl β-D-maltoside (DDM). The suspension was stirred for 3 h at 4 °C and centrifuged at 145,000 × g for 1 h at 4 °C to obtain the solubilized membrane proteins. Samples were concentrated at this step. Nonionic (Triton X-100 and DDM), ionic (sodium deoxycholate), and zwitterionic detergents were also tested for solubilization at 1–3 mg of detergent per mg of proteins and at various pH values (pH 7, pH 4, and pH 2). When specified, nonsolubilized membrane pellets were resuspended in 1 M NaBr, stirred for 2 h at room temperature, and spun at 145,000 × g for 1 h at 4 °C to remove peripherally bound proteins.

**Purification of the Supercomplex and Proteins**

For the supercomplex purification, concentrated solubilized proteins were layered on top of a 43-ml 10–20% glycerol gradient formed in buffer A, 0.1% DDM, and centrifuged at 145,000 × g overnight at 4 °C. Fractions 1–9 (5 ml each) were collected from the top of the tube and concentrated. The heaviest fraction of glycerol gradient (18–20%) was applied, after concentration, to a Superose 6 gel filtration (FPLC, 25 ml; GE Healthcare) equilibrated in buffer A, 0.02% DDM. The flow was 0.2 ml/min. Molecular weight standards were analyzed under identical conditions. Fractions (0.5 ml) with specific spectroscopic signals of cytochromes and cyt c oxidase were pooled and concentrated. From 600 mg of solubilized proteins, the purification yield was 1.5%.

Cytochromes Cyt1, Cyt2, and RcY were purified as described previously (6, 11). Cyt c oxidase from *A. ferrooxidans* was kindly supplied by D. Lemesle (CNRS, Marseilles, France). Cyt2 was purified from solubilized membranes using 10–30% sucrose gradient formed in a buffer containing 50 mM sodium acetate, pH 5, 0.01% DDM, 0.05% aminocaproic acid, and 5% glycerol (buffer B). Fractions corresponding to 28–30% sucrose, collected from the top of the tube, were concentrated and loaded onto a SP-Sepharose column. After washing steps, elution was performed by two steps with 100 mM ± glycine, pH 2.4. Fractions obtained were analyzed by SDS-PAGE.

**Co-immunoprecipitation**

5 mg of antibodies directed against Cyc2 or ORF1 were covalently immobilized on CNBr-Sepharose 4B matrix (Amerham Biosciences) following the instructions of the manufacturer. The column was equilibrated in buffer A, 100 mM NaCl, 0.1% DDM, and 400 µg of solubilized membrane proteins were loaded onto the column. After washing steps, elution was performed by two steps with 100 mM glycine, pH 2.4. Fractions obtained were analyzed by SDS-PAGE.

**Analytical Procedures**

**Electrophoreses**—Proteins were loaded on 4% polyacrylamide stacking, 10% running SDS or native gels, or 5–15% Blue Native gels according to Ref. 12 (MiniProtean3 apparatus, Bio-Rad). After migration, proteins were revealed with Coomassie Blue, 3,3',5,5'-tetramethylbenzidine (TMBZ, specific for hemes) (13), specific staining for cyt c oxidase and for bc complex, or antibodies.

**Western Blots**—Proteins separated on denaturing or native gels were transferred to nitrocellulose membrane (Electron) with the Fast blot apparatus (Biometra) at 5 mA/cm² for 45 min. Primary antibodies used were as follows: anti-Cyc1, anti-Cyc2, anti-CoXb, anti-ORF1, and anti-RcY rabbit sera. The secondary antibody used was the goat peroxidase-conjugate anti-rabbit IgG (Sigma). Proteins were detected using the SuperSignal West Pico Chemiluminescent substrate (Pierce) following manufacturer’s guidelines. Restore Western blot Stripping Buffer (Pierce) was used for stripping antibodies from blots to enable several reprobings on the same membrane.

