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Review

The bacterial cytochrome cbb_3 oxidases

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Abstract

Cytochrome cbb_3 oxidases are found almost exclusively in Proteobacteria, and represent a distinctive class of proton-pumping respiratory heme-copper oxidases (HCO) that lack many of the key structural features that contribute to the reaction cycle of the intensely studied mitochondrial cytochrome *c* oxidase (CcO). Expression of cytochrome cbb_3 oxidase allows human pathogens to colonise anoxic tissues and agronomically important diazotrophs to sustain N_2 fixation. We review recent progress in the biochemical characterisation of these distinctive oxidases that lays the foundation for understanding the basis of their proposed high affinity for oxygen, an apparent degeneracy in their electron input pathways and whether or not they acquired the ability to pump protons independently of other HCOs.

Keywords: Microaerobic metabolism; Cytochrome cbb_3 oxidase; Heme-copper oxidase; *Pseudomonas*

1. Introduction

Understanding the mechanistic basis of energy transduction by cytochrome *c* oxidase (C*c*O), the enzyme that terminates the electron transfer chain of mitochondria [1] and member of the heme-copper oxidase (HCO) superfamily, has fascinated bioenergeticists ever since Mitchell [2] formulated the chemiosmotic theory over 40 years ago. More recently, the discovery of less complex protonmotive HCOs that catalyse the same four-electron reduction of dioxygen to water in Eubacteria [3–5] opened the way for the function of individual residues to be tested by site-specific mutagenesis. These experiments have been important in interpreting the structures of both the mitochondrial and bacterial cytochrome aa_3-type oxidases.

Genetic and physiological studies, and more recently the emerging sequences of the bacterial genomes, have established that many bacteria exhibit a high degree of respiratory flexibility [6]. In particular aerobically respiring bacteria can express, according to changes in environmental conditions, a number of different enzymes all of which can clearly be identified as HCOs [7]. Some of these enzymes are not only quite distinct from the well-known aa_3-type CcOs, but can also, under certain physiological conditions, have functions other than terminal electron transfer and energy conservation. These include ion-transport, oxygen scavenging and maintaining redox homeostasis [7].

2. The cytochrome cbb_3 oxidases

Representatives of the HCO superfamily found in Eubacteria are usually considered to belong to one of two main groups: quinol oxidases, for example cytochrome bo_3 from *Escherichia coli* [4] and CcOs such as the aa_3-type oxidases from *Paracoccus denitrificans* [3] and *Rhodobacter sphaeroides* [5]. Both classes of oxidase catalyse the four-electron reduction of dioxygen to water and use the liberated free energy to translocate protons across the periplasmic membrane. The main difference between these two well-known classes of HCO is the presence of a metal centre, known as Cu_A or purple copper, in the hydrophilic domain of subunit II of C*c*Os. Cu_A contains two copper atoms in a mixed valence

Abbreviations: C*c*O, cytochrome *c* oxidase; CO, carbon monoxide; EPR, electron paramagnetic resonance; FNR, fumarate and nitrate reduction (regulatory protein); FTIR, Fourier transform infrared; HCO, heme-copper oxidase; HPLC, high-performance liquid chromatography; NIR, near infrared; NOR, nitric oxide reductase; RT-MCD, room temperature magnetic circular dichroism; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylene-diamine

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configuration [8,9] and is the primary acceptor of electrons from cytochrome *c* [10]. Quinol oxidases, in contrast, accept electrons directly from a quinol derivative in the cytoplasmic membrane.

In recent years a third highly diverged group of HCOs, the cytochrome cbb_3 oxidases, has been described in the Proteobacteria [11,12]. Cytochrome cbb_3 oxidases have been purified from several organisms including *P. denitrificans* [13], *R. sphaeroides* [14], *Rhodobacter capsulatus* [15] and *Bradyrhizobium japonicum* [16]. Rather uncertain yields and a tendency for the purified complex to dissociate made detailed biochemical characterisation difficult. However, some progress was made and there is considerable evidence from these earlier studies that cytochrome cbb_3 oxidases are quite distinct from other bacterial HCOs in terms of their strategy for receiving electrons, the heme prosthetic group present in the active site and their affinity for oxygen. For instance the experimentally determined K_m for dioxygen for the enzyme from *B. japonicum* is in the order of 7 nM [16], which is consistent with its function in the bacteroid.

