Structural and functional analysis of \textit{aa}_3\text{-type} and \textit{cbb}_3\text{-type} cytochrome \textit{c} oxidases of \textit{Paracoccus denitrificans} reveals significant differences in proton-pump design

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Summary

In \textit{Paracoccus denitrificans} the \textit{aa}_3\text{-type} cytochrome \textit{c} oxidase and the \textit{bb}_3\text{-type} quinol oxidase have previously been characterized in detail, both biochemically and genetically. Here we report on the isolation of a genomic locus that harbours the gene cluster \textit{ccoNOQP}, and demonstrate that it encodes an alternative \textit{cbb}_3\text{-type} cytochrome \textit{c} oxidase. This oxidase has previously been shown to be specifically induced at low oxygen tensions, suggesting that its expression is controlled by an oxygen-sensing mechanism. This view is corroborated by the observation that the \textit{ccoNOQP} gene cluster is preceded by a gene that encodes an FNR homologue and that its promoter region contains an FNR-binding motif. Biochemical and physiological analyses of a set of oxidase mutants revealed that, at least under the conditions tested, cytochromes \textit{aa}_3, \textit{bb}_3 and \textit{cbb}_3 make up the complete set of terminal oxidases in \textit{P. denitrificans}. Proton-translocation measurements of these oxidase mutants indicate that all three oxidase types have the capacity to pump protons. Previously, however, we have reported decreased H^+/e^- coupling efficiencies of the \textit{bb}_3\text{-type oxidase under certain conditions. Sequence alignment suggests that many residues that have been proposed to constitute the chemical and pumped proton channels in cytochrome \textit{aa}_3 (and probably also in cytochrome \textit{bb}_3) are not conserved in cytochrome \textit{cbb}_3. It is concluded that the design of the proton pump in cytochrome \textit{cbb}_3 differs significantly from that in the other oxidase types.

Introduction

In mitochondrial respiration the reduction of oxygen to water is catalysed by the \textit{aa}_3\text{-type} cytochrome \textit{c} oxidase. This membrane-bound protein complex is the last component of a linear respiratory pathway in which electrons from NADH and succinate are transferred, in a sequence of redox reactions, to oxygen. The aerobic respiration in bacteria, on the other hand, often proceeds via a more complex electron-transfer network. Alternative respiratory pathways enable a bacterium to adjust the composition and/or the efficiency of oxidative phosphorylation in response to changes in environmental conditions (Anraku and Gennis, 1987; van der Oost et al., 1994; Garcia-Horsman et al., 1994a; van Spanning et al., 1995a).

In addition to a number of alternative quinone-reducing dehydrogenases, the terminal oxidases appear to represent an important site of flexibility in respiratory networks. To date, the best-characterized bacterial respiratory system is the one in \textit{Escherichia coli}. In this bacterium two distinct quinol oxidases (cytochromes \textit{bo}_3 and \textit{bd}) are expressed during aerobic growth, but a cytochrome \textit{c} branch is absent (Anraku and Gennis, 1987). \textit{Paracoccus denitrificans}, in turn, possesses three distinct terminal oxidases. An \textit{aa}_3\text{-type} cytochrome \textit{c} oxidase (Raitio et al., 1987; 1990; Steinrucke et al., 1987; van Spanning et al., 1990; van der Oost et al., 1991), as well as a \textit{bb}_3/\textit{ba}_3\text{-type} quinol oxidase, hereafter called cytochrome \textit{bb}_3, have been characterized previously (de Gier et al., 1994; Richter et al., 1994). In addition, the expression of an alternative cytochrome \textit{c} oxidase has been suggested (Bosma, 1989; de Gier et al., 1992; 1994; Raitio and Wikström, 1994).

In the present study, a \textit{P. denitrificans} \textit{Δaa}_3/\textit{bb}_3 mutant...
was used for the purification of an alternative cytochrome c oxidase: cytochrome cbb₃. Using the N-terminal amino acid sequence of one of its subunits, a genomic locus, ccoNOQP, was isolated that contained a gene cluster with four open reading frames. Sequence analysis of the flanking regions revealed the presence of a ccoGH cluster with four open reading frames. Sequence analysis of the acid sequence of one of its subunits, a genomic locus, downstream of which are homologous to HemN and FNR. Open reading frames were found, the derived sequences ccoNOQP, c₁₂₄₈ can best be explained as the products of multiple oxidase mutants of Paracoccus Aaa₃/bb₃. Besides cytochrome aa₃ and cbb₃, no additional cytochrome c oxidase is present. The currently generated set of oxidase mutants of P. denitrificans was used to demonstrate that all three terminal oxidases have the capacity to pump protons. It is discussed, however, that in the cbb₃-type cytochrome c oxidase the coupling between oxygen reduction and proton translocation may be less tight than in cytochromes aa₃ and bb₃/bo₃.

Results

Purification of the cbb₃-type cytochrome c oxidase

In a previous study, the simultaneous deletion of cytochromes aa₃ and bb₃ in P. denitrificans (Δaa₃/bb₃) has been reported. Analysis of this mutant revealed (i) the presence of an alternative cytochrome c oxidase, (ii) the presence of only protohaem IX (haem B) in membrane extracts, and (iii) an increased expression of cytochromes c and b. No quinol oxidase activity was detectable in this mutant (de Gier et al., 1994). Here, we report the isolation of a cb-type cytochrome c oxidase from membranes of the Paracoccus Δaa₃/bb₃ mutant. Both the activity and the subunit composition of the oxidase complex turned out to be rather unstable during the purification, as described in the Experimental procedures. As will be discussed below, a cytochrome c subunit with an apparent molecular mass of 45 kDa is lost during the purification of Paracoccus cytochrome cbb₃ (as described in the Experimental procedures).

