The High-Molecular-Weight Cytochrome *c* Cyc2 of *Acidithiobacillus ferrooxidans* Is an Outer Membrane Protein

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A high-molecular-weight *c*-type cytochrome, Cyc2, and a putative 22-kDa *c*-type cytochrome were detected in the membrane fraction released during spheroplast formation from *Acidithiobacillus ferrooxidans*. This fraction was enriched in outer membrane components and devoid of cytoplasmic membrane markers. The genetics, as well as the subcellular localization of Cyc2 at the outer membrane level, therefore make it a prime candidate for the initial electron acceptor in the respiratory pathway between ferrous iron and oxygen.

Acidithiobacillus ferrooxidans, one of the most studied bioleaching bacteria, derives the energy required for its growth mainly from the oxidation of reduced sulfur compounds and/or ferrous iron (Fe^{2+}) (22). Although various redox proteins from this organism have been identified and characterized, the electron pathway from Fe^{2+} to oxygen has not been established. Indeed, several different pathways have been proposed (3, 6, 17, 18, 19, 23, 24, 43). In each case, the terminal electron acceptor is assumed to be a cytochrome oxidase anchored to the cytoplasmic membrane, and transfer of electrons is postulated to occur through a series of periplasmic carriers, including the copper protein, rusticyanin, and at least one *c*-type cytochrome. However, the nature and the subcellular localization of the primary electron acceptor from Fe^{2+} still remain in question (3, 6, 14, 17, 18, 19, 23, 24, 43). In its natural habitat, *A. ferrooxidans* derives ferrous iron from pyrite, which is insoluble. As previously pointed out (22, 24), an excreted and/or an outer membrane (OM) electron transporter can be assumed to be required to link the extracellular pyrite to the periplasmic redox proteins. Based on several arguments, we postulated that the high-molecular-weight cytochrome *c* encoded by the *cyc2* gene (Cyc2) would be located in the OM and could therefore be the first electron carrier in the Fe^{2+} oxidation respiratory pathway (3). First of all, the *cyc2* gene is cotranscribed with the genes encoding an *aa*₃-type cytochrome *c* oxidase, a 21-kDa *c*₄-type cytochrome, and rusticyanin, suggesting that these proteins belong to the same pathway (3). Further, the putative signal peptide is removed from Cyc2 in *A. ferrooxidans*, suggesting its translocation to the periplasm (2, 10). Additionally, from the analysis of its amino acid sequence, Cyc2 was predicted to be acid stable and to be located in the OM (3). In support of the postulated role of Cyc2, we present here direct

evidence that the processed holocytochrome is localized in the OM of *A. ferrooxidans*.

Prediction of Cyc2 secondary structure. Cyc2 appears to lack any hydrophobic segments long enough to span the inner membrane (IM) as α -helices, based on studies of secondary structure prediction by using multivariate linear regression combination (http://npsa-pbil.ibcp.fr/cgi-bin/secpred_mlr.pl) (20), GOR secondary structure prediction (<http://molbiol.soton.ac.uk/compute/GOR.html>) (15), and prediction of secondary structural content from amino acid composition with analytic vector decomposition (<http://www.bork.embl-heidelberg.de/SSCP>) (8, 9) algorithms. Rather, this protein is predicted to be composed mainly of β -sheets, a characteristic of many OM proteins (41).

Preparation and characterization of OM fractions from *A. ferrooxidans*. To determine the subcellular localization of Cyc2 in *A. ferrooxidans* ATCC 33020, an efficient procedure to resolve membrane fractions was required. Repeated attempts to resolve the crude membranes into the IM and OM fractions by isopycnic sucrose gradients from French press lysates were unsuccessful.