**Far-Western blotting** was performed essentially as described (14). A total amount of 10 µg of purified cyt c oxidase, Cyc1, Cyc2, RcY, and ORF1 was analyzed by SDS-PAGE and electrotransferred to a nitrocellulose membrane. The membrane was then incubated for 1 h in blocking buffer 1 (0.05% Tween/phosphate-buffered saline) and for another 1 h in blocking buffer 2 (5% milk) to allow partial renaturation of the proteins on the nitrocellulose membrane. The membrane was briefly washed with phosphate-buffered saline followed by incubation with...
New Two Membrane-spanning Respirasome from an Acidophile

JOURNAL OF BIOLOGICAL CHEMISTRY

SEPTEMBER 19, 2008 • VOLUME 283 • NUMBER 38

25805

from reduced minus “as-prep” spectra using the following wavelength pairs and adsorption coefficients (mm$^{-1}$ cm$^{-1}$): heme $a$, $\Delta\varepsilon_{600-640}$ nm = 11.6; heme $b$, $\Delta\varepsilon_{562-577}$ nm = 22; heme $c$, $\Delta\varepsilon_{552-554}$ nm = 19.1 (18).

**Optical Spectra**

UV-visible spectra were recorded on a Cary 50 Bio (Varian) spectrophotometer at 20 °C according to the method of Dutton (19) at pH 4.2 in 50 mM ammonium acetate, 0.01% DDM. The following redox mediators were present at a concentration of 4 $\mu$M: ferrocene monocarboxylic acid, ferrocene dicarboxylic acid, p-benzoquinone, 1–2-naphthoquinone, and potassium ferricyanide. The purified cytochrome $c$ (16 $\mu$M) was titrated.

**EPR**

EPR spectra were recorded at liquid helium temperatures with a Bruker ESP 300e X-band spectrometer fitted with an Oxford Instruments cryostat and temperature control system. EPR spectra were recorded on enriched cytochrome Cyc2 (2 mg/ml) in buffer B and on the supercomplex (11 mg/ml of proteins) in buffer A, 0.01% DDM, 2 mM EDTA. Reduction was achieved with a solution of FeSO$_4$ at the final concentration of 2 mM.

**RESULTS**

**Stable Association of Soluble and Membrane-bound Proteins Involved in Electron Transfer**—To investigate the organization of the Fe(II) oxidation pathway, we prepared and solubilized membranes using various detergents and detergent/protein ratios at different pH values as described under “Experimental Procedures.” DDM proved to be very suitable for membrane solubilization at pH 7 at a detergent concentration of 3 mg/mg of protein.

UV-visible absorption spectra and electrophoresis analysis showed the presence of cytochrome(s) $c$ and membrane proteins as cytochrome(s) $b$ and $aa_3$-type cyt $c$ oxidase in the DDM supernatant (Fig. 1A). In addition, using specific antibodies it was found the presence of proteins known to be soluble in the periplasm (Cyc1 and RcY), which suggests a strong affinity of these proteins for membranes and/or for their membrane-bound physiological partners (Fig. 1B).

To determine whether periplasmic proteins interact with membrane proteins, the solubilized proteins were separated by a 10–20% glycerol density gradient. Nine fractions from the gradients were analyzed by absorption spectra. As controls, purified cytochrome Cyc1, RcY, and cyt $c$ oxidase were subjected also to density gradients in the same conditions. In accordance with their molecular weights, cytochrome Cyc1 (21 kDa) and RcY (16 kDa) are mainly present in the lightest fractions at 12% glycerol, whereas cyt $c$ oxidase (150 kDa) migrates...
in the intermediary fractions at 15.5% glycerol. Absorption spectra of the fractions showed a different distribution of the same proteins when coming from the DDM supernatant. Indeed, total c-type cytochromes, cyt c oxidase, as well as cytochrome(s) b are mainly found in the heaviest fractions (about 18–20% glycerol, Fig. 2). These data give a clear indication that, in the DDM supernatant, cytochromes c and cyt c oxidase are shifted toward the heaviest fractions of the gradient suggesting the presence of high molecular weight proteins complex(es). The controls with purified proteins exclude aggregation or polymerization as no proteins were found in the 18–20% glycerol; Fig. 2). These data give a clear indication that, in the DDM supernatant, cytochromes c and cyt c oxidase are shifted toward the heaviest fractions of the gradient suggesting the presence of high molecular weight proteins complex(es). The controls with purified proteins exclude aggregation or polymerization as no proteins were found in the 18–20% glycerol fraction.