Analysis of the purified cytochrome c oxidase by a Coomassie brilliant blue-stained SDS-PAGE gel indicates that it consists of two components with apparent molecular masses of 45 kDa and 30 kDa (Fig. 1A). Only the fast-migrating component has been identified as a cytochrome c because of the presence of covalently bound haem, as demonstrated by haem-stained SDS-PAGE (not shown). Sequencing of this 30 kDa subunit revealed the N-terminal sequence AILKHKVILEKATLLLVSFLVVR. The optical spectrum of this oxidase preparation indicates that it involves a cb-type cytochrome (Fig. 1B). However, the relatively small amount of cytochrome c in this spectrum relative to a previous preparation (de Gier et al., 1994) can best be explained as the loss of a second haem c-containing subunit which co-migrated with the 45 kDa polypeptide, as demonstrated below.

Isolation and characterization of the ccoNOQP cluster

Based on the N-terminal sequence of the 30 kDa subunit, degenerate primers were designed (see the Experimental procedures, primers 171 and 172) and used to perform the polymerase chain reaction (PCR) on genomic DNA from the Δaa₃/bb₃ mutant. A PCR product of the expected size (0.1 kb) was obtained. Sequence analysis of the PCR product indicated that the DNA sequence corresponded to the N-terminal peptide of the 30 kDa subunit (not shown). With the PCR product as a probe, the complete ccoNOQP cluster could be cloned (Fig. 2A). Fragments were subcloned into M13mp18/19 for sequence analysis. The complete DNA sequence has been submitted to the GenBank database Data Library (Accession Number Bankit2589 U34353).

Four open reading frames (ORFs) in the isolated genomic locus share a high degree of homology with the ccoNOQP gene cluster from Rhodobacter capsulatus (Thöny-Meyer et al., 1994) and to the fixNOQP operons of Rhizobium meliloti (Kahn et al., 1993; D. Kahn, 1993, EMBL Data Library Accession No. 221854), Bradyrhizobium japonicum (Preisig et al., 1993; Fig. 3) and Azorhizobium caulinodans (Mandon et al., 1994). As P. denitrificans is not known to be able to fix molecular nitrogen, the ccoNOQP rather than the fixNOQP nomenclature

Fig. 1. A. SDS–PAGE of purified cytochrome cbb₃ (approx. 7.5 µg of protein). The molecular mass standards are: 97.4, 66.2, 45.0, 31.0, 21.5, and 14.4 kDa.

B. Absorption spectrum of purified cytochrome cbb₃ after reduction with Na-dithionite (approx. 0.01 mg protein ml⁻¹).

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has been adopted for these genes. The four ORFs have a GC-rich codon preference which is typical of *P. denitrificans*. The ccoNOQP genes are tightly clustered, with the exception of the ccoQ and ccoP genes which are 41 bases apart. As no transcription-termination signal was found here, the four genes are probably transcribed as a single operon, as has been demonstrated for *R. meliloti* (Batut et al., 1989). The proposed initiation codons of all four genes are six to eight bases downstream of putative Shine–Dalgarno sequences. A putative anaerobox (TTGAC-N_{4-5}-ATCAA) is located upstream of ccoN. Its sequence is very similar to the binding sites of FNR in *E. coli* and of FixK in *R. meliloti* and *B. japonicum* (TTGAT-N_{4-5}-ATCAA; Spiro, 1994), and identical to the anaerobox found upstream of the *A. caulinodans* fixNOQP operon (Mandon et al., 1994).

Attempts to sequence the N-terminus of subunit I (CcoN, apparent molecular mass 45 kDa (Fig. 1A)) were unsuccessful, probably because of blocking of the first amino group. The N-terminal methionine of CcoN (Fig. 3A) has been chosen because the corresponding ATG codon is downstream of a Shine–Dalgarno sequence, and because no alternative in-frame initiation codons are present. Moreover, the size of the gene correlates with that of ccoN/fixN in the aforementioned bacteria. The putative anaerobox is located at positions −110 and −124 of the chosen start codon. Based on sequence alignment with the subunits I of the well-characterized cytochromes aa₃ from *Rhodobacter sphaeroides* and *P. denitrificans*, as well as with the subunit I of cytochrome bo₃ from *E. coli* (Hosler et al., 1993), four histidines in the CcoN sequence (H271, H321, H322 and H409) are the most probable ligands of the binuclear catalytic centre formed by a penta-co-ordinated haem B and a copper atom, CuB (Fig. 3A). The ligands of the hexa-co-ordinated haem B are H411 and (probably) H122 in the *Paracoccus* CcoN (Fig. 3A).

CcoO has an N-terminal amino acid sequence that corresponds to the N-terminal sequence determined for the 30 kDa subunit (Fig. 3B). Apart from this hydrophobic, putative membrane-spanning fragment, CcoO contains a hydrophilic, probably periplasmic, domain with a C₉₃G₉₃M motif which is the consensus for haem C binding (Fig. 3B). Apart from this motif, CcoO/FixO does not share much similarity with the available cytochrome c sequences (Moore and Pettigrew, 1990). CcoQ is a small protein with one hydrophobic region (Fig. 3C); apart from CcoQ and FixQ, no significant homologues have been found in sequence databases. CcoP appears to be a membrane-anchored di-haem cytochrome. Its C-terminal hydrophilic domain, probably facing the periplasmic side of the plasma membrane, contains two motifs (Gₓₓ₃Fxₓ₃Cₓₓ₃Hₓₓ₃M) that are well conserved among soluble mono-haem cytochromes of photosynthetic bacteria and eucaryotic algae (Mandon et al., 1994). In contrast to the FixP/CcoP sequences from other sources, an N-terminal hydrophilic extension is present in the *Paracoccus* sequence (Fig. 3D), probably located at the cytoplasmic side of the membrane.