One method, widely used to prepare OM fractions of *A. ferrooxidans* (7, 21, 25, 32, 37, 40), is the selective disaggregation and solubilization of the IM components by the anionic detergent Sarkosyl (12). However, no conclusive evidence demonstrating the OM nature of Sarkosyl-insoluble fractions or excluding their possible contamination with IM components has been presented to date; generally, the efficiency in OM isolation has been inferred from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein profiles. In our studies, the Sarkosyl-insoluble fraction, although featuring some typical properties of an OM fraction, was significantly contaminated with at least one IM protein (cytochrome *c* oxidase) (data not shown). The failure to resolve efficiently the OM and IM of *A. ferrooxidans* could be due to the composition of its OM, which is particularly rich in lipids (37). We concluded that this procedure could not be used with confidence to localize components specifically to the OM of *A. ferrooxidans* and, therefore, that the localization of proteins

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FIG. 1. Characterization of membrane fractions from *A. ferrooxidans* ATCC33020 by SDS-15% PAGE (27) (A and C) and by Western immunoblotting (B). Total membranes (lanes 1) and OMs (lanes 2) were prepared from *A. ferrooxidans* cells grown in iron medium (3) according to the method of Mizuno and Kageyama (31). In brief, cells were pelleted, washed three times with basal salts, and resuspended in 3 ml of 20% sucrose at 4°C. Spheroplasts were obtained by adding sequentially 1.5 ml of 2 M sucrose, 1.7 ml of 0.1 M Tris-HCl (pH 8.0), 0.13 ml of 1% EDTA, 0.15 ml of 10 mg of lysozyme ml⁻¹, and 10 µg of DNase ml⁻¹. The mixture was incubated at 30°C for 1 h with occasional shaking. The spheroplasts were removed by centrifugation at 15,000 × g for 15 min; OMs were recovered from the supernatant by centrifugation at 100,000 × g for 90 min at 4°C and then resuspended with distilled water. The remaining spheroplasts were resuspended in 5 mM MgCl₂ and passed three times through a French press. Total membranes were then recovered by centrifugation at 100,000 × g for 90 min at 4°C after removal of the unbroken cells and debris by low-speed centrifugation. After SDS-PAGE, gels were stained for total proteins with Coomassie blue (A) or for hemoproteins with *o*-dianisidine (C) (13). The positions of Cyc2 and Omp40 are indicated by arrows in panel A. (B) Western immunoblots were performed with antisera raised against Omp40 (21), purified CoxB, and purified Cyc2 (Bonnefoy et al., unpublished) and visualized with a chemiluminescence kit (ECL Western blotting reagents from Amersham Pharmacia Biotech)

based only on the analysis of Sarkosyl-insoluble fractions is questionable.

As an alternative approach, we employed a technique relying on the release into the medium of OM fragments during spheroplast formation (31). This phenomenon seemed likely to occur in *A. ferrooxidans*, since electron micrographs from a frozen-etched preparation of spheroplasts have shown that the OM was disrupted and partially detached from the cells (1). *A. ferrooxidans* cells were treated with lysozyme and EDTA to produce spheroplasts. After removal of the spheroplasts, the released membranes were pelleted by high-speed centrifugation, resuspended, and analyzed. The SDS-PAGE protein profile of the released membranes displayed a significantly less complex protein band pattern than that of the total membrane (TM) fraction prepared from lysed spheroplasts (Fig. 1A, lanes 1 and 2). Furthermore, while a TM fraction remained at the 40 to 50% interphase of a discontinuous 30 to 70% sucrose density gradient, the released membrane fraction migrated to the 60 to 70% interphase (data not shown), indicating a higher buoyant density as expected for an OM preparation.

Compared to the TM fraction, the released membranes were enriched for specific components of the OM. 2-Keto-3-deoxyoctonate (KDO), a component of the lipopolysaccharides (LPS), quantified by the thiobarbituric acid method (26), was 10-fold more abundant in the OM fraction (32.5 mg of KDO/g of protein) than in the TM fraction (3.2 mg of KDO/g of protein). These KDO levels are relatively low, as previously reported for *A. ferrooxidans* (44), possibly due to the preparation of membranes at a neutral pH. Indeed, a significant release of LPS has been observed when *A. ferrooxidans* cells are incubated at a pH of >3.5 (4). The released membranes were also enriched for Omp40, the major OM porin of *A. ferrooxidans* (21), as shown by Western immunoblotting with a specific antiserum (Fig. 1A and B).