Thus far, the enzyme from *B. japonicum* is the only cytochrome cbb_3 oxidase in which substrate affinity has been measured. However, given the degree of sequence conservation in the catalytic subunit (CcoN), and the fact that cytochrome cbb_3 is expressed by bacteria only under microaerobic conditions, it is highly probable that a similar high affinity for substrate will be demonstrated in enzyme isolated from other sources. This experimentally determined value for the *B. japonicum* cbb_3-type oxidase is considerably higher than that recorded for other HCOs. For example, cytochrome bo_3 oxidase from *E. coli* [17,18] has a K_m for dioxygen of the order of 0.15–0.35 μM, while the K_m of cytochrome aa_3 oxidase has been reported as being in the region of 0.1 μM [19,20] and elsewhere as high as 1 μM [21].

Proton translocation has been demonstrated in cell suspensions in which cytochrome cbb_3 oxidase is the only cytochrome bc_1-dependent oxidase present in the cytoplasmic membranes [13,22]. When proton translocation was coupled with the oxidation of succinate the H^+/e^- ratio approaches 1, however when succinate was replaced by *N*,*N*,*N*′,*N*′ -tetramethyl-*p*-phenylene-diamine (TMPD) and ascorbate the value of this ratio fell considerably [13,22]. It should be noted that a value of 1 has been reported for the cytochrome cbb_3 oxidase from *R. sphaeroides* using either succinate or ascorbate and TMPD as electron donors [23]. Complementary experiments in which the H^+/e^- stoichiometry associated with purified cytochrome cbb_3 oxidase reconstituted into phospholipid vesicles [24,25] was measured suggest a variable number, usually between 0.2 and 0.5, which is significantly lower than maximum efficiency (1 H^+/e^-) exhibited by cytochrome aa_3 oxidase. Physiological studies are also consistent with the notion that cytochrome cbb_3 is less efficient at transducing energy than cytochrome aa_3 [13].

2.1. Genes required for the expression of cytochrome cbb_3 oxidase

Genes encoding a cytochrome cbb_3 oxidase were initially identified in *B. japonicum* and designated *fixNOQP* (*ccoNOQP*) since their expression is required both to support symbiotic N_2 fixation, which is energetically demanding, whilst ensuring that the O_2 labile nitrogenase is not compromised [26]. More recently, homologous genes have been identified in other Proteobacteria, for example *R. capsulatus* [27], *Azorhizobium caulinodans* [28], and three human pathogens *Campylobacter jejuni* [29], *Helicobacter pylori* [30] and *Neisseria meningitidis*, in which cytochrome cbb_3 is the only respiratory oxidase encoded by the genome (Fig. 1; Table 1). This suggests that expression of this oxidase is required for the successful colonisation of anoxic tissues and may be an important determinant of pathogenicity.

The *ccoNOQP* operon is always found close to a second gene cluster, known as *fixGHIS* (*ccoGHIS*) whose expression is necessary for the assembly of a functional cbb_3 oxidase [31,32]. The *ccoGHIS* operon comprises four tightly linked open reading frames first identified, cloned and sequenced in *Sinorhizobium meliloti* [33]. Like the *ccoNOQP* operon, expression is strongly induced in cells grown under microaerobic or anaerobic conditions. On the basis of their derived amino acid sequences each of the four proteins encoded by the *ccoGHIS* operon are thought to be membrane-bound and it has been suggested that they may function in concert as a multisubunit complex [31]. Moreover, it has been proposed, on the basis of sequence similarities between CcoI and a Cu-transporting P-type ATPase (CopA) of *Enterococcus hirae* and the presence in CcoG of two cysteine-rich motifs, resembling those encountered in the [4Fe–4S] cluster of bacterial ferredoxins, that these two proteins may play a role in the uptake and metabolism of copper required for the assembly of the dinuclear centre of cytochrome cbb_3 oxidase [31].

The almost exclusive presence of cytochrome cbb_3 oxidase in the Proteobacteria contrasts with the universal distribution of the HCO catalytic centre throughout all domains of life [11,34]. This suggests that cytochrome cbb_3 oxidase may represent a modern enzyme that has evolved independently to fulfill a specialized role in microaerobic energy metabolism [34] rather than being an evolutionary remnant related to the primordial oxidase that is proposed to have evolved some 2 billion years ago [35]. Interestingly, the cytochrome cbb_3 oxidase from *Pseudomonas stutzeri* has the highest NO reductase activity of any known dioxygen reducing HCO [36]. This is perhaps not surprising as cytochrome cbb_3 oxidase is also a close relative of the specialist bacterial nitric oxide reductase (NOR), also a HCO, with which it shares both phylogenetic origins [37,38] and a number of structural features [34,39]. Note that the elemental composition of the dinuclear centre of bacterial NOR differs from the oxygen reducing HCOs in that it contains non-heme iron in the active site rather than Cu_B.