Flanking regions

Upstream of the ccoNOQP cluster of *P. denitrificans* is an ORF that potentially codes for a polypeptide of 278 amino acids (orf278 in Fig. 2A). It shares a rather high degree of homology (28% identical amino acids, not shown) with orf277, which is located adjacent to the fixNOQP operon.
### Fig. 3. Alignments of the *P. denitrificans* ccoNOQP-encoded polypeptides.

A. CcoN of *P. denitrificans* (cytochrome cbb3), FixN of *B. japonicum* (cytochrome cbb3; Preisig et al., 1993), CtaDII of *P. denitrificans* (cytochrome a+; Ratio et al., 1990), and CyoB of *E. coli* (cytochrome bo3; Chepuri et al., 1990). Transmembrane helices are shaded and conserved residues discussed in the text are indicated.

B. CcoO of *P. denitrificans* and FixO of *B. japonicum* (Preisig et al., 1993). Identical residues are boxed.

C. CcoQ of *P. denitrificans* and FixQ of *B. japonicum* (Preisig et al., 1993). Identical residues are boxed.

D. CcoP of *P. denitrificans* and FixP of *B. japonicum* (Preisig et al., 1993). Identical residues are boxed.

of *B. japonicum*. No homologue has yet been reported in other members of the Rhizobiaceae family. As no mutants are available, a link between this gene product and cytochrome *cbb*₃ remains to be demonstrated. Further upstream, two genes are located that encode homologues of *E. coli* HemN and FNR (Fig. 2A). In the latter bacterium both polypeptides play an important role during oxygen limitation: HemN catalyses an oxygen-independent bypass in haem biosynthesis (Plunkett *et al.*, 1993), and FNR is a transcription activator of genes involved in anaerobic metabolism (Spiro and Guest, 1990).

Without exception, the gene cluster *fixGHlS* is located downstream of the *fixNOQP* genes of the Rhizobiaceae family (Kahn *et al.*, 1993; Preisig *et al.*, 1993; Mandon *et al.*, 1994) and *ccoNOQP* genes of *R. capsulatus* (Thöny-Meyer *et al.*, 1994). In *R. melliloti*, a number of transposon insertions in *fixGH* have been isolated which had a ‘fix-minus’ phenotype (Kahn *et al.*, 1989). Also, in *Paracoccus* at least part of this cluster is located adjacent to the *ccoNOQP* genes. Two genes homologous to *fixG* and *fixH* (Fig. 2A) have been found downstream of *ccoNOQP* but, for the reasons given above, these genes are designated *ccoG* and *ccoH*. In all instances an anaerobox is located in the *fixG* and in the *ccoG* promoter regions (Fig. 2A), suggesting expression of this oxidase type at low oxygen tensions. The *ccoG* product is a ferredoxin-like protein, with two typical 4Fe4S-binding motifs. FixGHIS has been suggested to be a polypeptide complex consisting of, at least, a redox protein (FixG) and a cation pump (FixL) (Kahn *et al.*, 1989).

**Mutagenesis of ccoNO**

Insertion mutants were generated by substituting part of the *ccoNOQP* operon by a kanamycin-resistance (*Km*) cassette, deleting the 3′ half of *ccoN* and the two 5′ codons of *ccoO* (Fig. 2B). The *ccoNOQP* operon was deleted in wild-type *Paracoccus* (Pd1222) and in the *ctaDi/ctaDII* double mutant Pd92.20 (∆aa₃), yielding Pd27.21 (∆cbb₃) and Pd93.12 (∆aa₃/cbb₃), respectively. Southern blot analysis of chromosomal DNA of selected *Km* clones confirmed the appropriate recombination (not shown). Analysis of the mutant strains generated, grown on minimal medium with succinate, did not show significant spectroscopic changes relative to their respective parent strain. SDS–PAGE and subsequent haem staining of cytoplasmic membranes showed that the 30 kDa cytochrome *c* is absent, and the 45 kDa cytochrome *c* is only just detectable in the ∆cbb₃ mutant strains (Fig. 4).

**Oxygen consumption**

The oxygen-consumption rates in cell suspensions of wild type and oxidase mutants were measured polarographically, with endogenous as well as exogenous substrates (succinate or ascorbate with *N,N,N′,N′*-tetramethyl-p-phenylenediamine (TMPD)). The oxidation rates are shown in Table 1. In separate assays the electron flow to oxygen via cytochrome *c* reductase (the *bc₁* complex) was inhibited by addition of either antimycin A or myxothiazol. The endogenous respiration of the wild type was clearly affected by these inhibitors. In contrast, the endogenous respiration of the ∆aa₃ mutant and the ∆cbb₃ mutant was only slightly inhibited, and that of the ∆aa₃/cbb₃ mutant strain appeared to be unaffected.

The effect of the cytochrome *bc₁* inhibitors on succinate oxidation in the ∆cbb₃ mutant and in the ∆aa₃ mutant was much less than in the wild type. Again, in the ∆aa₃/cbb₃ mutant the succinate oxidation appeared to be unaffected by these inhibitors. More or less equal values of ascorbate/TMPD oxidation indicated the presence of cytochrome *c* oxidase(s) in the wild type, as well as in the ∆cbb₃ and the ∆aa₃ mutant. In the ∆aa₃/cbb₃ mutant, the endogenous respiration was not enhanced at all upon the addition of ascorbate/TMPD, indicating that no cytochrome *c* oxidase was expressed.

