Redox Components of Cytochrome bc-type Enzymes in Acidophilic Prokaryotes

I. CHARACTERIZATION OF THE CYTOCHROME bc1-TYPE COMPLEX OF THE ACIDOPHILIC FERROUS ION-OXIDIZING BACTERIUM THIOBACILLUS FERROOXIDANSA

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The redox components of the cytochrome bc1 complex from the acidophilic chemolithotrophic organism Thio- bacillus ferrooxidans were investigated by potentiometric and spectroscopic techniques. Optical redox titrations demonstrated the presence of two b-type hemes with differing redox midpoint potentials at pH 7.4 (~169 and +20 mV for bL and bH, respectively). At pH 3.5, by contrast, both hemes appeared to titrate at about +20 mV. Antimycin A, 2-heptyl-4-hydroxyquinoline N-oxide, and stigmatellin induced distinguishable shifts of the b hemes’ a-bands, providing evidence for the binding of antimycin A and 2-heptyl-4-hydroxyquinoline N-oxide near heme bL (located on the cytosolic side of the membrane) and of stigmatellin near heme bH (located on the periplasmic side of the membrane). The inhibitors stigmatellin, 5-(n-undecyl)-6-hydroxy-4,7-dioxobenzothiazole, and 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone affected the EPR spectrum of the Rieske iron-sulfur center in a way that differs from what has been observed for cytochrome bc1 or bH complexes. The results obtained demonstrate that the T. ferrooxidans complex, although showing most of the features characteristic for bc1 complexes, contains unique properties that are most probably related to the chemolithotrophy and/or acidophilicity of its parent organism. A speculative model for reverse electron transfer through the T. ferrooxidans complex is proposed.

Thiobacillus ferrooxidans is the main bacterium used in the industrial extraction of copper and uranium from ores, using the microbial leaching technique (1). The sole energy-producing process used by this obligate chemolithotrophic bacterium for growth and cell maintenance involves the oxidation of reduced sulfur compounds and/or ferrous iron under acidic conditions, using O2 as the oxidant (2–4). When grown on Fe(II), a comparatively small amount of energy is released by the oxidative reaction. The bacterium nevertheless fixes its own CO2, and Fe(III) oxidation must therefore be coupled to reduction of the NAD(P) required for this fixation and other anabolic processes. It has been suggested that an uphill electron transfer, established at the expense of the energy derived from the oxidation of Fe2+ by oxygen, may be involved in the reduction of NAD(P)+ from Fe3+ (for a review, see Ref. 5).

Various respiratory chain components have been detected in T. ferrooxidans. The optical spectra measured at 77 K on whole cells indicated the presence of multiple cytochromes c, one cytochrome b, and cytochrome oxidase (6). Using optical and EPR techniques combined with potentiometric and kinetic studies, Ingledew and Cobley (7) have detected an a1-type cytochrome, multiple cytochromes c, cytochrome b, two copper-containing centers, high and low spin ferric hemes, and a ferredoxin center. In these experiments, only the γ-band of cytochrome b was detected at pH 3.2. The presence of ubiquinone-8 has also been reported (8). Some of these compounds have been isolated and purified: a soluble 14-kDa cytochrome c552 (9), a soluble c4-type cytochrome (10), the blue copper protein rusticyanin (11–13), an iron-sulfur protein Fe2+/cytochrome c552 oxidoreductase (14, 15), an a1-type cytochrome oxidase (16), and three membrane-bound cytochromes c: a high molecular mass cytochrome c, the size of which depends on the strain (17–19), and a 30-kDa (19) and a 22.3-kDa (18) or 21-kDa (17, 19) cytochrome c.