On spheroplast suspension, evidence has been obtained that electrons arising from ferrocytochrome take two pathways as follows: the first one being the classical energy-producing cytochrome c-cytochrome oxidase-oxygen pathway (downhill), and the second one corresponding to a protonmotive force-dependent pathway via the bc₁ complex and the complex I (uphill) (3). We have tested the oxidation of exogenous ferrocytochrome c to determine the presence of proteins from downstream and uphill pathways in the 18–20% glycerol fraction (Fig. 3). When added to 100 μg of 18–20% fraction at pH 4.8, exogenous cytochrome c was oxidized. After addition of stigmatellin, a specific complex bc₁ inhibitor, either before or after ferrocytochrome c, the rate of oxidation was inhibited at 44%. The oxidation of ferrocytochrome is then abolished in presence of KCN, an inhibitor of cyt c oxidase. These results show the presence of proteins or complexes constituting both the uphill and downhill pathways in 18–20% glycerol fraction.

**New Respiratory Supercomplex Spanning Both the Inner and Outer Membranes**—As our previous results suggest the existence of large complexes containing membrane and soluble proteins, we realized co-immunoprecipitation with antibodies raised against Cyc2 provided evidence of the existence of this organization. An immune complex was obtained from DDM supernatant, and 11 proteins were identified by mass spectrometry after separating them on a denaturing gel (Table 1). Four of these proteins are products of genes included in the rus operon in.
the Acidithiobacillus genome: Cyc2, Cyc1, RcY, and a protein (ORF1) of unknown function. This operon contains also four genes encoding the four subunits of the *aa*₃ *cyt c* oxidase not identified by mass spectrometry, but the presence of subunit II (CoxB) is detected by immunoblotting using specific antibodies (data not shown). At this step, it was impossible to know if other bound proteins are truly complex subunits or contaminants. This experiment demonstrates the existence of a supercomplex including both soluble (Cyc1 and RcY) and membrane-bound proteins (Cyc2 and *cyt c* oxidase). To our knowledge, this is the first time that the unknown protein ORF1 is detected interacting with metalloproteins supposed to be involved in the iron oxidation pathway.

The complex was then purified as described under “Experimental Procedures.” Low temperature optical spectroscopy showed the presence of *bc*- and *b*-type cytochromes and the *aa*₃ *cyt c* oxidase (Fig. 4). *b*-Type hemes are substoichiometric to *c*-type hemes (around 1/10) and can be discerned as a shoulder on the red flank of the α-peak of the *c*-type hemes.

To visualize the physical interaction between all the proteins eluted in the same fraction from the gel filtration chromatography, the complex was allowed to migrate on a Blue Native (BN) gel (Fig. 5A). Staining with Coomassie Blue and TMBZ revealed the presence of two bands with a molecular mass of about 300 kDa (Fig. 5A, lanes 1 and 2, respectively, bands 1 and 2). These two bands present a *cyt c* oxidase activity (Fig. 5A, lane 3) that is specifically inhibited by KCN (data not shown). As the presence of cytochrome *b* was detected by UV-visible spectroscopy, the *bc* complex was searched directly in-gel using a specific complex III heme staining as described under “Experimental Procedures.” Two bands (Fig. 5A, lane 4) were revealed with weak intensity compared with the *cyt c* oxidase activity. This result indicates the presence of a *bc* complex (or a part) in the supercomplex. All these results demonstrate that two multi-plant complexes with similar molecular weights, each containing an active oxidase and cytochrome(s), are present in the purified fraction. The presence of two bands could be a consequence of a difference in proteins stoichiometry as demonstrated below.

**Identification of the Complex Components**—The composition of the two proteins bands (Fig. 5A) was determined by a proteomic approach, including trypsin digestion of the two individual bands excised from the BN gel and analysis by ion trap mass spectrometry. The major band (Fig. 5A, band 1) contains all components encoded by the *rps* operon (Cyc1, Cyc2, ORF1, CoxA, and CoxB) except RcY (Table 2). Moreover, in agreement with the optical spectra and in-gel detection, the components of the *bc*₁ complex (cytochromes *c₁* and *b*) as well as the diheme cytochrome *CycA₂*, whose genes are present in *pet*₁ operon, were identified. The outer membrane protein Omp40 is also found in the complex, and its presence after isolation of the supercomplex by both co-immunoprecipitation and purification steps suggests that it is not an artifact. Similarly, the association of ORF1 with the electron transfer com-

