Evidence for a cytochrome *bcc*–*aa*₃ interaction in the respiratory chain of *Mycobacterium smegmatis*

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Spectroscopic analysis of membranes isolated from *Mycobacterium smegmatis*, along with analysis of its genome, indicates that the cytochrome *c* branch of its respiratory pathway consists of a modified *bc*₁ complex that contains two cytochromes *c* in its *c*₁ subunit, similar to other acid-fast bacteria, and an *aa*₃-type cytochrome *c* oxidase. A functional association of the cytochrome *bcc* and *aa*₃ complexes was indicated by the findings that levels of detergent sufficient to completely disrupt isolated membranes failed to inhibit quinol-driven O₂ reduction, but known inhibitors of the *bc*₁ complex did inhibit quinol-driven O₂ reduction. The gene for subunit II of the *aa*₃-type oxidase indicates the presence of additional charged residues in a predicted extramembrane domain, which could mediate an intercomplex association. However, high concentrations of monovalent salts had no effect on O₂ reduction, suggesting that ionic interactions between extramembrane domains do not play the major role in stabilizing the *bcc*–*aa*₃ interaction. Divalent cations did inhibit electron transfer, likely by distorting the electron-transfer interface between cytochrome *c*₁ and subunit II. Soluble cytochrome *c* cannot donate electrons to the *aa*₃-type oxidase, even though key cytochrome *c*-binding residues are conserved, probably because the additional residues of subunit II prevent the binding of soluble cytochrome *c*. The results indicate that hydrophobic interactions are the primary forces maintaining the *bcc*–*aa*₃ interaction, but ionic interactions may assist in aligning the two complexes for efficient electron transfer.

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INTRODUCTION

Aerobic respiration in bacteria involves electron transfer through a series of membrane protein complexes, which are designed to transform the energy of exergonic electron transfer into a voltage gradient across the cytoplasmic membrane (Hosler & Shapleigh, 2002). This potential is utilized by the ATP synthase to produce ATP. The respiratory electron-transfer chain can be divided in two: a quinone-reducing and a quinol-oxidizing side (Thony-Meyer, 1997; Hosler & Shapleigh, 2002). Electrons are transferred from cytoplasmic electron carriers (e.g. NADH₂) to membrane-bound reductases (e.g. NADH-dehydrogenase), which in turn reduce quinone. The quinol then passes electrons in two possible directions, directly to a quinol-O₂ oxidoreductase, or through the cytochrome *c* pathway. The latter consists of a quinol-cytochrome *c* oxidoreductase (the cytochrome *bc*₁ complex) and a cytochrome *c* oxidase (terminal oxidase). Thus, the two ways terminal oxidases accept electrons are directly from quinol or from cytochrome *c*.

The wide variety of bacterial terminal oxidases fall into two groups based upon the structure of their active sites. The active sites of the haem-copper family contain a five-coordinate haem and a type 2 copper, while the active sites of the *bd*-type oxidases are composed of two closely associated haems. Some haem-Cu oxidases oxidize cytochrome *c*, others quinol, and all are proton pumps. The *bd*-type oxidases all oxidize quinol and do not function as proton pumps, but these enzymes do have a high affinity for O₂ (D'Mello *et al.*, 1996). Regardless of proton pumping, all terminal oxidases function as energy-transducing devices by consuming the protons for water production only from the inner surface of the membrane. Many bacteria synthesize one or more cytochrome *c* pathways plus a *bd*-type quinol oxidase. The *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* genomes encode genes for two terminal oxidases, a *bd*-type quinol oxidase and a haem-Cu-type cytochrome *c* oxidase (Cole *et al.*, 1998; Kana *et al.*, 2001; <http://www.tigr.org>). The presence of a second *bd*-type oxidase has been proposed for *M. smegmatis* based on gene sequence homologies (Matsoso *et al.*, 2005).