**Proton translocation**

The proton-pumping capacity of the set of oxidase mutants was measured by using the oxygen-pulse method as described elsewhere (Raitio and Wikström, 1994; de Gier *et al.*, 1994). In the proton-translocation assay, succinate was used as the electron-donating substrate and either oxygen or potassium ferricyanide was used as the terminal electron acceptor (Table 2). The main goal was to study the proton-pumping capacity of the *cbb₃*-type cytochrome *c* oxidase. The H⁺/e⁻ ratios of the ∆aa₃ mutant and the ∆bb₃/aa₃ mutant clearly show that the *cbb₃*-type cytochrome *c* oxidase does have the capacity to translocate protons. In Fig. 5, representative proton-translocation traces of the ∆bb₃/aa₃ mutant are shown. The H⁺/e⁻ ratios of the ∆cbb₃ mutant and the ∆aa₃/cbb₃ mutant strain are shown.

**Fig. 4.** SDS–PAGE and subsequent haem staining of membrane fractions of the wild type (*wt*) and the cytochrome *cbb*₃ mutant (∆cbb₃) of *P. denitrificans* after microaerobic cultivation. The *c*-type cytochromes indicated are subunits of the *bc₁* complex (FbcC), subunits of cytochrome *cbb*₃ (CcoP, 45 kDa; CcoO, 30 kDa), cytochrome *c₅₉* (CycM), and a subunit of the NO reductase (NorC).
Table 1. Oxygen-consumption measurements of whole-cell suspensions of different Paracoccus denitrificans strains: Pd1222 (wild type), Pd92.20 (Δaa3 mutant), Pd27.21 (Δcbb3 mutant), and Pd93.12 (Δaa3/cbb3 mutant).

<table>
<thead>
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<th>Strain</th>
<th>Substrate inhibitor</th>
<th>Endogenous</th>
<th>Succinate</th>
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<tr>
<td></td>
<td></td>
<td>AA</td>
<td>myx</td>
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<tr>
<td>Wild type</td>
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<td>1.5</td>
<td>0.9</td>
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<tr>
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<tr>
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<td>1.2</td>
</tr>
<tr>
<td>Δaa3/cbb3 mutant</td>
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<td>2.2</td>
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</tr>
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Rates after addition of exogenous substrate have been corrected by subtraction of the corresponding endogenous rates. Values are the means of three independent assays (nmol O2 per mg of protein per s). Inhibitors: antimycin A (AA), myxothiazol (myx).
express cytochrome c oxidase activity (Table 1) (van der Oost et al., 1995). Hence, it is concluded that, at least under a variety of tested conditions, these three types form the complete set of terminal oxidases expressed in 

**P. denitrificans.**

### Characterization of cytochrome cbb3

The products of the isolated 

**P. denitrificans** ccoNOQP gene cluster share a high degree of homology with the cytochrome c oxidase of 

**R. capsulatus** (Thöny-Meyer et al., 1994) and the ‘high-affinity’ oxidase from several members of the Rhizobiaceae (Kahn et al., 1993; Preisig et al., 1993; Mandon et al., 1994) (Fig. 3). Although the cbb3-type oxidase apparently represents a distant member of the haem-copper oxidase family (van der Oost et al., 1993; Preisig et al., 1993; Mandon et al., 1994) (Fig. 3). The alignment shown in Fig. 3A suggests that H271, H321, and H322 are the most probable ligands of CuB, H409 of the penta co-ordinated haem B, and H411 and H122 of the hexa co-ordinated haem B in 

**P. denitrificans** CcoN.

Garcia-Horsman et al. (1994b) have recently reported a thorough biochemical analysis of the cbb3-type oxidase from 

**R. sphaeroides** and have demonstrated the presence of a binuclear centre typical of haem–copper oxidases and the absence of CuA. Moreover, metal analysis revealed the ratio of haemC:haemB:copper to be 3:2:1. This agrees very well with the sequence data of cytochrome cbb3; two haems B and a copper ion are located in subunit I (CcoN), and three haems C reside in the two cytochrome c subunits (one in CcoO and two in CcoP). Bosma (1989) has reported a 30 kDa cytochrome c associated with the alternative oxidase from 

**P. denitrificans** with an 

Em7 value of 322 mV. Gray et al. (1994) purified cytochrome cbb3 from 

**R. capsulatus** and reported a 28 kDa cytochrome c (CcoO) with an 

Em7 value of 320 mV, a 32 kDa cytochrome c (CcoP) with an 

Em7 value of 265 mV, and a low-spin cytochrome b with an 

Em7 value of 385 mV. These data may indicate that CcoO is the direct electron donor of the redox centres (haems B and CuB3) in CcoN. Indeed, the purified 2 subunit CcoNO complex (Fig. 1) retains TMPD oxidase activity. Apparently CcoO is less tightly bound to the cytochrome cbb3 core and may play a role as electron carrier from the bc1 complex to the CcoO–CcoN complex, which would be in agreement with the reported midpoint potential values.

### Expression

Comparison of membrane proteins from the wild type and from the Δcbb3 mutant of 

**P. denitrificans** by haem-stained SDS–PAGE (Fig. 4) identified the 30 kDa and 45 kDa cytochromes c as the CcoO and CcoP subunits of cytochrome cbb3, respectively. Previously, Bosma (1989) performed a detailed analysis of the expression of c-type cytochromes in chemostat-grown cells of 

**P. denitrificans**, using the same haem analysis (reviewed by van Spanning et al., 1995a). Bosma demonstrated that both the 30 kDa (CcoO) and the 45 kDa (CcoP) cytochromes c were abundantly present in membrane fractions when cells were grown in either nitrate-limited (anaerobically) or oxygen-limited (microaerobically) conditions. In succinate-limited (aerobic) cultures, on the other hand, the 45 kDa cytochrome c was absent and the 30 kDa cytochrome c was barely detectable (Bosma, 1989). It was concluded that the cbb3-type oxidase plays an important role at decreased oxygen tensions.