**FIGURE 5. Supercomplex subunit composition.** A, analysis of the purified supercomplex by a 5–15% BN gel associated with Coomassie Blue staining (lane 1), TMBZ staining (lane 2), *cyt c* oxidase activity staining (lane 3), and *bc* complex heme-specific staining (lane 4). On the left, arrows indicate band 1 and band 2, corresponding to multiprotein complexes. B, analysis of the purified supercomplex by a 10% SDS gel associated with Coomassie Blue staining (lane 1) and Western blotting with antibodies raised against RcY, Cyc2, subunit II of the *cyt c* oxidase (CoxB), and Cyc1 (lane 2). Molecular mass markers are indicated in kDa.

**TABLE 2 Identification of proteins from band 1, by ion trap mass spectrometry, after separation by BN gel**

| Protein name | NCBI entry | Gene | Coverage* | Peptides* | Massa |
|--------------|------------|------|-----------|-----------|-------|
| Cyc2         | 3282057    | cyc2 | 19        | 6         | 49.7  |
| CoxB (subunit II *aa*₃ cytochrome oxidase) | 3282060 | CoxB | 40        | 7         | 28.4  |
| CoxA (subunit I *aa*₃ cytochrome oxidase) | 3282061 | CoxA | 9         | 3         | 70.0  |
| ORF1         | 15209320   | orf1 | 15        | 6         | 16.7  |
| Cyc1         | 2851568    | cyc1 | 18        | 2         | 20.0  |
| CYC42       | 14251201   | CycA | 9         | 2         | 23.7  |
| Cytochrome *c₁* | 8547221 | PetC | 12        | 2         | 25.2  |
| Cytochrome *b* | 8547220 | PetB | 11        | 4         | 45.5  |
| OmpA-like outer membrane protein | 29467513 | FopA | 15        | 2         | 20.2  |
| Major outer membrane protein | 4138616 | omp40 | 30       | 14        | 40.0  |
| CydA (subunit I bound ubiquinol oxidase) | 57157696 | CydA | 8         | 3         | 60.8  |
| CsoS1A       | 4836667    | csoS1A | 28       | 10        | 12.2  |
| CsoS1B       | 4836668    | csoS1B | 58       | 6         | 11.5  |
| ATP synthase B chain | 728929 | AtpF | 45       | 9         | 17.9  |

*a* Coverage % means protein sequence coverage by the matching peptides.

*b* Peptides means the number of different peptides matching the protein sequence.

*a* Mass means the theoretical molecular mass of the identified protein calculated from the amino acid sequence without possible processing or modifications.
New Two Membrane-spanning Respirasome from an Acidophile

plex is real as this protein was detected in both experiments. Although an active oxidase is part of the complex, two subunits (CoxC and CoxD) were not detected by mass spectrometry, and one (CoxA) was detected only by three peptides, probably because of the high hydrophobicity of the peptides generated by trypsin digestion of these proteins. The c-type cytochromes are identified with only two peptides because of the covalent binding of hemes to the polypeptidic chain. The third component of bc₁ complex (the Rieske protein) is known to be easily lost during purification (10). CsoS1A and CsoS1B are components of the carboxysomal supercomplex that plays an important role in the global carbon cycle (20). As a link exists between the uphill pathway and CO₂ assimilation in A. ferrooxidans, the presence of these proteins is unlikely to be due to chance. The B chain of ATP synthase and subunit I of quinol oxidase are probably contaminants as only one subunit of these enzymes is detected. The second band detected on BN gel (Fig. 5A, band 2) showed mainly the same components. These results confirm the presence of two forms of the same multiprotein complex with different stoichiometry for each component, and exclude the existence of two different complexes in the same purification fraction.

As a consistency check, we used antibodies for detection of specific components after separation of the supercomplex on a native PAGE. Cyc1, Cyc2, CoxB, and ORF1 but no RcY were detected in the same band. On SDS-PAGE, Cyc1, Cyc2, ORF1, CoxB, and RcY were revealed as shown in Fig. 5B. Heme staining confirmed the presence of cytochromes of about 50 kDa (Cyc2), 20 kDa (Cyc1), and 25 kDa (c₁ and Cycc₂) (data not shown). Comassie Blue staining shows six major and several minor bands. Analysis by MALDI-TOF mass spectrometry confirmed the composition of the supercomplex (data not shown).