In mitochondria, soluble cytochrome *c* is reduced by cytochrome *c*₁ of the *bc*₁ complex and oxidized by the Cu_A centre in subunit II of an *aa*₃-type cytochrome *c* oxidase. Eubacterial cytochrome *c* oxidases have evolved a variety of

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Abbreviations: DM, dodecyl β-D-maltoside; DMNQ, dimethylnaphthoquinone; DMNQH₂, dimethylnaphthoquinol; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

cytochrome *c*–cytochrome *c* oxidase interactions, at least partly in response to constraints imposed by different cell architectures. Gram-negative bacteria contain a periplasmic space analogous to the intermembrane space of mitochondria. Correspondingly, the mitochondrial-like cytochrome *c* oxidases of many α -proteobacteria, including *Rhodobacter sphaeroides* and *Paracoccus denitrificans*, interact with soluble *c*-type cytochromes with homology to mitochondrial cytochrome *c* (Hosler *et al.*, 1992; Castresana *et al.*, 1994; Maneg *et al.*, 2004). However, these oxidases also interact reversibly with a membrane-anchored cytochrome *c*, also related to mitochondrial cytochrome *c* (Hosler *et al.*, 1992; Daldal *et al.*, 2001; Maneg *et al.*, 2004). Gram-positive bacteria do not possess a periplasmic space or an outer membrane; thus the cytochrome *c*-binding surface of the oxidase is topologically outside the cell. In apparent response to this, *Bacillus subtilis* synthesizes a *caa*₃-type cytochrome *c* oxidase in which a cytochrome *c* domain is fused to the extramembrane portion of subunit II of the enzyme complex (Bengtsson *et al.*, 1999). The acid-fast bacteria, including *Mycobacterium* spp., have structural similarities to both Gram-positive and Gram-negative bacteria. The genomes of the *Corynebacterium*, *Mycobacterium* and *Rhodococcus* species examined encode neither soluble cytochrome *c* nor an independent membrane-bound cytochrome *c* (Niebisch & Bott, 2001; Sakamoto *et al.*, 2001; Sone *et al.*, 2003). Analysis of the *Corynebacterium glutamicum* genes encoding its *bc*₁ complex and its haem-Cu-type oxidase revealed two modified components (Sakamoto *et al.*, 2001). The *qcrC* gene of the *bc*₁ complex encodes an extra region predicted to lie in the extramembrane domain that includes a binding site for a second haem *c*. This cytochrome *c*₁ can be considered as a fusion between two cytochrome *c* proteins (Sone *et al.*, 2001). The gene for subunit II of the oxidase encodes an additional 30 amino acids containing many charged residues. The hypothesis was put forth that these enlarged domains mediate an interaction between the *bcc* complex and the terminal oxidase that precludes the necessity for an independent cytochrome *c*. This has been verified in *C. glutamicum* by activity assays and the co-isolation of the *bcc* complex and an *aa*₃-type cytochrome oxidase complex (Niebisch & Bott, 2003). As shown by Sakamoto *et al.* (2001), the genes for the *bc*₁ complex and the haem-Cu oxidase of mycobacteria are very similar to those of corynebacteria, suggesting a similar scenario.

Here, we have analysed isolated cytoplasmic membranes of *M. smegmatis* and found that a detergent-resistant association of the predicted *bcc* complex and an *aa*₃-type oxidase constitutes the cytochrome *c*-dependent pathway of the respiratory chain. The results demonstrate that hydrophobic interactions provide the primary interactive force between the *bcc* and *aa*₃ complexes while ionic interactions may play a role in aligning the two complexes for efficient electron transfer.

METHODS

Bacterial strains and culture conditions. *Escherichia coli* DH10B was cultured in LB broth (De Bruijn & Rossbach, 1994). *M.*

smegmatis LR222 (Beggs *et al.*, 1995) and derivatives were routinely cultured in tryptic soy broth (Difco) containing 0.5% Tween 80 at 37 °C.

Membrane isolation. This followed the procedure of Lee *et al.* (1992). Briefly, harvested *M. smegmatis* cells were suspended in 50 mM KH₂PO₄, pH 7.2, 1 mM EDTA and 1 mM phenylmethanesulfonyl fluoride (Sigma). Cell lysis was achieved by three passes through a French pressure cell at 4 °C and 16 000 p.s.i. (110.4 MPa). The lysate was centrifuged at 3000 *g* for 15 min to remove cell debris. The resulting supernatant was centrifuged at 100 000 *g* for 1 h to pellet the membrane fraction, washed in 50 mM KH₂PO₄, pH 7.2, 1 mM EDTA, and repelleted. The resulting membranes were used in spectral studies and activity measurements.