To assess the degree of resolution of the released membrane fraction from IM components, cytochrome *c* oxidase and *b*-type cytochromes were monitored as IM markers (23). Contrary to what was observed with TM fractions, reduced minus oxidized difference optical spectra of the released OM fraction exhibited no absorption peaks corresponding to *aa*₃ cytochrome oxidases (442 and 597 nm) or to *b*-type cytochromes (430, 530, and 560 nm) (Fig. 2A and B). Furthermore, no detectable cytochrome *c* oxidase enzymatic activity (28) was detected in the released membrane fraction compared to the TM fraction (6 µmol of cytochrome *c* oxidized min⁻¹ g⁻¹ by using exogenous reduced horse heart cytochrome *c* as the substrate). Finally, Western immunoblotting with an antiserum raised against the periplasmic domain of the subunit II of the *aa*₃ cytochrome oxidase, CoxB, showed only traces of this protein compared to TM levels (Fig. 1B). Based on these results, we conclude that the released membrane fraction represents a well-resolved OM fraction.

Identification and characterization of OM cytochromes. To detect cytochromes *c*, TM and OM preparations were analyzed

according to the manufacturer's instructions. The retarded migration of the bands in the OM fraction in panels A and B is possibly due to a higher content in LPS. The LMW Electrophoresis Calibration kit from Bio-Rad was used as molecular weight markers (MW).

FIG. 2. Spectrophotometric characterization of membrane fractions of *A. ferrooxidans*. (A) Room temperature optical spectra in the α , β , and Soret regions. Spectra were obtained as the difference between dithionite-reduced minus H_2O_2 -oxidized samples. TM and released OM fractions were suspended at 2.4 and 2.5 mg ml^{-1} , respectively, in 20 mM β -alanine buffer (pH 3.5). (B) Optical spectra recorded at liquid nitrogen temperature (77 K) in the α -band region by using a dual-wavelength DW2000 SLM Aminco spectrophotometer with a slit of 1 nm. Wavelengths were calibrated with a Holmium filter. Spectra were obtained as the difference between dithionite-reduced minus Na_2IrCl_6 -oxidized samples. TM and OM fractions were suspended at 1.4 mg ml^{-1} in 20 mM β -alanine buffer (pH 3.5).

by different procedures. Room temperature spectra showed absorption peaks corresponding to the γ , β , and α bands (420, 522, and 552 nm) of *c*-type cytochromes in both preparations (Fig. 2A). To resolve possible individual components of the α peak, low-temperature (77 K) difference spectra were recorded (Fig. 2B). Three spectrally distinct α peaks at 545, 548, and 552 nm (Fig. 2B) were clearly distinguished in the OM. Heme staining with *o*-dianisidine (13) of proteins resolved on SDS-PAGE revealed at least five putative *c*-type cytochromes in the TM fraction (Fig. 1C, lane 1), suggesting that these cytochromes are closely associated to the IM and/or the OM. Strikingly, two of these *c*-type cytochromes, the 46- and 22-kDa bands, were associated with the OM fraction (Fig. 1C, lane 2).

The 46-kDa cytochrome *c* reacted with a Cyc2-specific antiserum (Fig. 1B). Furthermore, microsequencing of the 46-kDa band (473A; Applied Biosystems, Warrington, United Kingdom) yielded an amino-terminal sequence (LPSFARQ) that corresponded to that of the mature Cyc2. The 22-kDa putative *c*-type cytochrome reacted neither with the Cyc2 specific antibodies nor with antibodies directed against the periplasmic *c*₄-type cytochrome (data not shown). Because it was not pos-

sible to sequence the amino-terminal region, its identity remains unclear. However, the presence of three peaks in the low-temperature spectra (Fig. 2B) suggests that this 22-kDa band corresponds to a dihemic cytochrome because Cyc2 is known to be monohemic (2).