The electron transfer chain from Fe2+ to O2 is presently considered to involve a Fe2+/cytochrome c oxidoreductase, rusticyanin, at least one cytochrome c (the 14-kDa soluble cytochrome c and/or the c1-type cytochrome), and an a1-type cytochrome. Ingledew (5) has put forward the idea that the uphill electron transfer from Fe2+ to NAD+ may involve a putative bc1 complex, according to a chemiosmotic mechanism, possibly via a Q-cycle mechanism operating in reverse. This mechanism requires the presence of two separate and independent quinone reaction sites: the ubiquinol oxidation site (Qp site) located on the positive side of the membrane and the ubiquinone reduction site (Qn site) located on the negative side of the membrane (20–22). The Qp site is formed by cytochrome b (in the vicinity of heme b1) and the iron-sulfur protein and is the target of specific inhibitors like myxothiazol, stigmatellin, DBMIB, and UHDBT (for a review, see Ref. 23). The binding of myxothiazol and stigmatellin results in an alteration of the absorbance spectrum of heme b1, which is shifted toward the red. The binding of stigmatellin, DBMIB, and UHDBT further induces spectral changes in the EPR spectrum of the Rieske cluster. The Qn site is associated with cytochrome b (in the vicinity of heme b3) and is the target of other specific

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1 The abbreviations used are: Qp and Qn sites, quinone-binding sites (for a review, see Ref. 5).
Cytochrome bc Complex from Thiobacillus ferrooxidans

EXPERIMENTAL PROCEDURES

Organism and Culture Growth Procedure—T. ferrooxidans was kindly supplied by Dr. D. Morin (Bureau des Recherches Géologiques et Minières (BRGM), Orléans, France). The strain was isolated from drainage water at the Salsigne sulfur mine (France). Large-scale growth of the organism was performed at pH 1.6 in the 9 K medium described by Silverman and Lundgren (26), supplemented with 1.6 mM CuSO4,5H2O, using a homemade polypropylene fermentor with a capacity of 400 liters. Cells were harvested according to Bodo and Lundgren (27) and stored as pellets at −70 °C. About 12 g of cell (wet weight) were obtained from 300 liters of cell culture.

Preparation of Membrane Fragments and Spheroplasts—Membrane fragments were prepared as described by Elbehti and Lesme Le-Meur (19). For the preparation of spheroplasts, cell paste (5 g, wet weight) was washed three times in 0.5 M NaCl at 0 °C and resuspended in 20% (w/v) glucose. The cell suspension was incubated at 37 °C in a shaking water bath for 2 h. The cells were centrifuged at 6000 × g for 10 min and washed three times in 30 mM Tris-HCl (pH 8). After each washing step, one freezing (in liquid nitrogen) and thawing (room temperature) step was performed. The cells were then resuspended in 30 mM Tris-HCl (pH 8), 10 mM EDTA, and 20% glucose and incubated for 20 h at 37 °C in the presence of lysozyme (1 mg/ml). After being centrifuged at 16,000 × g for 15 min, the spheroplasts obtained were washed twice in 20 mM β-alanine/H3SO4 buffer (pH 3.5) and 20% glucose and finally resuspended in the same buffer. The Gram coloration method was used to verify formation of spheroplasts (28). Protein concentrations were measured using the method of Lowry et al. (29).

Optical and EPR Spectroscopy—The visible and UV absorption spectra were determined with an Aminco DW2 recording spectrophotometer using 1-cm light path cuvettes. The heme content was determined from the reduced-minus-oxidized difference spectrum using the following millimolar extinction coefficients for the a-peak: Δε505-540 nm = 16.5 mM−1 cm−1 (cytochrome c); Δε505-550 nm = 34 mM−1 cm−1 (cytochrome b), and Δε503-630 nm = 21 mM−1 cm−1 (cytochrome oxidase).