Difference spectra. To determine the cytochrome *aa*₃ content, membranes were dissolved in 3% dodecyl β -D-maltoside (DM; Anatrace) in 50 mM KH₂PO₄, pH 7.2, 1 mM EDTA and dithionite-reduced minus ferricyanide-oxidized spectra ($\epsilon_{605-630} = 24 \text{ mM}^{-1} \text{ cm}^{-1}$) were recorded at room temperature using a Hitachi U-3000 UV-visible spectrophotometer (Hiser *et al.*, 2000).

Activity assays. Rates of oxygen consumption were determined using a Clark-type oxygen electrode in a magnetically stirred chamber at 25 °C in 50 mM KH₂PO₄, pH 6.5, 1 mM EDTA. Isolated membranes containing 21.5 pmol cytochrome *aa*₃ were added to each reaction. Quinol-driven O₂ reduction activity was determined by using dimethylnaphthoquinol (DMNQH₂) as the electron donor. DMNQH₂ was generated in the reaction using DT-diaphorase (generously provided by Dr Gary Cecchini of UCSF) to mediate the reduction of 2,3-dimethyl 1,4-naphthoquinone (DMNQ, 0.3 mM; also from Dr Cecchini) by NADH₂; each reaction was initiated by the addition of 0.2 mM NADH₂. Cytochrome *c* oxidase activity was determined using 0.7 mM tetramethyl-*p*-phenylenediamine (TMPD) plus 3 mM sodium ascorbate as electron donors. The addition of detergent, salts, or inhibitors to any reaction preceded the addition of the electron donor.

Genetic and molecular manipulations. All cloning and DNA manipulations followed standard molecular techniques (Sambrook *et al.*, 1989). *M. smegmatis* sequence data were obtained from The Institute for Genomic Research (TIGR) through the website at <http://www.tigr.org>. The wild-type *bd*-type oxidase gene cluster was obtained by PCR from *M. smegmatis* LR222 genomic DNA. PCR was carried out using *pfuTurbo* polymerase (Stratagene) in a Bio-Rad Gene Cycler. The oligonucleotide primers (supplied by Invitrogen Life Technologies) were: 5'-ATCGTGGTGGGCGTGTGGCTCAT-3' (forward) and 5'-CGGGTCGGGCGGGGTGTCT-3' (reverse). PCR products were isolated from agarose and cloned into pCR4Blunt-TOPO (Invitrogen Life Technologies), forming pCR4SMbd. Constructs were verified as correct by sequencing the ends of the cloned DNA using sequencing primers for pCR4Blunt-TOPO. Electroporation of *M. smegmatis* LR222 followed the procedure of Zhu *et al.* (1998).

Creation of a cytochrome *bd* deletion strain. A 1610 bp *EcoRI*–*Sma*II fragment, containing a portion of the genes for subunits I and II of the *bd*-type oxidase, was cloned from pCR4SMbd into pUC18 (Invitrogen), forming pUCSMbd. The kanamycin-resistance cassette from Tn903 on a *Bam*HI fragment was cloned into a *Bam*HI site, thus disrupting the gene for subunit I of the *bd*-type oxidase. The resulting construct, pUCSMbdKan, was digested with *EcoRI* and the linear fragment carrying the mutant allele was electroporated into LR222. Mutants were selected on plates containing 15 μg kanamycin ml^{-1} and were confirmed by PCR of genomic DNA using primers lying outside the mutated gene region and difference spectra of membrane extracts. The oligonucleotide primers used for mutagenesis verification were: 5'-ATCGTGGTGGGCGTGTGGCTCAT-3' (forward) and

5'-TCAGTGGTGGTGGTGGTGGCTCGACCGCTGGACAA-3' (reverse). One mutant strain, named JAM1, was chosen for further study.

RESULTS

Construction of an *M. smegmatis* strain expressing only the cytochrome *c* pathway

Spectroscopic analysis of the cytochromes present in membranes isolated from *M. smegmatis* LR222 membranes shows the presence of a *bd*-type oxidase (~ 630 nm) containing haems B and D plus a cytochrome *c* oxidase containing haem A (~ 600 nm) (Fig. 1b and Kana *et al.*, 2001). The presence of these two oxidases predicts that the respiratory chain of *M. smegmatis* is branched at the level of menaquinol, which can pass its electrons to either the *bd* oxidase or a cytochrome *c* branch composed of the *bcc* complex and an *aa*₃-type oxidase. In order to characterize the cytochrome *c* branch of the respiratory chain, a strain deleted in the *bd*-type oxidase was created (see Methods). Verification that the *bd*-type oxidase was absent from this strain, called JAM1, was obtained in three ways. First, the genomic copy of the gene for subunit I of the *bd*-type oxidase that is interrupted by the kanamycin-resistance cassette should be approximately 1 kb larger than the wild-type gene. PCR was performed on genomic DNA from JAM1 and wild-type *M. smegmatis* using primers that would hybridize outside the mutated gene region. Agarose gel electrophoresis revealed a 1 kb difference between JAM1 and the wild-type PCR