To confirm the localization of Cyc2 *in situ*, protease digestion of surface proteins with proteinase K was performed with intact *A. ferrooxidans* cells (11). Cyc2 and Omp40 were significantly degraded after a 2-h incubation (Fig. 3A and B) while no degradation of an IM marker, CoxB, was observed (Fig. 3C). These results not only support the conclusion that Cyc2 is localized in the OM, but they also indicate that some domains of this cytochrome are exposed to the external environment where they may interact with insoluble substrates such as pyrite. Further support for the OM localization of Cyc2 is that, when expressed in *Escherichia coli* cells, the holo-cytochrome

FIG. 3. Localization of Cyc2 in *A. ferrooxidans* ATCC 33020 cells *in situ* by proteinase K digestion of surface proteins (41). Iron-grown bacteria (3) were pelleted and resuspended in phosphate-buffered saline containing 10 mM MgCl_2 at a final concentration of 4×10^9 bacteria ml^{-1} . Proteinase K was added to a final concentration of 100 $\mu\text{g ml}^{-1}$, and the mixture was incubated for 30, 60, 90, and 120 min at 37°C. Digestion was terminated by the addition of 1 mM phenylmethylsulfonyl fluoride plus Laemmli sample buffer (27). The samples were immediately boiled at 100°C for 5 min and then subjected to SDS-15% PAGE (27). (A) Protein digestion was assessed by immunodetection on Western blots with anti-Cyc2 polyclonal antiserum (Bonnefoy et al., unpublished) and visualized with a chemiluminescence kit (ECL Western blotting reagents from Amersham Pharmacia Biotech) according to the manufacturer's instructions. Omp40, an OM protein (B), and CoxB, an IM protein (C), were used as positive and negative controls, respectively, and analyzed with the corresponding antisera. Control experiments were performed without any protease added.

was targeted to the OM (V. Bonnefoy et al., unpublished results).

OM *c*-type cytochrome(s) have been previously described in some neutrophilic microorganisms that also interact with insoluble metals or minerals for energy metabolism, including *Desulfovibrio vulgaris* (42), *Geobacter sulfurreducens* (16), *Shewanella putrefaciens* (33, 34), and *Shewanella frigidimarina* (36). Furthermore, in *S. putrefaciens*, two of these OM *c*-type cytochromes were shown to be required for the activity of the terminal metal reductase (5, 34). To our knowledge, this is the first report of OM cytochromes *c* from an acidophilic microorganism.

In nature, *A. ferrooxidans* must extract electrons from pyrite, an insoluble mineral sulfide. It seems unlikely that iron would be oxidized inside the cell because of the rapid autooxidizability of soluble ferrous iron and the highly insoluble nature of ferric oxide products at the pH of the cytoplasm or of the periplasm (pH 6.5 and 3, respectively). Therefore, the site of ferrous oxidation has been inferred to be outside the cell (24) and, indeed, ferric oxides produced during cell growth appear to be deposited outside the cell membranes. Ingledew et al. (24) proposed that electrons derived from the oxidation of ferrous iron are conducted through an extracellular polynuclear iron complex, bound to the phospholipid head groups of the OM, to an acceptor in the periplasmic space. Another hypothesis is that an electron transfer protein would be responsible for the direct oxidation of ferrous iron to ferric iron. Several candidates have been postulated (3, 6, 14, 17, 18, 19, 23, 24, 43), but all of them were periplasmic or bound to the IM, except for Cyc2, which is shown here to be localized in the OM. Therefore, the pathway of electron transfer from ferrous iron to oxygen to drive ATP synthesis is likely Cyc2 rusticyanin → Cyc1(*c*₄-type cytochrome) → *aa*₃ cytochrome *c* oxidase, as we had previously proposed (3). An attractive model for this pathway would be an electron wire spanning both the OM and the IM to conduct electrons from pyrite to oxygen, as has been proposed for the Hmc complex of *Desulfovibrio vulgaris* subsp. *vulgaris* Hildenborough (38), and the metal reductase complex of the *Shewanella* genus (5, 36). The genes encoding the electron transporters involved in these complexes are cotranscribed and the corresponding proteins are located in different cellular compartments, as in the present case. According to such a model, the proteins encoded by the *cyc2cyc1ORF1coxBACD* operon (that is, Cyc2, Cyc1, ORF1, the *aa*₃-type cytochrome oxidase, and rusticyanin) may also form a transmembrane electron wire allowing electron transfer from the OM Cyc2 to the IM cytochrome oxidase. At this stage, however, we cannot exclude the involvement of excreted redox proteins, as suggested for *G. sulfurreducens* (39), or the participation of exogenous extracellular molecules (29, 30, 35), which could transport electrons from the insoluble minerals to the OM.

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