In the promoter regions of all presently known cco/fixNOQP and cco/fixGH(IS) clusters is a sequence motif that closely resembles the binding site of the well-known anaerobic/microaerobic transcription activator FNR (Spiro and Guest, 1990). In 

**R. meliloti**, the ‘oxygen state’ is sensed by the haem protein FixL. In the absence of oxygen, this protein is autophosphorylated (FixL-P). Subsequently, the phosphate is transferred from the sensor to the regulator, FixJ, inducing its activation (FixJ-P). The latter modification results in transcription activation of 

fixK, the gene that encodes an FNR homologue. FixK, in turn, binds at the anaerobox upstream of the fixNOQP and fixGHIS clusters, thereby activating their transcription (David et al., 1988; Batut et al., 1989; Kahn et al., 1989; Fischer, 1994). To date, no FixLJ system has been demonstrated in any non-nitrogen-fixing organism. However, a FixK-like protein, NNR, has been identified in 

**P. denitrificans** as a transcription activator of nitrite reductase and nitric oxide reductase, two polypeptide complexes that are involved in anaerobic respiration (van Spanning et al., 1995b). Mutagenesis of the nrr gene, however, did not affect expression of cytochrome cbb3. Here we show that in the flanking region of the cco locus of 

**Paracoccus**; a gene is located that potentially codes for a second transcription regulator: fnrP (Fig. 2A). Unlike NNR and FixK, this FNR homologue has an N-terminal cysteine cluster which, in the case of 

**E. coli** FNR, has been demonstrated to be involved in the binding of iron. The redox state of this iron ion determines whether the regulator is active or not (Spiro and Guest, 1990).

### Energy conservation

Cytochromes aa3 and bb3 from 

**P. denitrificans** are proton-
pumping terminal oxidases (van Verseveld et al., 1981; Solioz et al., 1981; Puustinen et al., 1989). Analysis of proton translocation in cell suspensions of the Paracoccus oxidase mutants offers the unique opportunity to measure proton translocation of individual oxidases in whole-cell suspensions. During succinate oxidation, a minimum $H^+/e^-$ stoichiometry of 2 is theoretically expected when ubiquinol is oxidized directly by cytochrome $bb_3$ (Fig. 6). On the other hand, a ratio of $3H^+/e^-$ is the theoretical maximum when ubiquinol is oxidized via cytochrome $bc_1$ and cytochrome $aa_3$ (Fig. 6).

Measurements of the set of P. denitrificans mutants are in perfect agreement with the theoretical values (Table 2). Succinate oxidation by cytochrome $bb_3$ in the $\Delta aa_3/cbb_3$ mutant results in 2 $H^+/e^-$ . In the conditions used for analysis of proton translocation (0.4–1.7 μM $O_2$), ubiquinol appears to be oxidized preferentially via the cytochrome $c$ branch ($bc_1$ complex, cytochrome $c$, cytochrome $c$ oxidase) rather than by the quinol oxidase cytochrome $bb_3$. This observation may be due to the low affinity for oxygen that has been measured for cytochrome $bb_3$ ($K_m$ 10–20 μM $O_2$; de Gier, 1995). This may explain why, in the $\Delta bb_3$ mutant, electrons mainly flow to cytochrome $aa_3$, resulting in a stoichiometry of $3H^+/e^-$ . The proton pumping capacity of cytochrome $bb_3$, as first reported by Raitio and Wikström (1994) in a $\Delta aa_3$ mutant, has been confirmed here with the $\Delta aa_3/bb_3$ mutant during electron transfer from succinate to oxygen (Table 2).

The conclusion that cytochrome $bb_3$ does translocate protons, however, is in conflict with the previous measurements by de Gier et al. (1994). In the latter study an $H^+/e^-$ stoichiometry of 2 has been measured during succinate oxidation of the $\Delta aa_3/bb_3$ mutant, suggesting that cytochrome $bb_3$ does not pump protons. The main technical difference is that, in the experiment presented here, HEPES (0.5 mM) rather than glycylglycine (1.5 mM) has been used as buffer. The 'decoupling' of cytochrome $bb_3$ in glycylglycine (de Gier et al., 1994) has been confirmed by analysis of cells from a single batch in both buffers (not shown). The apparent buffer sensitivity of cytochrome $bb_3$, a phenomenon that is not observed in the case of cytochromes $aa_3$ and $bb_3$, is not understood at present (see below).

The finding that all three terminal oxidases of Paracoccus ($aa_3$, $cbb_3$ and $bb_3$) couple the reduction of oxygen to the translocation of protons with maximal efficiency (1 $H^+/e^-$ ) also does not agree with analyses of growth efficiency in chemostat cultures of (wild-type) P. denitrificans, grown under a variety of conditions. The maximal growth efficiency is observed only under conditions in which cytochrome $aa_3$ is expressed (reviewed by Stouthamer, 1991). To address the discrepancy between the latter physiological studies and the above-described proton-pumping capacity of cytochrome $cbb_3$, we are currently cultivating a number of Paracoccus oxidase mutants under well-defined conditions. Preliminary data from a comparison of the growth yields of these mutants suggest that the energy-transducing efficiency of cytochrome $cbb_3$ is indeed lower than that of cytochrome $aa_3$ (de Gier, 1995).