products (Fig. 1a), indicating that the gene for subunit I was interrupted. Second, the 630 nm signal indicative of the *bd*-type oxidase is absent in membranes isolated from JAM1 (Fig. 1b). Third, the cyanide sensitivity of the O₂ reduction activity catalysed by isolated membranes was significantly altered in JAM1. Typically, a haem-Cu cytochrome *c* oxidase is more sensitive to cyanide than *bd*-type oxidases (Junemann, 1997; Thony-Meyer, 1997). Fig. 2 shows that the O₂ reduction activity catalysed by JAM1 membranes lacking the *bd*-type oxidase is considerably more sensitive to cyanide than wild-type membranes containing both the *aa*₃-type and the *bd*-type oxidases. The data are consistent with apparent *K*_i values of ~ 1 μ M and ~ 1 mM, as previously observed for the *aa*₃-type and *bd*-type oxidases of *C. glutamicum*, respectively (Sone, 1990; Kusumoto *et al.*, 2000).

The *bcc* complex and the *aa*₃ oxidase of *M. smegmatis* form a detergent-resistant electron-transfer system

Using isolated, intact membranes, exogenous NADH₂ supports electron transfer through the entire respiratory chain to oxygen (Fig. 3a). An NADH-dehydrogenase reduces the menaquinone pool (Thony-Meyer, 1997; Hosler & Shapleigh, 2002). The addition of the detergent DM to a final concentration of 16 mg (mg total protein)⁻¹ was sufficient to completely abolish this NADH-dependent activity by dissolving the membrane and dispersing the electron-transfer complexes (Fig. 3a). Addition of the exogenous quinol DMNQH₂ restored O₂ reduction activity to dissolved membranes originally isolated from both wild-type *M. smegmatis* and JAM1 (Fig. 3b). In the wild-type membranes, the *bd*-type oxidase can oxidize DMNQH₂ and reduce O₂. However, the presence of quinol-driven O₂ reduction in solubilized JAM1 membranes indicates a

Fig. 1. Verification of the insertional inactivation of the *bd*-type oxidase. (a) PCR products from *M. smegmatis* LR222 and JAM1 (LR222*bd::kan*) genomic DNA using primers lying outside the mutated gene region. Lanes: 1, molecular mass markers (sizes indicated to the left); 2, *M. smegmatis* LR222 (wild-type); 3, JAM1. (b) Difference spectra of membrane extracts from late-exponential-phase cells: (1) *M. smegmatis* LR222 and (2) JAM1. Arrows indicate the peaks for the *aa*₃-type oxidase and *bd*-type oxidase.

Fig. 2. Cyanide inhibition of NADH₂-driven O₂ reduction activity of isolated membranes. *M. smegmatis* LR222 ($\blacktriangle$) and JAM1 ($\circ$). Membranes were mixed with increasing molar concentrations of KCN prior to the addition of NADH₂. The results are expressed as the percentage of the initial rate when no cyanide was present.

Fig. 3. The effect of detergent (dodecyl maltoside, DM) on O₂ reduction activity in membranes from *M. smegmatis* LR222 (black bars) and JAM1 (white bars). The results are expressed as the rate of oxygen consumption. Turnover numbers (e⁻ s⁻¹) for O₂ reduction activity from JAM1 membranes are reported in the parentheses above the white bars. (a) Membrane-associated NADH₂-driven O₂ reduction activity with and without a low concentration of detergent. (b) Quinol-driven O₂ reduction activity, using DMNQH₂, with increasing detergent concentrations.

detergent-resistant association of the cytochrome *bcc* and *aa₃* complexes since only the former can oxidize quinol and only the latter can reduce O₂.

As the DM concentration was increased, the O₂ reduction activity by both wild-type and JAM1 membranes gradually declined. Finally, at 640 mg DM (mg total protein)⁻¹, the activity exhibited by JAM1 membranes showed a sharp decrease in comparison to wild-type. At this point, the residual O₂ reduction activity observed using the wild-type membranes must be largely due to the *bd*-type oxidase.