The supercomplex was likewise purified from membranes washed with NaBr. The same composition was obtained indicating that disruption of any or putative ionic or hydrophobic interaction is not sufficient to dissociate the superplex. In particular, components of bc₁ complex are still present and stoichiometrically heterogeneous especially between the oxidase and bc₁ complex (10/1). However, the presence of the bc₁ complex components after drastic washing suggests that our preparation contains mostly proteins involved in the downhill pathway but also probably molecular association with proteins involved in the uphill pathway.

Characterization and Iron Oxidase Activity of Cyc2—It was proposed that Cyc2 is the first electron acceptor in the downhill pathway (7). To specify the role of this cytochrome, we purified it from the membranes of A. ferrooxidans, obtaining highly enriched Cyc2. The identity of the protein was confirmed by N-terminal sequencing and liquid chromatography coupled to tandem mass spectrometry analysis after treatment with trypsin. As observed on a TMBZ-stained SDS-PAGE, no other cytochrome is present in this fraction (data not shown). The EPR spectrum of this fraction shows a signal at g = 3.31 corresponding to the g₂ signal of the untreated oxidized Cyc2 (Fig. 6A). When Fe(II) was added to this sample, the signal at g = 3.31 completely disappeared, demonstrating that Cyc2 is reducible by Fe(II) (Fig. 6B), reduction instantaneously visualized by a modification of the cytochrome color upon addition of FeSO₄.

In line with this property, the redox potential of purified Cyc2 was determined by spectrometric titration to be 560 mV at pH 4.8 (data not shown). This is the higher redox potential determined to date for a cytochrome c.

Iron Oxidase Activity of the Purified Supercomplex—A functional association of proteins involved in the downhill Fe(II) pathway should possess iron oxidase activity. To test whether this complex is reducible by Fe(II), we recorded EPR spectra of the highly enriched supercomplex in the presence of Fe(II). In this condition, a signal at g = 4.1 typical of Fe(III) appears (data not shown). This EPR signal, much more intense for the supercomplex than for the control (Fe(II) solution only), suggests oxidation of Fe(II) by the supercomplex. This experiment shows that the supercomplex can catalyze electron transfer from Fe(II) to O₂ available in the sample. Moreover, the purified supercomplex presents an O₂ consumption activity of 212 units/mg when Fe(II) is used as electrons donor (Table 3). This confirms that the purified structure transfers electrons from...
Fe(II) to O₂. Addition of KCN, an aa₃-type oxidase inhibitor, caused a significant decrease in O₂ consumption, which showed that O₂ was specifically reduced by this enzyme. As demonstrated before (4, 6), purified cyt c oxidase cannot oxidize Fe(II) in the same conditions (Table 3), although this purified enzyme is catalytically active with ferrocytochrome c as electron donor (data not shown). The involvement of cytochromes included in the complex in electron transfer has been verified by stopped-flow experiments. Cyc1 and cyt c oxidase are not reducible by Fe(II) (4, 6), and the kinetics of reduction of RCY have shown that this protein is not the first electron acceptor. Using Fe(II) as reaction substrate, a reduction of c-type cytochrome(s) was observed at 552 nm. As this reduction is faster than reduction of the smaller complex, RCY-Cyc1 in the same conditions (data not shown), one or more proteins present in the complex must promote the electron transfer. Our experiments showed that the multiprotein complex is functional in the iron oxidation pathway and strongly suggested that Cyc2 carries the Fe(II) oxidase activity. All attempts to spectrometrically obtain a bc complex activity with the supercomplex (in both forward and reverse directions using the classical test of bc complex as well as oxidation of ferrocytochrome c) were unsuccessful. Lemesle-Meunier et al. (4, 10) have already reported the loss of one of hemes b or the Rieske subunit during the purification steps, which could explain the loss of the activity.