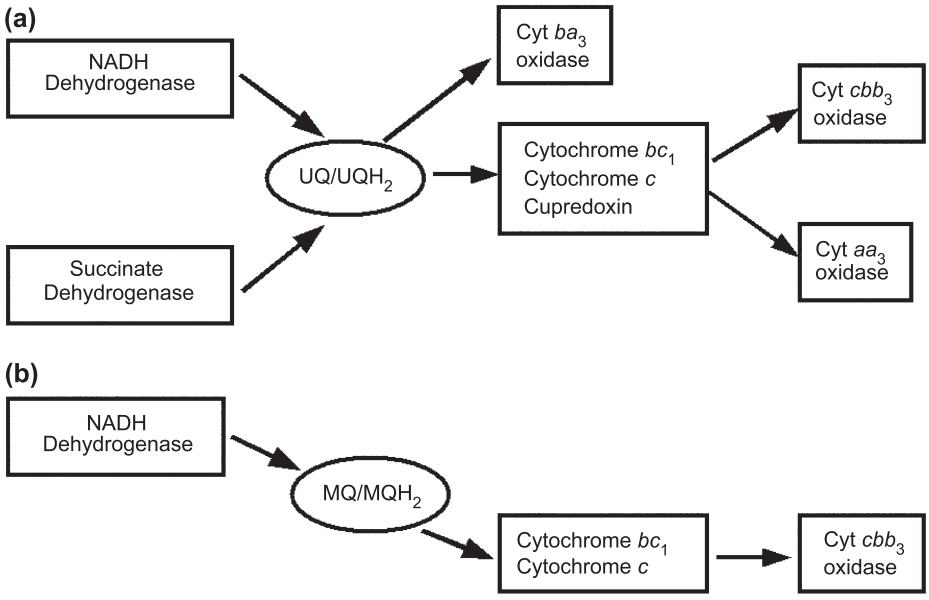

Fig. 1. Organisation of the aerobic respiratory chains of (a) *P. denitrificans* and (b) *H. pylori*. In *P. denitrificans* different terminal heme-HCOs, including cytochrome cbb_3 oxidase are expressed in part according to the oxygen tension of the external environment. In contrast, in *H. pylori*, the cytochrome cbb_3 oxidase provides the only means for the organism to respire aerobically.

2.2. Organisation of the cytochrome cbb_3 oxidase complex

All members of the HCO superfamily contain a highly conserved catalytic subunit (subunit I), represented in cytochrome cbb_3 oxidases by CcoN [34,35,38]. Subunit I comprises at least 12 transmembrane helices and contains the active site, a dinuclear centre formed by the iron of a high-spin heme, to which substrate and other exogenous ligands can bind, and an adjacent copper ion (Cu_B) [1]. Also found in subunit I is the binding site for a second heme that is low-spin and bis-histidine coordinated and which serves to transfer electrons to the active site. Transmembrane helices II, VI, VII and X of subunit I harbour six absolutely conserved histidine residues that are diagnostic of the entire superfamily and which serve to ligate both heme irons and Cu_B [40].

We have recently reported the sequence of a 3.6 kbp region of the *P. stutzeri* genome that spans four open reading frames (*ccoN*, *ccoO*, *ccoQ* and *ccoP*) [41]. The amino termini of CcoN, CcoO and CcoP obtained by direct sequencing of the polypeptides are in good agreement with the derived sequence from the corresponding genes [41]. A consensus sequence (TTGAT-N4-GTCAA), that recognises members of the fumarate and nitrate reduction (FNR) family of transcriptional regulators [42], is located − 88 and − 102 bp upstream of the *ccoN* start codon. This is consistent with the observation that FnrA is required by *P. stutzeri* for the expression of cytochrome cbb_3 under microaerobic conditions [43]. The presence in our derived amino acid sequence, and that of all other cytochrome cbb_3-type oxidases, of the six canonical histidine residues that ligate the metal centres of CcoN is consistent with the topological model, based upon a minimum of 12 transmembrane helices, of the catalytic subunit of cytochrome cbb_3 oxidase that has been proposed for the *B. japonicum* enzyme [44,45]. These helices correspond to the 12 authentic transmembrane helices of subunit I seen in the X-ray structures of aa_3-type oxidases [46,47] (Fig. 2).