If the quinol-driven activity exhibited by detergent-solubilized JAM1 membranes is due to a detergent-resistant association of the cytochrome *bcc* and *aa₃* complexes, then cytochrome *bc₁* complex inhibitors should prevent O₂ reduction. Fig. 4 shows this to be the case. The addition of micromolar amounts of antimycin A and myxothiazol, well-established inhibitors of the cytochrome *bc₁* complex (Gao *et al.*, 2003), totally abolished quinol-driven O₂ reduction by dissolved JAM1 membranes. The quinol-driven O₂ reduction activity of dissolved wild-type membranes in the presence of antimycin A and myxothiazol is attributed to the *bd*-type oxidase since these inhibitors do affect *bd*-type oxidases but to a lesser degree (Bertsova *et al.*, 1997).

The *aa₃*-type oxidase of *M. smegmatis* does not oxidize soluble cytochrome *c*

Bacterial cytochrome *c* oxidases often oxidize soluble cytochromes *c* in addition to membrane-bound cytochromes *c* (Hosler *et al.*, 1992; Assempour *et al.*, 1998; Maneg *et al.*, 2004). The activity of the cytochrome *c* oxidase can be measured independently by reducing the *c*-type cytochromes

Fig. 4. Inhibition of quinol-driven O₂ reduction activity by *bc₁*-complex inhibitors on membranes from *M. smegmatis* LR222 (black bars) and JAM1 (white bars). Inhibition by antimycin A (A) at 35.7 µg ml⁻¹ and myxothiazol (M) at 35.7 µM is shown. Turnover numbers (e⁻ s⁻¹) are reported in parentheses.

with ascorbate plus the mildly hydrophobic one-electron donor TMPD (Drachev *et al.*, 1976; Jurtshuk *et al.*, 1981). Neither the addition of soluble horse heart cytochrome *c* (Fig. 5) nor soluble cytochrome *c₂* of *R. sphaeroides* (data not shown) increased the rate of ascorbate/TMPD-driven O₂ reduction to a rate greater than that supported by ascorbate/TMPD alone (Fig. 5). This suggests an inability of soluble cytochrome *c* to access its binding site on subunit II of the oxidase.

The additional amino acid residues present in subunit II of the *aa₃*-type oxidase of *M. smegmatis* contain numerous

Fig. 5. O₂ reduction activity of isolated JAM1 membranes driven by different electron donors. The rate of O₂ consumption by JAM1 membranes using 0.2 mM DMNQH₂, 0.7 mM TMPD, and 0.7 mM TMPD, 1 mM horse heart cytochrome *c* (TMPD+HHc) as electron donors was compared in the presence of dodecyl maltoside (DM). The rate of oxidase activity is reported as turnover number (e⁻ s⁻¹).

charged residues, as in subunit II of the aa₃-type oxidase of *C. glutamicum* (Sakamoto *et al.*, 2001). To test whether or not the charged residues participate in ionic interactions required for the association of the cytochrome *bcc* and aa₃ complexes, the effect of monovalent and divalent salts on the quinol-driven O₂ reduction activity of JAM1 membranes was assessed (Fig. 6). Interestingly, high concentrations of monovalent salts had no inhibitory effect on quinol-driven O₂ reduction activity, indicating that disruption of any putative ionic interactions is not sufficient to dissociate the *bcc* complex from the aa₃-type oxidase. However, the addition of divalent cations, as 0.5 M MgCl₂ or MnSO₄, essentially abolished DMNQH₂-supported O₂ reduction (Fig. 6). The inhibitory effects of MgCl₂ were enhanced when the detergent concentration was simultaneously increased. In Fig. 7 the effect of 16 and 160 mg DM (mg total protein)⁻¹ in the presence of a monovalent and a divalent salt on quinol oxidase activity is compared. While the detergent had no increased inhibitory effect in combination with 1 M KCl, 160 mg DM (mg protein)⁻¹ along with 0.1 M MgCl₂ inhibited O₂ reduction by about 59 % as compared to the rate in the absence of salt at this detergent concentration. An explanation for the effect of divalent salts is presented below.