Organization of Proteins Involved in Downhill Pathway Inside the Supercomplex—To specify interaction of proteins within the supercomplex, we performed far-Western blotting experiments using purified cyt c oxidase, RCY, Cyc1, Cyc2, and ORF1. Purified RCY or cyc1 were then incubated with nitrocellulose membranes on which different proteins of the complex were transferred after SDS-PAGE. After removal of unspecific binding by washing, bound RCY or Cyc1 were detected with anti-RCY antibody (A) and anti-Cyc1 (B) antibody. Bovine serum albumin (BSA) serves as a negative control and bait proteins (RCY and Cyc1) as positive controls. The arrowheads point to extra bands corresponding to the binding of RCY to Cyc1 and to Cyc2 (A) and the binding of Cyc1 to ORF1, to RCY, and to subunit II (CoxB) of cyt c oxidase (B). Molecular mass markers are indicated in kDa.

DISCUSSION

Envelope-spanning structures have been studied in Gram-negative bacteria (secretion systems, pili, and efflux pumps) and mitochondria (translocases), but very few of these complexes have been purified, and none has an electron transferring func-
New Two Membrane-spanning Respirasome from an Acidophile

tion (21, 22). Here we have described the existence of a stable supercomplex associating proteins and complex located in three cellular sites (outer and inner membranes and periplasm).

For the first time, EPR studies indicate that the uncharacterized monoheme outer membrane cytochrome Cyc2 is responsible for iron oxidation and is probably therefore the primary electron acceptor from Fe(II). Outer membrane-bound c-type cytochromes have been frequently implicated as electron transfer proteins that interact directly with metals in the environment (23–25). Despite minimal sequence identity (15%), Cyc2 shares many properties with the cytochrome \( c_{572} \), recently identified from a natural acidophilic microbial community (25). Both proteins have structures that are primarily composed of \( \beta \)-strands, are monoheme, and catalyze the iron oxidation. The redox potential of Cyc2 is to our knowledge the most oxidizing redox potential observed in c-type cytochromes so far and is most probably in line with a functioning of this cytochrome in the downhill electron transport from Fe(II)-Fe(III) couple toward reduction of molecular oxygen.

OMP40 is also an outer membrane protein that is present in the supercomplex. It has been proposed that this protein, which shows high similarity with the porin family, is involved in the interaction between the bacterium and the substrate (26).

ORF1 is also present in the supercomplex. It has the cupredoxin-like fold found in some copper ion-binding proteins, and three amino acids (Cys-168, His-175, and Met-180) could be involved in copper-binding site. Comparison with cytochrome oxidases isolated or associated in supercomplex does not permit the function of ORF1 to be elucidated. However, this protein does not have a motif such as a series of methionine- and histidine-rich residues found in many proteins involved in copper homeostasis (27, 28). In the same way, ORF1 does not present any homology with the SurF1 protein family. This protein is involved in cyt \( c \) oxidase maturation as well as in the formation of the oxidase-\( bc \) supercomplex in eukaryotic cells, and a SurF1 homologue is found in the bacterial quinol oxidase operon, suggesting that SurF1 is associated with this primitive oxidase, which belongs to the same superfamily as cyt \( c \) oxidase (29, 30). When membranes were treated with 1 M NaBr before solubilization, ORF1 was found to be exclusively bound to the membrane and absent from the wash fraction, indicating that its association with lipids or its protein partner is strong.

We have previously demonstrated that Cyc1, from \( \text{ras} \) operon, and Cyc\( c_{42} \), from petl operon, interact with RcY and are reduced by this protein (6, 11). By far-Western experiments, we demonstrate for the first time a direct protein-protein interaction between RcY and Cyc2. In the same way, a direct interaction between Cyc1 and ORF1 is also demonstrated. All our results demonstrate the position of RcY between Cyc2 and Cyc1 in \( A. \text{ferrooxidans} \) as it is proposed in the model of the supramolecular organization presented in Fig. 8. We could not show, in our experimental conditions, any interaction between the two cytochromes Cyc2 and Cyc1. This situation might be specific for \( A. \text{ferrooxidans} \). Indeed, genome analysis of various bacteria such as \( B. \text{thaailandensis} \) E264, \( R. \text{solanacearum} \), \( A. \text{cryptum} \), \( D. \text{aromatica} \), \( T. \text{denitrificans} \), \( R. \text{ferrireductens} \), and \( X. \text{campestris} \) indicated that the gene encoding a cytochrome homologous to Cyc2 is always located directly next to a gene encoding a diheme cytochrome \( c \) similar to Cyc1 or Cyc\( c_{42} \) from \( A. \text{ferrooxidans} \) (probably in the same operon) suggesting that these two cytochromes could be involved in a same electronic pathway (data not shown). As no gene coding for a RcY homologue could be found in these genomes, we can speculate that these two cytochromes interact \( \text{in vivo} \) in these organisms. Recently, Jeans \textit{et al.} (25) have identified a small periplasmic soluble cytochrome \( c \), the cytochrome \( c_{572} \) in a natural acidophilic microbial community in addition to the cytochrome \( c_{572} \) and hypothesized that it could serve as a periplasmic electron transfer protein from Fe(II) oxidation (by \( c_{572} \) at the cell surface) to protein complexes localized on the cytoplasmic membrane, implying a direct association between both cytochromes.