The latter conclusion would be consistent with the aforementioned observation that the $H^+/e^-$ stoichiometry of cytochrome $cbb_3$ is not a fixed number, and may be sensitive to, for example, buffer composition (de Gier et al., 1994; Table 2). Moreover, proton pumping analysis of the $\Delta aa_3/bb_3$ mutant with ascorbate/TMPD as substrate, either in HEPES or in glycylglycine, results in $H^+$-values that never exceed 50% of the ratio that is measured in the presence of cytochrome $aa_3$ (de Gier et al., 1994; Raitio and Wikström, 1994). This apparent variability in the $H^+/e^-$ ratio of cytochrome $cbb_3$ might be the result of certain structural deviations of subunit I within the regions that have been demonstrated to be important for the coupling between oxygen reduction and proton pumping (see below).

**Residues involved in proton translocation**

A major challenge of cytochrome oxidase research is elucidation of the proton-translocation mechanism at a
molecular level. This requires the identification of residues that are involved in the transfer of consumed (scalar) and/or pumped (vectorial) protons. Among the highly conserved residues in subunits I of cytochrome aa₃ and cytochrome bo₃, a large number of polar residues have been substituted by means of site-directed mutagenesis (reviewed by Hosler et al., 1993). An aspartic acid in the domain between transmembrane helices II and III (loop II–III) has recently been proposed to participate in the influx of protons into the oxidase core (Garcia-Horsman et al., 1995; Fetter et al., 1995). Substitution of this conserved aspartate with an asparagine (D124N; numbering for P. denitrificans cytochrome aa₃, CtaDII, is used throughout) in both cytochromes aa₃ and bo₃ results in decoupling of proton pumping from electron transfer. In addition, substitution of two conserved asparagine residues (N113, N131) results in a decrease in the H⁺/e⁻ ratio. Interestingly, proton pumping is recovered in a double mutant of E. coli CyoB, D124N/N131D (Garcia-Horsman et al., 1995). It has been suggested that this cytoplasmic loop plays an important role as part of the entry to the pumped proton pathway, and moreover, that there should be a second channel, the chemical proton pathway. Residues that may be part of the latter pathway are a tyrosine (Y280) in helix VI, as well as two threonines (T344, T351) and a lysine (K354) in helix VIII. Non-polar substitutions for each of these residues result in a severe loss of oxidase activity (Hosler et al., 1993). Both T351 and K354 have been proposed to participate in a proton-conducting channel towards the binuclear centre (Hosler et al., 1993; Fetter et al., 1995). Spectroscopic analyses suggest that T344 is in close proximity to the binuclear centre. Y280 may be a ligand to CuB, at least during part of the catalytic cycle of oxygen reduction (Hosler et al., 1993). In addition, one of the invariant histidines that has been identified as a CuB ligand has recently been proposed to play a key role in the proton-translocation machinery. In the 'histidine cycle' model, a CuB co-ordinating histidine has been proposed to move back and forth in the binuclear 'pocket', thereby passing two protons from the inside to the outside (Morgan et al., 1994).

Analysis of the recently resolved structure of P. denitrificans cytochrome aa₃ (Iwata et al., 1995) supported many of the conclusions that were based on previous studies of the E. coli and R. sphaeroides oxidases (Hosler et al., 1993; Fetter et al., 1995; Garcia-Horsman et al., 1995). Indeed, two suggested proton channels have been envisaged in the structural model (Iwata et al., 1995). A pathway for consumed protons, from the cytoplasmic surface to the oxygen-binding site, appears to be made up of S291, K354, T351, the hydroxyl group of haem a₃, and, ultimately, Y280, which may donate a proton to oxygen (Fig. 3A, Table 3). With the exception of K354, all residues in this channel appear to be connected by hydrogen bonds, either directly or via putative solvent molecules. A second series of hydrophilic residues may constitute the pathway for pumped protons. A gate is formed by D124, N119, and T203; subsequently, a number of hydrophilic residues, again with additional putative solvent molecules, may form a pathway to E278 (Table 3). Beyond E278 the proton pathway is less clear. However, as proposed before (Morgan et al., 1994), the structural model suggests that one of the histidine ligands, H325, may not be in a fixed position, but rather may switch between different conformational states. One option is that two subsequent protons, approaching the catalytic site via E278, convert the H325 imidazolate, via imidazole, into imidazolium. In the latter state, the positively charged H325 moves away from the binuclear centre, and no longer ligates CuB. The two protons of H325 imidazolium would leave the system via the exit pathway, in which groups of the haem a₃ molecule and/or residues in the periplasmic loop XI–X may be involved (Table 3), and H325 imidazole would return to its original position (for details, see Iwata et al., 1995).

As cytochrome cbb₃ has the capacity to pump protons (Raitio and Wikström, 1994; Table 2), comparison of its sequence with that of the much better characterized cytochrome aa₃ is of particular interest. From the alignment presented in Fig. 3A, it is obvious that the sequences from CcoN/FixN show some significant deviation from both the aa₃-type and the bo₃-type oxidases, especially...

Table 3. Comparison of residues proposed to be involved in the chemical and the pumped proton channels in cytochrome aa₃ (Hosler et al., 1993; Fetter et al., 1995; Iwata et al., 1995), with counterparts in cytochrome cbb₃, based on the alignment presented in Fig. 3.