DISCUSSION

The cytochrome *c* pathway in *M. smegmatis* has been proposed to consist of a cytochrome *bcc* complex and an aa₃-type cytochrome *c* oxidase, based on genomics (Kana *et al.*, 2001). The work presented here provides the biochemical evidence for this proposal. The gene sequence of cytochrome *c*₁ of the *M. smegmatis* bc₁ complex indicates the presence of a second cytochrome *c*, as previously seen for *C. glutamicum* and *Rhodococcus rhodochrous* (Niebisch & Bott, 2001; Sakamoto *et al.*, 2001; Sone *et al.*, 2003). Analysis of the

Fig. 6. Monovalent and divalent salt inhibition of O₂ reduction activity catalysed by cytochrome *bcc*-aa₃. Quinol-driven O₂ reduction activity of *M. smegmatis* JAM1 membranes in the presence of 0.1 M (white bars) or 1 M monovalent salts (hatched bars) was assayed. Inhibition by divalent salts was also assayed at 0.5 M salt (black bars). The effect on activity by 0.1 M MnSO₄ was not determined. The rate of oxidase activity is reported as turnover number (e⁻ s⁻¹).

Fig. 7. Combined effects of detergent and salt on the O₂ reduction activity catalysed by cytochrome *bcc*-aa₃. Quinol-driven O₂ reduction activity of *M. smegmatis* JAM1 membranes with no added salt (black bars), 1 M KCl (white bars) and 0.1 M MgCl₂ (hatched bars) was compared in the presence of two concentrations of dodecyl maltoside (DM). The rate of oxidase activity is reported as turnover number (e⁻ s⁻¹).

complete *M. tuberculosis* genome and the partial genome of *M. smegmatis* indicates the absence of any independent membrane-bound or soluble cytochromes *c* in these species, as in other acid-fast bacteria (Niebisch & Bott, 2001; Sakamoto *et al.*, 2001; Sone *et al.*, 2003). Therefore, the likely candidate for the cytochrome *c* that interacts with the aa₃-type oxidase is this second haem C of cytochrome *c*₁ of the *M. smegmatis* *bcc* complex. Our results provide strong evidence for the functional association of the cytochrome *bcc* complex and the aa₃-type cytochrome *c* oxidase of *M. smegmatis*. First, exogenous quinol supports O₂ reduction activity in the presence of detergent concentrations sufficient to solubilize the membrane. This is similar to the quinol oxidase activity of the isolated cytochrome *bcc*-aa₃ complex of *C. glutamicum*. Confirmation that the quinol is being oxidized by the *bcc* complex, and not by an unusual reaction of the aa₃-type terminal oxidase, is shown by our finding that known inhibitors of the bc₁ complex inhibit quinol-driven O₂ reduction. Together, these results indicate direct electron transfer between the two complexes of the cytochrome *c* pathway.

The data of Fig. 3(b) may be interpreted as disruption of the cytochrome *bcc*-aa₃ interaction by high concentrations of detergent. However, ascorbate/TMPD-driven O₂ reduction activity was not inhibited by the same high levels of detergent (Fig. 5). Significant TMPD oxidation generally requires the association of a cytochrome *c* with the oxidase. For example, at the concentration of TMPD used, the mitochondrial aa₃-type oxidase exhibits a turnover number of less than 2 s⁻¹ (Crisson & Nicholls, 1992). The purified aa₃-type oxidase of *C. glutamicum*, which contains no cytochrome *c*, oxidizes TMPD at the slow rate of <1 s⁻¹ (Sakamoto *et al.*, 2001). Thus, the retention of higher rates of TMPD oxidation at high detergent concentrations

(Fig. 5) suggests a continued association of cytochrome c_1 with the oxidase. The inhibition of quinol-driven O_2 reduction by high detergent concentrations, then, could be due partly to the separation of the cytochrome bcc - aa_3 complexes and partly to disruption of the cytochrome bcc complex, leaving cytochrome c_1 associated with the aa_3 -type oxidase.