Despite these conserved metal ligands and a conserved secondary structure, CcoN contains neither heme O nor heme A in the active site. Several analyses of the non-covalently bound hemes present in the cbb_3 oxidase complex by high-performance liquid chromatography (HPLC), have shown that only heme B is present [14,15,48]. Consequently the dinuclear centre of cytochrome cbb_3 oxidases must consist of a *b*-type heme, heme b_3, magnetically coupled to Cu_B. This observation is of considerable interest because unlike heme O and heme A, heme B does not have a hydroxyethylfarnesyl substituent on the porphyrin macrocycle which may have implications for the architecture of the dinuclear centre.

A further difference between CcoN and the subunits I of other Eubacterial oxidases, for example the well characterised cytochrome aa_3- and cytochrome bo_3-type oxidases, is suggested by careful alignment of their primary amino acid sequences [13,34]. These analyses clearly show that many of the ionisable residues that form two structurally defined proton uptake channels within cytochrome aa_3 oxidase, known as the D- and K-channels, responsible for moving protons from the face of the inner membrane to the buried dinuclear centre during turnover, are absent in CcoN [13]. In particular, Tyr-280 (residue numbering corresponds to the *P. denitrificans* cytochrome aa_3 sequence) that lies at the top of the K-channel is absent. In cytochrome aa_3 post-translational modification of Tyr-280 covalently links it to His-284, one of the Cu_B ligands [49],

Table 1
Occurrence of cytochrome cbb_3 oxidase in selected bacterial species

	cbb_3-type oxidase	NOR	cytochrome oxidase[1]	*bd*-type oxidase
Pseudomonas aeruginosa PAO1	✔	✔	✔	✔
Pseudomonas putida KT2440	✔		✔	✔
Pseudomonas syringae pv. Tomato str., *DC3000*	✔		✔	✔
Caulobacter crescentus CB15	✔		✔	✔
Ralstonia solanacearum	✔	✔	✔	✔
Neisseria meningitidis MC58	✔	✔		
Neisseria meningitidis Z2491	✔	✔		
Neisseria meningitidis serogroup C	✔	✔		
Neisseria gonorrhoeae	✔	✔		
Helicobacter pylori 26695	✔			
Helicobacter pylori J99	✔			
Vibrio cholerae	✔			✔
Campylobacter jejuni subs. *jejuni NCTC 11168*	✔			✔
Campylobacter jejuni RM1221	✔			✔
Gemmata obscuriglobus UQM 2246	✔		✔	
Cytophaga hutchinsonii	✔		✔	

The listed genomes were interrogated using a BLAST search using the derived amino acid sequences of the catalytic subunits (subunit I) from cytochrome cbb_3 oxidase (CcoN) of *P. aeruginosa*, nitric oxide reductase (NorB) of *P. stutzeri* and cytochrome aa_3 oxidase of (CtaD) *P. denitrificans*. The genomes were also interrogated with the derived amino acid sequence of subunit I of the structurally unrelated cytochrome *bd* oxidase-I (CydA) of *E. coli*. GenBank accession numbers for the subunits used in the search are: AE004583, P98008 CAA68821, P11026. Discrimination between cbb_3 oxidase, NOR and cytochrome oxidases (NB no differentiation was made between the catalytic subunits of cytochrome aa_3 type oxidases and bo_3-type quinol oxidases) was based on conserved residues in transmembrane helix VI, which diagnose each class of HCO. All of the species to which the searched genomes belong are Proteobacteria except the last two. *C. hutchinsonii* belongs to the Bacteroidetes/Chlorobi group, whilst *G. obscuriglobus* belongs to the Planctomycetes group. These represent the only two examples of CcoN's presence outside the Proteobacteria of the currently available finished and unfinished genomes (223 eubacteria, 18 archaebacteria) available in March 2003.

to form a site with the potential to stabilise a radical species during oxygen reduction [50]. There is no obvious replacement for this unusual structure in the cytochrome cbb_3-type oxidases, which again might suggest different organisation of the active site.