The spectral shift of the hemes was measured as the spectral difference between the dithionite-reduced enzyme in the presence and absence of saturating amounts of inhibitor. Spheroplasts were first reduced by adding a few grains of sodium dithionite, and the base line was used as a reference for the full redox difference during the titration (at pH 7.4) representing the low (−215 mV) and bH (20 mV) and imply a ratio of ∼1:1 assuming similar extinction coefficients. At pH 3.5, by contrast, a good fit could already be obtained using only a single n = 1 component with a midpoint potential of 20 mV. These results suggest that the two cytochrome b hemes exhibit rather close redox midpoint potentials at pH 3.5. The data points for the c-type hemes were fitted with four components with midpoint potentials of −169 and 22 mV, each contributing approximately equally to the total absorbance. These would correspond to cytochrome bH (−170 mV) and bH (20 mV) and imply a ratio of ∼1:1 assuming similar extinction coefficients. At pH 3.5, by contrast, a good fit could already be obtained using only a single n = 1 component with a midpoint potential of 20 mV. These results suggest that the two cytochrome b hemes exhibit rather close redox midpoint potentials at pH 3.5. The data points for the c-type hemes were fitted with four components with midpoint potentials of −169 and 22 mV, each contributing approximately equally to the total absorbance. These would correspond to cytochrome bH (−170 mV) and bH (20 mV) and imply a ratio of ∼1:1 assuming similar extinction coefficients. At pH 3.5, by contrast, a good fit could already be obtained using only a single n = 1 component with a midpoint potential of 20 mV. These results suggest that the two cytochrome b hemes exhibit rather close redox midpoint potentials at pH 3.5. The data points for the c-type hemes were fitted with four components with midpoint potentials of −169 and 22 mV, each contributing approximately equally to the total absorbance. These would correspond to cytochrome bH (−170 mV) and bH (20 mV) and imply a ratio of ∼1:1 assuming similar extinction coefficients. At pH 3.5, by contrast, a good fit could already be obtained using only a single n = 1 component with a midpoint potential of 20 mV. These results suggest that the two cytochrome b hemes exhibit rather close redox midpoint potentials at pH 3.5. The data points for the c-type hemes were fitted with four components with midpoint potentials of −169 and 22 mV, each contributing approximately equally to the total absorbance. These would correspond to cytochrome bH (−170 mV) and bH (20 mV) and imply a ratio of ∼1:1 assuming similar extinction coefficients. At pH 3.5, by contrast, a good fit could already be obtained using only a single n = 1 component with a midpoint potential of 20 mV. These results suggest that the two cytochrome b hemes exhibit rather close redox midpoint potentials at pH 3.5. The data points for the c-type hemes were fitted with four components with midpoint potentials of −169 and 22 mV, each contributing approximately equally to the total absorbance. These would correspond to cytochrome bH (−170 mV) and bH (20 mV) and imply a ratio of ∼1:1 assuming similar extinction coefficients.

For redox potentiometry, optical titrations were carried out at 25 °C as described by Dutton (30), using a Kontron Uvikon 922 double-beam spectrophotometer equipped with a Spectralon integrating sphere. Membrane fragments were suspended in the same buffers (pH 7.4 and 3.5) as mentioned above; pH values were controlled at the beginning and end of each redox titration. The following redox mediators were used: 1,1'-phenylenediamine, ferrocenemonomocarboxylic acid, 1,4-benzoquinone, N,N,N',N'-tetramethyl-p-phenylenediamine, 2,5-dimethyl-p-benzoquinone, 1,2-naphthoquinone, 1,4-naphthoquinone, phenazine ethosulfate, indigo carmine, anthraquinone-2,6-disulfonylate, and anthraquinone-2-sulfonate. Reductive titrations were carried out using sodium dithionite, and oxidative titrations were done using sodium hexachloroiridate (Na2Cl6Ir).

RESULTS

Electrochemical Properties of the b- and c-type Hemes of T. ferrooxidans—Membrane fractions prepared from the BRGM strain of T. ferrooxidans were examined for their content of the various cytochromes at pH 7.4. The optical difference spectra shown in Fig. 1 demonstrate the presence of cytochrome a (597 nm), cytochrome(a) c (552 nm), and cytochrome b (560 nm). It can be seen that b-type hemes are substoichiometric to c-type hemes and can only be discerned as a shoulder on the red flank of the c-type hemes’ a-peak in the full redox difference spectrum.