<table>
<thead>
<tr>
<th>aa₃</th>
<th>Location</th>
<th>cbb₃</th>
<th>Consensus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical channel</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a₃-hydroxyl</td>
<td>Haem</td>
<td>No hydroxyl</td>
<td></td>
</tr>
<tr>
<td>Y280</td>
<td>TM-VI</td>
<td>G</td>
<td>+</td>
</tr>
<tr>
<td>T351</td>
<td>TM-VIII</td>
<td>S</td>
<td>+</td>
</tr>
<tr>
<td>K354</td>
<td>TM-VIII</td>
<td>G</td>
<td></td>
</tr>
<tr>
<td>S291</td>
<td>TM-VI</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td>Pump channel</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D399</td>
<td>Loop-IX/X</td>
<td>N</td>
<td>+</td>
</tr>
<tr>
<td>L393-carbonyl</td>
<td>Loop-IX/X</td>
<td>M</td>
<td>+</td>
</tr>
<tr>
<td>a₃-protonate</td>
<td>Haem</td>
<td>D₃-protonate</td>
<td>+</td>
</tr>
<tr>
<td>a₃-formyl</td>
<td>Haem</td>
<td>No formyl</td>
<td></td>
</tr>
<tr>
<td>H325</td>
<td>TM-VII</td>
<td>H</td>
<td>+</td>
</tr>
<tr>
<td>E278</td>
<td>TM-VI</td>
<td>A</td>
<td>–</td>
</tr>
<tr>
<td>N113</td>
<td>TM-II</td>
<td>X</td>
<td>–</td>
</tr>
<tr>
<td>N131</td>
<td>Loop-II/III</td>
<td>X</td>
<td>–</td>
</tr>
<tr>
<td>N199</td>
<td>TM-IV</td>
<td>V</td>
<td>–</td>
</tr>
<tr>
<td>T203</td>
<td>TM-V</td>
<td>T</td>
<td>+</td>
</tr>
<tr>
<td>D124</td>
<td>Loop-II/III</td>
<td>X</td>
<td>–</td>
</tr>
</tbody>
</table>

In transmembrane helix II (TM-II) and the interconnecting loop between helices II and III (loop II–III) a reliable alignment is not possible; in this case the cbb₃ residues are marked ‘X’.
with respect to the residues that have been predicted to be part of either one of the two proton channels (Table 3). Although alignment of the oxidase sequences at their N-termini is difficult, it is clear that the invariant aspartate residue in loop II–III of cytochrome aa3 is not conserved in cytochrome cbb3 (Table 3). In helix VI of cytochrome cbb3, the tyrosine (Y280) and the glutamate (E278) that are conserved in all aa3- and b03-type oxidases (and appear to be essential in the aforementioned proton pump model) are substituted by a glycine and an alanine, respectively. In helix VIII, the threonines are conserved as serines, but a glycine is found at the position in loop 11–111 of cytochrome aa3 is not conserved in cytochrome cbb3 (and probably also in cytochromes bo3 and b2b3) are conserved in all aa3- and b03-type oxidases (and appear to be essential in the aforementioned proton pump model) are substituted by a glycine and an alanine, respectively. In helix VIII, the threonines are conserved as serines, but a glycine is found at the position in loop 11–111 of cytochrome aa3 is not conserved in cytochrome cbb3. These proton-conducting pathways are undoubtedly an essential component of the proton-translocation machinery. Hence, it is concluded that the design of the proton pump in cytochrome cbb3 differs significantly from that present in the other oxidase types. Although site-directed mutagenesis would provide more definitive evidence, it is tempting to assume that these deviations in cytochrome cbb3 might correlate with its apparently variable H+/e− coupling.

Experimental procedures

Bacterial strains, plasmids and growth conditions

The strains of P. denitrificans and E. coli as well as the plasmids used in this study are listed in Table 4. Cells of wild-type P. denitrificans (Pd1222) and mutants were cultivated in aerobic batch cultures (0.5 l bottles with 100 ml of culture, on a rotary shaker at 30°C), with either brain–heart infusion (BHI) broth or minimal medium supplemented with 25 mM succinate, as described previously (van Spanning et al., 1990). For microaerobic cultivation, cultures were not shaken. E. coli strains were cultivated in YT medium at 37°C. When appropriate, antibiotics were added: rifampicin (Rif, 40 μg/ml), streptomycin (Sm, 50 μg/ml), tetracycline (Tc, 12.5 μg/ml), kanamycin (Km, 50 μg/ml), tetracycline (Tc, 12.5 μg/ml), kanamycin (Km, 50 μg/ml) and ampicillin (Amp, 100 μg/ml).

Purification of the cbb3-type cytochrome c oxidase from P. denitrificans

Cells from succinate-grown Pd93.11 (Δaα3Δbβ3) were harvested in the late log phase, and resuspended in 20 mM Bis-Tris propane (pH 7.3), 2 mM EDTA and 0.5 mM PMSF. Membranes were prepared by passage of the cell suspension twice through a French Pressure Cell and subsequent centrifugation (1.5 h, 150000 × g). Membranes were solubilized in 20 mM Bis-Tris propane (pH 7.3), with 1.0% (w/v) dodecyl maltoside and 0.15 mM PMSF (30 min, 4°C). The suspension contained approx. 10 mg protein per ml and was cleared by ultracentrifugation (30 min, 150000 × g). The solubilized membrane proteins were applied to a Q-Sepharose HP (Pharmacia) column in 20 mM Bis-Tris propane (pH 7.3), with 0.03% (w/v) dodecyl maltoside, and bound protein was eluted with a linear NaCl gradient (0.0–1.0 M). Oxidase activity was measured spectrophotometrically with TMPD as described previously (Lubben et al., 1994). Fractions with cytochrome c oxidase activity eluted at 0.3 M NaCl. Pooled fractions were applied onto a sucrose gradient, as described previously (Gray et al., 1994). Fractions with TMPD oxidase activity were applied to a Chelating–Sepharose FF (Pharmacia) column, saturated with Cu2+. A linear ammonium chloride gradient (0.0–1.0 M) in 20 mM Tris (pH 8.0), 0.5 M NaCl, 15 mM PMSF and 0.03% (w/v) dodecyl maltoside was used to elute bound proteins. Cytochrome c oxidase activity eluted at 0.3 M ammonium chloride. As a last purification step the active fractions were loaded onto a Q-Sepharose HP column (Pharmacia) in 20 mM Bis-Tris propane (pH 7.3), with 0.03% (w/v) dodecyl maltoside, and bound protein was