We showed that the isolated supramolecular structure has iron oxidase and oxygen reductase activities, indicating functional electron transfer between metalloproteins. The term “respirasome” to describe it is justified as the purified supercomplex contains all the components required for electron transfer from primary donor to terminal acceptor (21, 31). It is becoming more and more apparent that protein complexes are not exceptional but constitute a basic working principle of the cell. The interdependence among respiratory complexes was first noticed when cytochrome \( bc \) complex was shown to interact tightly with cyt \( c \) oxidase (21). This phenomenon might not be surprising considering that direct electron transfer between two complexes could be facilitated by physical interactions. Large macromolecular structures are believed to optimize electron transfer by minimizing the effect of diffusion on electron transports (21, 32). In acidophiles, spatial organization associated with other parameters (33) (high percentages of basic proteins in the periplasm, mechanisms of pH homeostasis, etc.) could participate for an improvement of the stabilization of the system.

In \( A. \text{ferrooxidans} \), the controlled bifurcation of electrons from the exergonic electron transfer chain toward the endergonic uphill electron flow is of major importance, and the question arises as to how the balance of reducing equivalents between these two pathways is detected and controlled \( \text{in vivo} \). Our results indicate the presence of \( \text{a} \text{bc}_{1} \text{complex in the supercomplex, but we did not measure a reverse as well as a direct } \text{bc}_{1} \text{complex activity probably because of the loss of the Rieske protein. Although our results show that the } \text{bc} \text{ complex is largely substoichiometric in the supercomplex as compared with cyt } \text{c oxidase, this work provides evidence for a possible physical association, in the cell, of proteins involved in downhill and uphill pathways. All together, these data suggest that next to a regulation at the expression level, the association of complexes also might be part of the adaptation of the primary metabolism to a change in environmental conditions.}

\textbf{Acknowledgments—We thank M. Bauzan (Fermentation Plant Unit IBSM, Marseilles, France) for growing the bacteria; S. Lignon and D. Moinier (Proteomic Analysis Center, Marseilles, France) for proteomic analysis; V. Bonnefoy (LCB, Marseilles, France) for kindly supplying the anti-RcY, Cyc2, ORF1, CoxB, and Cyc1 antibodies; and W. Nitschke, E. Lojou, P. Tron, M. L. Cardenas, and A. Cornish-Bowden for helpful discussions.}
REFERENCES