Fig. 2 shows the results of a redox titration of b-type (at 562 nm; circles) and c-type (at 550 nm; diamonds) hemes carried out on membrane preparations at pH 7.4 (closed symbols) and 3.5 (open symbols). In the case of cytochrome b and at pH 7.4, the data could be fitted with two components with midpoint potentials of −169 and 22 mV, each contributing approximately equally to the total absorbance. These would correspond to cytochrome bH (−170 mV) and bH (20 mV) and imply a ratio of ∼1:1 assuming similar extinction coefficients. At pH 3.5, by contrast, a good fit could already be obtained using only a single n = 1 component with a midpoint potential of 20 mV. These results suggest that the two cytochrome b hemes exhibit rather close redox midpoint potentials at pH 3.5. The data points for the c-type hemes were fitted with four components with midpoint potentials of 150, 360, 410, and 485 mV at pH 7.4 and 240, 350, 430, and 560 mV at pH 3.5.

Fig. 2 inset shows differences in spectra recorded during the titration (at pH 7.4) representing the low (−238 mV minus −127 mV; spectrum A) and high (−127 mV minus +103 mV; spectrum B) potential components of cytochrome b as well as the full redox difference attained during the titration (spectrum C). A slight shift toward shorter wavelengths was observed going from the low to the high potential spectra of cytochrome b (see below).

Inhibitor Binding to the Thiobacillus Cytochrome bc Complex—The typical inhibitors of the bc1 complexes (antimycin A, HQNO, myxothiazol, and stigmatellin) have been found to inhibit a proton-motive force-dependent uphill electron transfer inhibitors like antimycin A, funiculosin, HQNO, and diuron. The binding of these Q₁ site inhibitors to the bc complexes induces a red or a blue (in the case of funiculosin) shift in the absorbance spectrum of heme b₃. These shift properties have been used to measure the binding of the inhibitors to their specific sites in the bc complexes (24).

We have recently provided evidence for the presence of a bc₁ complex in T. ferrooxidans based on the observation of the typical EPR spectrum of the Rieske center, which is one of the crucial redox centers of bc₁-type complexes (25). This was the first and so far only evidence for the existence of a bc₁ complex in an acidophilic proteobacterium. In the present study, we characterized the redox centers of the enzyme and the effect of the binding of the specific bc₁ complex inhibitors on cytochrome b and the Rieske iron-sulfur protein in more detail.
from reduced cytochrome c to NAD$^+$ in spheroplasts from *T. ferrooxidans*. The study of inhibitor-induced changes in spectral properties of the redox site allows the detection of binding of the respective inhibitors and the identification of the affected heme center by its characteristic absorption maximum (24, 31). Typically, difference spectra between dithionite-reduced bc$_1$-containing samples in the presence and absence of inhibitor yield symmetrical S-shaped curves crossing the base line at a wavelength corresponding to the maximum of the absorption band of the heme affected by the inhibitor binding (31–35).

Binding experiments were performed at acidic and neutral pH values (3.5 and 7.4, respectively) on *Thiobacillus* spheroplast preparations reduced by an excess of dithionite. Spheroplasts are closed vesicles, and the internal pH is always constant (almost neutral around pH 6.5) whatever the external pH (36). Fig. 3 (A and B) shows the effect of antimycin A, HQNO, and stigmatellin on the optical spectra of cytochrome b recorded at pH 7.4 and 3.5, respectively.

At pH 7.4, the S-shaped symmetrical curves obtained upon adding antimycin A and HQNO crossed the base line at $\lambda_{max} = 562$ nm, showing a maximum at 566 nm and a minimum at 559 nm (Fig. 3A). This shape reflects a shift of a heme α-band with a maximum at $\lambda_{max} = 562$ nm toward longer wavelengths (bathochromic effect). These features are consistent with the results of experiments carried out on the purified mitochondrial bc$_1$ complex and on submitochondrial particles of various origins regarding the red shift of the heme 562 nm absorption band induced by these inhibitors (31–33, 37, 38). At pH 3.5, the S-shaped curves induced upon adding antimycin A and HQNO also crossed the base line at $\lambda_{max} = 562$ nm, but the curves were less symmetrical than those obtained at pH 7.4, with minima at 553 and 552 nm for antimycin and HQNO, respectively, and maxima at 566 nm for both these inhibitors (Fig. 3B).