Cytochrome cbb_3 oxidases appear to utilize cytochrome *c* and not quinol [15,51] as an electron donor and yet they lack a Cu_A site [14]. Instead the complex contains two membrane-anchored subunits that contain *c*-type hemes; CcoO (23 kDa) and CcoP (35 kDa). Inspection of the derived amino acid sequences reveals that one and two conserved CXXCH motifs are present in CcoO and CcoP, respectively, diagnosing the covalent binding sites of three *c*-type hemes between them. In principle one or both of these subunits could serve to mediate the transfer of electrons derived from the bc_1 complex to the catalytic subunit. Despite the presence of a single heme *c* binding site, CcoO exhibits minimal similarity to known *c*-type cytochromes with the notable exception of NorC, a membrane anchored cytochrome *c* subunit of NOR [37,52]. Thus far, analysis of the derived amino acid sequence of CcoP has not revealed any similarities with other diheme *c*-type cytochromes.

The cytochrome cbb_3 oxidase operon includes a fourth gene, *cco*Q, which is predicted to encode a small membrane bound polypeptide. The presence of CcoQ in a purified complex has so far only been unequivocally demonstrated, immunologically, in *B. japonicum* and its function remains unclear [45]. In-frame deletion mutants of *cco*Q constructed in *B. japonicum* [53] and *R. sphaeroides* [54] have no apparent effect upon the assembly or the activity of cytochrome cbb_3 oxidase although the cytochrome *c* quantity appeared somewhat decreased in the *Cco*Q mutant of *B. japonicum* [53]. There is some recent evidence to suggest that in *R. sphaeroides* CcoQ serves as a "transponder" in an as yet undefined signal transduction pathway that controls the expression of photosynthesis-related genes in response to the flux of electrons through the cytochrome cbb_3 oxidase. It has been suggested that this specific role for CcoQ may perhaps be related to the presence of two histidine residues that are conserved in *R. sphaeriodes* and *R. capsulatas*, but which are not present in non-photosynthetic species [54,55].

2.3. Cytochrome cbb_3 oxidase from *P. stutzeri*

Recently a method of purifying cytochrome cbb_3 oxidase in high yield from *P. stutzeri*, a facultative

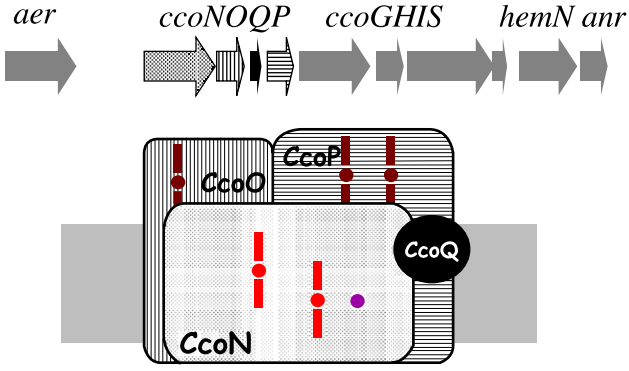

Fig. 2. Organisation of the *ccoNOQP* operon in *P. stutzeri*. The *ccoNOQP* operon of *P. stutzeri* encodes the four structural proteins that comprise the cytochrome cbb_3 oxidase complex. Although CcoQ is shown as an integral part of the complex there is no evidence for its presence in the cytochrome cbb_3 oxidase purified from *P. stutzeri* which is a three subunit (CcoNOP) complex [37]. In the schematic of the membrane bound complex *b*-type hemes are coloured red, *c*-type hemes brown and Cu_B purple.

anaerobic bacterium capable of denitrification, has been described [48]. Initial biochemical characterisation of this enzyme used a combination of SDS-PAGE, oxygen uptake measurements, heme determination, sedimentation analysis and mass spectrometry [41,48]. The results show the *P. stutzeri* cytochrome cbb_3 oxidase to be a stable three-subunit complex (CcoNOP) containing the expected cofactors, which will serve as a paradigm for this class of HCO. Recent work in our laboratory has focused upon using this enzyme in a range of spectroscopic and ligand-binding experiments that will provide a framework for future investigations into its structure and mechanism.

3. Recent spectroscopic studies of cytochrome cbb_3 oxidase

Past difficulties in obtaining cytochrome cbb_3 oxidase in high yields has made the spectroscopic study of this class of HCO rather difficult. The electron paramagnetic resonance (EPR) spectra of the fully oxidised enzymes purified from *R. capsulatus* [15] and *R. sphaeroides* [14] provided clear evidence for the presence of low-spin ferric heme and confirmed the absence of Cu_A [14,15]. Moreover, these EPR spectra lacked signals that could account for one equivalent of magnetically isolated high-spin ferric heme b_3. Since elemental analysis of both enzymes showed the presence of one equivalent of copper the implication was that the heme-copper dinuclear centre was both intact and magnetically coupled. In the case of the enzyme isolated from *R. sphaeroides*, evidence for an intact heme-copper dinuclear centre was also obtained using Fourier transform infrared (FTIR) spectroscopy to examine the CO complex of the fully reduced cytochrome cbb_3 oxidase. The light *minus* dark difference spectrum at cryogenic temperatures indicated that as expected the CO is bound to ferrous heme b_3 ($\upsilon = 1950\ cm^{-1}$) before illumination, but after illumination it migrates to Cu_B ($\upsilon = 2065\ cm^{-1}$).