Unlike the prototype aa_3 -type oxidases of *Rhodobacter sphaeroides* and *P. denitrificans* (Hosler *et al.*, 1992; Witt *et al.*, 1998), the addition of soluble cytochrome c fails to stimulate the activity of the aa_3 -type oxidase of *M. smegmatis*, suggesting that the soluble cytochrome c cannot bind at the electron-transfer site on subunit II. Even so, of the four carboxylate residues that mediate the interaction of soluble cytochrome c with subunit II of cytochrome c oxidase in mitochondria and in *R. sphaeroides*, three are conserved in *M. smegmatis* cytochrome aa_3 (Wang *et al.*, 1999; Zhen *et al.*, 1999). A conserved tryptophan has been shown to mediate electron transfer between the haem edge of cytochrome c and Cu_A in subunit II (Wang *et al.*, 1999). This tryptophan and surrounding residues are present in the aa_3 -type oxidase of *M. smegmatis*. Conservation of the electron-transfer interface on subunit II suggests that the geometry of the productive interaction between the second cytochrome c of the bcc complex with its binding site on the aa_3 -type oxidase is likely to be similar to that between soluble cytochrome c and mitochondrial-like cytochrome oxidases. A tight association of the cytochrome bcc complex with the aa_3 -type oxidase could prevent the binding of exogenous cytochrome c . However, the homologous aa_3 -type oxidase of *C. glutamicum*, in a preparation in which the cytochrome bcc complex is absent, also fails to oxidize soluble horse heart or yeast cytochrome c (Sakamoto *et al.*, 2001). Thus, it seems more likely that additional residues predicted to be present in the extramembrane domain of subunit II, as compared to the mitochondrial-like aa_3 -type oxidases, interfere with the binding of soluble cytochrome c . Interestingly, Weinstein *et al.* (2005) state that the aa_3 -type oxidase of *M. tuberculosis* readily oxidizes bovine and yeast soluble cytochrome c ; actual rates were not reported. The genomic evidence suggests that the cytochrome bcc - aa_3 interaction in *M. tuberculosis* should be very similar to that of *M. smegmatis* and *C. glutamicum*.

We have probed the forces that mediate the interaction between the cytochrome bcc and aa_3 complexes using both detergent and monovalent and divalent salts. Monovalent salts disrupt the association of soluble cytochrome c with the mitochondrial-like cytochrome c oxidase (Hosler *et al.*, 1992; Witt *et al.*, 1998), but the association of the bcc complex with the aa_3 -type oxidase is resistant to KCl or NaCl. This argues against ionic interactions being primarily responsible for the cytochrome bcc - aa_3 interaction, as originally suggested for the *C. glutamicum* system on the basis of the high number of charged residues present in the additional string of residues in subunit II of the oxidase (Sakamoto *et al.*, 2001). The aa_3 -type oxidase of *R.*

sphaeroides also interacts efficiently with a membrane-bound cytochrome c that mediates between the bc_1 complex and the oxidase (Hosler *et al.*, 1992; Daldal *et al.*, 2001). This cytochrome c -cytochrome oxidase interaction is easily disrupted by low levels of detergent, while the cytochrome bcc - aa_3 interaction in *M. smegmatis* membranes is not. Thus, the conclusion can be drawn that the association of the cytochrome bcc - aa_3 complexes depends largely upon hydrophobic interactions.

Results obtained by modifying ionic strength with monovalent salt do not exclude a role for ionic interactions in an association of the cytochrome bcc and aa_3 complexes that promotes optimal electron transfer. Divalent cations are capable of bridging negatively charged residues. Distortion of the electron-transfer interface between the second C haem of the cytochrome bcc complex and Cu_A in subunit II of the cytochrome aa_3 complex seems the likely explanation for the strong inhibition of quinol-driven O_2 reduction by Mg^{2+} and Mn^{2+} . Interestingly, this effect would not be evident in studies of the interaction of soluble cytochromes c with cytochrome oxidase since the ionic strength of the divalent salts would prevent the initial binding. The presence of divalent cations leads to further inhibition by high detergent concentrations, as would be expected if detergent loosens the cytochrome bcc - aa_3 interaction while divalent cations distort the cytochrome c_1 -subunit II interface.

Groups working on the cytochrome bcc - aa_3 interaction in *C. glutamicum* have worked to provide evidence for this interaction through protein purification. In one case, only the aa_3 -type oxidase was purified (Sakamoto *et al.*, 2001). Using lower detergent concentrations, Niebisch & Bott (2003) were able to purify a complex with quinol-driven O_2 reduction activity from *C. glutamicum* membranes. The isolated bcc - aa_3 complex, however, was partially dissociated. Our attempts to isolate a cytochrome bcc - aa_3 complex from *M. smegmatis* by lithium dodecyl sulfate (LDS) gel electrophoresis were not successful. It may be that delipidation of the cytochrome bcc and aa_3 complexes by all of these procedures destabilize the bcc - aa_3 interaction.

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