At pH 3.5, the difference spectra obtained after adding stigmatellin to reduced spheroplasts exhibited a symmetrical S-shape crossing the base line at $\lambda = 564–565$ nm, with a maximum at 569 nm and a minimum at 557 nm (Fig. 3B). Similar to what has been observed concerning the action of this inhibitor on submitochondrial particles or bc$_1$ complex isolated from beef heart mitochondria (34, 35), the red shift induced by stigmatellin therefore has a maximum and a minimum at higher wavelengths than those induced by antimycin and HQNO. This indicates that the heme affected by the binding of stigmatellin has an α-band with a maximum at a higher wavelength than the heme reacting to antimycin and HQNO, i.e. at $\lambda = 564–565$

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2 A. Elbehti and D. Lemesle-Meunier, manuscript in preparation.
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![EPR spectra recorded on membrane fragments (at a protein concentration of 100 mg/ml) from T. ferrooxidans reduced by ascorbate at pH 7.4 (spectra A-D) and at pH 3.5 (spectra A'–D') in the absence of inhibitor (spectra A and A') and in the presence of DEMIB (spectra B and B'), stigmatellin (spectra C and C'), and UHDBT (spectra D and D'). Instrument settings were as follows: temperature, 15 K; microwave frequency, 9.42 GHz; microwave power, 6.3 milliwatts; and modulation amplitude, 1.6 milliteslas (mT). Rieske centers typically start to saturate below 25 K (e.g. see Ref. 45). The lower temperature (15 K) was chosen in order to increase signal amplitude. Within experimental precision, no spectral distortions induced by the saturating conditions were observed.

At pH 7.4, only a very minor shift was observed in the presence of stigmatellin. The demonstration of the presence of two spectrally different hemes corroborates the slight shift observed during titration. The hemes sensitive to HQNO and antimycin A on one side and stigmatellin on the other side correspond to the high and low potential hemes, respectively.

Characteristics of the T. ferrooxidans Rieske Iron-Sulfur Protein—The EPR spectra of membrane preparations from T. ferrooxidans, reduced by ascorbate at pH 7.4 or pH 3.5, are shown in Fig. 4. The g value of the derivative-shaped g signal (gₓ = 1.90) and the fact that this center was reduced by a weakly reducing agent such as ascorbate are characteristic for the so-called Rieske center, representing one of the four redox centers of the bc complexes (21). A gᵧ signal is observed at g = 1.74 in the spectra at pH 7.4 (Fig. 4, spectrum A); lowering the pH to 3.5 resulted in broadening of this signal and a slight shift toward lower magnetic fields (spectrum A'). It has been shown previously that the shape of the EPR signal is affected by the redox state of the quinone pool in beef heart submitochondrial particles (39) and in chromatophores from Rhodobacter sphaeroides (40). In both cases, a significant sharpening of the gᵧ trough was seen to occur upon oxidation of the quinone pool.

Fig. 4 suggests that, at acidic pH values, the quinone pool in T. ferrooxidans membranes was at least partially reduced by the added ascorbate, whereas it remained oxidized at pH 7.4. This is in line with the fact that the Eₘ value of ubiquinone increased by 230 mV on going from pH 7.4 to pH 3.5, yielding a theoretical Eₘ,3.5 value of ~300 mV.

When the inhibitors stigmatellin, UHDBT, and DBMIB (known to bind at the Qₒ site in close vicinity of the Rieske center) were added, the shape and intensity of the Rieske EPR signals were altered, in particular the gᵧ signal (Fig. 4). At pH 7.4, DBMIB (spectrum B) and UHDBT (spectrum D) shifted the gᵧ signal to slightly lower fields. Addition of stigmatellin resulted in a distinct sharpening of the gₓ trough and of the gᵧ line (spectrum C). At pH 3.5, the gᵧ trough, which was rather broad in the absence of inhibitor (spectrum A'), was sharpened upon addition of all examined inhibitors (spectra B'–D'). The inhibitor-induced spectra at pH 3.5 appeared to be rather similar to those observed at pH 7.4.