1. Croal, L. R., Gralnick, J. A., Malasarn, D., and Newman, D. K. (2007) Annu. Rev. Genet. 38, 175–202
2. Rohwerder, T., Gehrke, T., Kinzler, K., and Sand, W. (2003) Appl. Microbiol. Biotechnol. 63, 239–248
3. Elbehti, A., Brasseur, G., and Lemesle-Meunier, D. (2000) J. Bacteriol. 182, 3602–3606
4. Brasseur, G., Levican, G., Bonnefoy, V., Holmes, D. H., Jedlicki, E., and Lemesle-Meunier, D. (2004) Biochim. Biophys. Acta 1656, 114–126
5. Appia-Ayme, C., Guiliani, N., Ratouchniak, J., and Bonnefoy, V. (1999) Appl. Environ. Microbiol. 65, 4781–4787
6. Malarte, G., Leroy, G., Lojou, E., Abergel, C., Bruschi, M., and Giudici-Orticoni, M. T. (2005) Biochim. Biophys. Acta 1656, 114–126
7. Brasseur, G., Bruscella, P., Bonnefoy, V., and Lemesle-Meunier, D. (2002) Biochim. Biophys. Acta 1555, 37–43
8. Elbehti, A., Nitschke, W., Tron, P., Michel, C., and Lemesle-Meunier, D. (1999) J. Biol. Chem. 274, 16760–16765
9. Giudici-Orticoni, M. T., Leroy, G., Nitschke, W., and Bruschi, M. (2000) Biochemistry 39, 7205–7211
10. Schägger, H. (2001) IUMRMB Life 52, 119–128
11. Thomas, D. E., Ryan, D., and Levin, W. (1976) Anal. Biochem. 75, 168–176
12. Edmonson, D. G., and Roth, S. Y. (1993) in Current Protocols in Molecular Biology (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds), pp. 1–6, Unit 20.6, John Wiley & Sons, Inc., New York
13. Wittig, I., and Schägger, H. (2007) Methods Cell Biol. 80, 723–741
14. Brugna-Guiral, M., Tron, P., Nitschke, W., Stetter, K., Burlat, B., Guigliarelli, B., Bruschi, M., and Giudici-Orticoni, M. T. (2003) Extremophiles 7, 145–157
15. Giúral, M., Tron, P., Aubert, C., Gloter, A., Ijobi-Nivol, C., and Giudici-Orticoni, M. T. (2005) J. Biol. Chem. 280, 42004–42015
16. Nye, B., and Bott, M. (2003) J. Biol. Chem. 278, 4339–4346
17. Dutton, P. L. (1978) Methods Enzymol. 54, 411–434
18. Yeates, T. O., Tsai, Y., Tanaka, S., Sawaya, M. R., and Kiefel, C. A. (2007) Biochem. Soc. Trans. 35, 8–11
19. чиске, N., Sepumi, N. B., Gordon, D. M., Saxena, S., Dancis, A., and Pain, D. (1999) J. Biol. Chem. 274, 2287–22854
20. Marlovits, T. C., Kubori, T., Sukhan, A., Thomas, D. R., Galan, J. E., and Unger, V. M. (2004) Science 306, 1040–1042
21. Marshall, M. J., Beliaev, A. S., Dohnalkova, A. C., Kennedy, D. W., Shi, L., Wang, Z. M., Boyanov, M. I., Iai, B., Kemner, K. M., McLean, J. S., Reed, S. B., Culley, D. E., Bailey, V. L., Simonson, C. J., Saffarini, D. A., Romine, M. F., Zachara, J. M., and Fredrickson, J. K. (2006) PLoS Biol. 4, 1234–1333
22. Weber, K. A., Achenbach, L. A., and Coates, J. D. (2006) Nat. Rev. Microbiol. 4, 752–764
23. Jeans, C., Singer, S. W., Chan, C. S., VerBerkmoes, N. C., Shah, M., Hettich, R. L., Banfield, J. F., and Theilen, M. P. (2008) ISME J. 2, 542–550
24. Arredondo, R., García, A., and Jerez, C. A. (1994) Appl. Environ. Microbiol. 60, 2846–2851
25. Arnedano, F., Banci, L. Bertini, I., Mangani, S., and Thompsett, A. R. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 3814–3819
26. Quaranta, D., McCarty, R., Bandarian, V., and Rensing, C. (2007) J. Bacteriol. 189, 5361–5371
27. Poyau, A., Buchet, K., and Godinot, C. (1999) FEBS Lett. 462, 416–420
28. Mick, D. U., Wagner, K., van der Laan, M., Frazier, A. E., Perschil, I., Pavlas, M., Meyer, H. E., Warscheid, B., and Rehling, P. (2007) EMBO J. 26, 4347–4358
29. Myllykallio, H., Drepper, F., Mattis, P., and Daldal, F. (2000) Trends Microbiol. 8, 493–494
30. Stroh, A., Anderka, O., Pfieffer, K., Yagi, T., Finel, M., Ludwig, B., and Schägger, H. (2004) J. Biol. Chem. 279, 5000–5007
31. Baker-Austin, C., and Doppson, M. (2007) Trends Microbiol. 15, 165–171