Table 4. Strains and plasmids.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Relevant characteristics</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. denitrificans</td>
<td>pUC19</td>
<td>AmpR lacZ’</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>Pd1222</td>
<td>pUC4KIXX</td>
<td>KmR (Tn5)</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>Pd92.20</td>
<td>M13mp18/19</td>
<td>pUC18/19 mcs, lacZ’</td>
<td>Sanger et al. (1980)</td>
</tr>
<tr>
<td>Pd26.21</td>
<td>pRK2020</td>
<td>TcR (Tn10)</td>
<td>Ditta et al. (1985)</td>
</tr>
<tr>
<td>Pd93.11</td>
<td>pGRDp1</td>
<td>oriV (colE1) AmpR ori’ SmR (Tn1831)</td>
<td>van Spanning et al. (1990)</td>
</tr>
<tr>
<td>Pd9220 derivative, ΔactaDI</td>
<td>pCco1</td>
<td>ccoNO::KmR</td>
<td>This work</td>
</tr>
<tr>
<td>Pd27.21</td>
<td>pCco2</td>
<td>ccoNO::KmR</td>
<td>This work</td>
</tr>
<tr>
<td>Pd93.12</td>
<td>pCco3</td>
<td>ccoNO::KmR</td>
<td>This work</td>
</tr>
<tr>
<td>Pd9220 derivative, ΔactaDI ΔactaDII ccoNO::KmR</td>
<td>pCco1 derivative, ccoNO::KmR</td>
<td>This work</td>
<td></td>
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<td>Pd93.11</td>
<td>pRtD27.21</td>
<td>ccoNO::KmR</td>
<td>This work</td>
</tr>
</tbody>
</table>
eluted with a linear NaCl gradient (0.0-1.0 M). Active fractions eluted at 0.3 M NaCl. The purified oxidase was analysed by SDS–PAGE and spectrophotometrically (Laemmli, 1970; de Gier et al., 1994). Haem staining was performed as described by Thomas et al. (1976).

DNA manipulation

General cloning techniques were carried out as described by Ausubel et al. (1993). Conjugations were performed as described previously (de Vries et al., 1989). The matings of Paracoccus host strains were performed either directly with an E. coli S17.1 derivative that carried the plasmid of interest or via a triparental mating using any E. coli strain transformed with the plasmid of interest, in combination with E. coli HB101(pRK2020) containing the 'helper plasmid'. Mutagenesis was performed using pGRPD1 as described by van Spanning et al. (1990).

The PCR was performed using Super-Taq polymerase (HT Biotechnology Ltd.), essentially as described by de Gier et al. (1994). Primers were synthesized according to the amino acid sequence of the N-terminal peptide of the 30 kDa subunit of the Paracoccus cbc complex (CooO) (nos 171 and 172). These were degenerate primers with a bias for G:C at the third position of a codon. The sequences of these primers are as follows: 171 (sense), 5'-AAAAAGTCTGCNNAT[T/C/A]CTNGA[A/G]CA[T/C]-3'; and 172 (antisense), 5'-AAAGAATC[T/G]ATATCGNNGAACNACNAG[A/G]AA-3'.

DNA sequencing was performed using the dye method (Sanger et al., 1977) on fragments subcloned into M13mp18/19 (Sanger et al., 1980) with the Taq dye terminator cycle sequencing kit, and the Taq dye primer cycle sequencing kit (Applied Biosystems). Sequence analysis was carried out on a 370A DNA sequencer (Applied Biosystems). Editing of the sequences was performed with the SEQUENCHER 2.1 Program (Gene Codes Corp.).

Oxygen-consumption analysis

Oxygen consumption by bacterial cell suspensions was performed polarographically with a Clark-type oxygen electrode, as described by de Gier et al. (1994).

Proton translocation

Proton translocation was studied by using the oxygen-pulse method in intact cell suspensions (de Gier et al., 1994; Raitio and Wikström, 1994). Cells were harvested in the late log phase from succinate batch cultures, washed twice and resuspended in 150 mM KCl (final OD560 is 100), and diluted four times in reaction medium (pH 7.4) in the stirred anaerobic reaction vessel (3.0 ml). The buffer contained 100 mM KCl, 100 mM KSCN, 0.5 mM HEPES (pH 7.4) and 30 μM rotenone, as described by Raitio and Wikström (1994). Succinate was used as the electron donor (final concentration 2.5 mM). The reactions were started with small pulses (5-20 μl) of air-saturated water (containing 258 μM C2 at 25°C) or 1.0 mM potassium ferricyanide, made anaerobic with argon. Pulses were calibrated with 1.0 mM HCl or 0.5 mM oxalic acid, made anaerobic with argon. Controls with the protonophore FCCP were routinely included to ascertain if the observed proton ejection was due to proton translocation.

Acknowledgements

We thank M. Wikström and H. V. Westerhoff for critical reading of the manuscript. This research was supported by the Netherlands Foundation for Chemical Research, with financial aid from the Netherlands Organization for the Advancement of Science. J.v.d.O. had a Fellowship of the Royal Netherlands Academy of Science. EMBO supported visits of J.W.d.G. to the laboratories of M. Wikström and M. Saraste.

References


Rhodobacter sphaeroides that lacks CuA. Biochemistry 33: 3113–3119.