The uphill electron transfer between Fe²⁺ and NAD⁺, required to allow CO₂ fixation via the Calvin cycle, has only been scanty studied so far. The only information on the compounds that may function in this respiratory chain was obtained by Ingledew and Cobley (7). In particular, these authors have performed redox titrations of the cytochromes in membrane preparations; clear results, however, were obtained only for cytochrome a₁. With respect to c-type hemes, their results indicated the presence of more than one species, but no clear inflection points were obtained. While titrating an a-type cytochrome at 440–479 nm, they found evidence for the presence of two additional components at pH 3.2 with Eₘ values of 280 and 150 mV, which they attributed to b-type hemes. No clear results were obtained from measurements carried out at pH 7.4 for the b- and c-type cytochromes. Electron paramagnetic resonance spectra of the electron transport particles revealed the presence of two copper-containing centers, high and low spin ferric hemes, and a ferredoxin center. The presence of a Rieske protein has not been detected in these studies.

We have recently shown that ferrous ion-grown cells from T. ferrooxidans contain a bc₁-type complex (25) functioning in reverse mode, as postulated by Ingledew (5). The present study reports on the characteristics of this bc₁ complex, allowing us to speculate about a possible reverse electron transfer mechanism.

Membrane-bound c-type Cytochromes—The global absorption band at 551–552 nm obtained from membrane preparations arises from three different c-type cytochromes with apparent molecular masses of 46, 30, and 21 kDa (19). The EPR spectrum of the 21-kDa cytochrome c was characteristic of a diheme cₓ-type cytochrome, and its terminal sequence was found to be identical to that of the soluble cytochrome cₓ present in the same strain of T. ferrooxidans (10). It is well known from studies on other species that cytochrome cₓ shows a strong affinity toward the membrane, and similar to what we found in Thiobacillus, this hemoprotein is generally detected both in the soluble and membrane fractions of cells (41). The EPR spectrum of the purified 46-kDa cytochrome did not resemble those typically found for cytochrome cₓ⁴. Moreover, Bonnelfoy and co-workers (42) have cloned and sequenced the gene coding for a c-type cytochrome, named cyc2, in the T. ferrooxidans ATCC 33020 strain, which is 67% identical and 86% similar to the N-terminal amino acid sequence of the 46-kDa cytochrome of our strain. This suggests that the cyc2 gene encodes a protein equivalent to the 46-kDa cytochrome of our strain (19, 42). No significant homologies to other cytochromes were detected in data banks, indicating that cyc2 does not correspond to cytochrome c₁. Moreover, the cyc2 gene is cotranscribed with cytochrome c₁ (cyc1), subunits 1–4 of cytochrome oxidase, and the rusticyanin genes, suggesting that all these components belong to the downhill electron transfer chain toward reduction of

DISCUSSION

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3. D. Lemesle-Meunier and P. Tron, unpublished results.
4. D. Lemesle-Meunier and W. Nitschke, unpublished results.
oxygen (43).5 Only the 30-kDa cytochrome therefore remains as a possible candidate for cytochrome c1. Since cytochrome c4 was present in the membrane fragments (as discussed above), it must contribute to the optical a-peak at 552 nm. By comparison with the reported midpoint potential of soluble cytochrome c4 from *T. ferrooxidans* (10), the components titrating at 360 and 410 mV at pH 7.4 and at 350 and 430 mV at pH 3.5 are attributed to the two hemes of cytochrome c4. It is, however, difficult at present to decide which of the two redox species at 240 and 560 mV (pH 3.5) and at 150 and 485 mV (pH 7.4) belongs to cytochrome c1 and which one to the 46-kDa cytochrome.

**Cytochrome b and Sensitivity to the Specific Inhibitors of the bc1 Complex**—The redox titration of cytochrome b at pH 7.4 shows the existence of a low midpoint potential heme bL (E_m = −169 mV) and a high midpoint potential heme bH (E_m = +20 mV); when the pH is lowered to 3.5, the heme bL midpoint potential increases in order to reach a value close to that of heme bH, which in turn is pH-independent. The two distinct types of bc1-specific inhibitors represented by antimycin A and HQNO on one hand and stigmatellin on the other hand, induce distinguishable red shifts in the absorption band of cytochrome b in this species. This demonstrates that the binding of antimycin and HQNO occurs on cytochrome b near a heme center with an a-band maximum at ~562 nm, whereas stigmatellin binds to another heme b center peaking at ~564 nm. The striking similarities to typical cytochrome bc1 complexes therefore provide evidence for the existence of a dihemic cytochrome b in *T. ferrooxidans*.

The experimental results furthermore show that antimycin and HQNO induced a red shift in the absorption band of cytochrome b irrespective of the pH of the medium, although yielding more symmetrical S-shaped curves at pH 7.4 than at pH 3.5, whereas stigmatellin induced a significant red shift only when the suspension medium was at pH 3.5; the red shift was comparatively weak at pH 7.4. These findings indicate that, at neutral pH, the binding domain for stigmatellin is modified and is no longer able to accommodate the inhibitor. This in turn suggests that the antimycin- and HQNO-binding domains are located on the cytoplasmic side of the membrane, which is in contact with a near-neutral pH (around pH 6.5) (36), whereas the stigmatellin-binding domain is located on the periplasmic side of the membrane, which is in contact with an acidic pH.

**The Rieske Iron-Sulfur Protein**—The EPR studies performed on a highly concentrated, ascorbate-reduced membrane preparation of *T. ferrooxidans* clearly showed the presence of a [2Fe-2S] Rieske center in this bacterium: the spectral parameters (g_σ = 1.90 and g_π = 1.74) and the inhibitor sensitivity are characteristic of this compound. The g_σ trough at g = 1.74 is at

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5 C. Appia and V. Bonnefoy, manuscript in preparation.
unusually high magnetic fields as compared with other cytochrome $bc_1$ complexes and rather resembles the spectral parameters reported for the chloroplast $b_{59}$ complex. The $g_z$ signal observed at pH 7.4 was seen to broaden and shift toward lower magnetic fields when the pH became more acidic (pH 3.5), most probably reflecting the fact that the quinone pool is oxidized at neutral pH, but reduced at acidic pH. It is noteworthy that in all $bc_1$ complexes studied so far, the $g_z$ signal of the Rieske protein is sharp in the presence of oxidized quinone, but shifts toward higher fields and broadens considerably when ubiquinone becomes reduced. In the $b_{59}$ complex, the $g_z$ trough was seen to shift toward lower fields in the presence of reduced plastocyanine. The $T. ferrooxidans$ Rieske center exhibited unusual characteristics with regard to inhibitor sensitivity at both studied pH values. In fact, the three inhibitors induced a shift of the $g_z$ trough to lower field positions, contrary to what is usually observed in both the $bc_1$ (34, 44) and $b_{59}$ (45) complexes. So far, similar downfield shifts of the $g_x$ signal of the Rieske protein have only been observed in *Helio bacterium chlorum* (46). In addition, the [2Fe-2S] center of the $bc_1$ complex is sharp in the presence of oxidized quinone, but shifts to a higher value of $g_x$ under acidic conditions and rather resembles the spectral parameters reported for the chloroplast $bc_1$ complex. This is consistent with the fact that higher redox potentials have been obtained with *T. ferrooxidans* than in usual neutrophilic systems, but turned out to be close to that found in neutrophilic systems, but turned out to be close to the results prior to publication, and R. Toci (Fermentation Plant Unit, Laboratoire de Chimie Bactérienne, Marseille, France) for growing *Thiobacillus* cells. We also thank S. Touloujian for technical assistance.

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