Recently, Stavrakis et al. [56] have made FTIR measurements on the CO adduct of the fully reduced enzyme from *P. stutzeri* at ambient temperatures. These authors report CO binding only to the b_3 heme resulting in a single isotopically sensitive band in the FTIR spectrum at 1956 cm^{-1}. The position and band width of this Fe(II)–CO vibrational mode is consistent with earlier resonance Raman experiments [57,58]; which indicated that the bound CO at the active site heme of cbb_3-type oxidases is in a conformation that corresponds to the so-called β-conformation of CO bound at the active site of cytochrome aa_3 [59]. Moreover, the kinetics associated with the decay of the band arising from the Cu(I)–CO species formed after transient illumination correspond to the kinetics of (re)formation of the Fe(II)–CO species [56]. This behaviour is quite unlike that reported for any other HCO and implies that no CO escapes from the active site after photolysis.

3.1. Identification of the axial ligands to the hemes

A more detailed spectroscopic analysis of the oxidised cytochrome cbb_3 oxidase complex (CcoNOP) from *P. stutzeri* using a combination of UV/vis, EPR and room temperature-magnetic circular dichroism (RT-MCD) spectroscopies has recently been reported [41]. The electronic absorption spectrum of fully oxidized *P. stutzeri* enzyme, as isolated, has a Soret maximum at 411 nm and two broad, but well-defined, features in the visible region between 530 and 550 nm. The spectrum also exhibits a weaker feature centred at 645 nm which represents one of a pair of ligand to metal charge transfer bands of the high-spin ferric heme b_3 (see Refs. [60,61]). The intensity of this feature varies somewhat between preparations, probably due to some degree of heterogeneity in the dinuclear centre; a phenomenon that is well described in other HCOs [62]. Complete reduction of the enzyme with excess dithionite under strictly anaerobic conditions causes the Soret maximum to shift to 417 nm and a shoulder at 420 nm to develop. Two features, at 551 and 521 nm, intensify as a result of reduction and at the same time each of them develops distinct shoulders at 559 and 528 nm, respectively. The maxima at 417, 521 and 551 nm are characteristic of ferrous *c*-type hemes, whilst the shoulders at 420, 528 and 559 nm are indicative of ferrous *b*-type heme.

RT-MCD spectroscopy in the visible region provides information on the oxidation- and spin-states of hemes and the results obtained with the oxidised cytochrome cbb_3 oxidase from *P. stutzeri* are consistent with the presence of four low-spin ferric hemes anticipated from sequence analysis; heme *b* together with the three *c*-type hemes of CcoO and CcoP. Information concerning the amino acid side-chains that serve as axial ligands to low-spin ferric hemes can be obtained from the RT-MCD spectrum in the near Infrared (NIR) region [63]. In the case of *P. stutzeri* cytochrome cbb_3 oxidase it was determined that the complex contained two hemes with bis-histidine coordination and two with His/Met coordination; an observation that implied that one of the *c*-type hemes of either CcoO or CcoP had bis-histidine axial coordination rather than the more usual His/Met ligation [64]. To discriminate between these possibilities we expressed the diheme CcoP subunit in *E. coli* and demonstrated, again using NIR-RT-MCD spectroscopy that the bis-histidine coordinated *c*-type heme was found in this subunit [64].

Although signals associated with each of the four low-spin ferric hemes can clearly be seen in the X-band EPR spectrum of the *P. stutzeri* cytochrome cbb_3 oxidase at 10 K, the complexity of the spectrum made assigning any given signal to a particular heme very difficult. However, by recording the EPR spectra of both the isolated CcoP subunit and the cbb_3 oxidase complex after partial reduction with ascorbate, it was possible to unambiguously assign the EPR signals associated with of each of the four hemes [41]. In common with earlier EPR studies of the cytochrome cbb_3 oxidases from *R. capsulatus* and *R sphaeroides*, there was no

indication of significant amounts of magnetically isolated high-spin ferric heme. Furthermore, there was no evidence in either of the three cases of the derivative shaped features at $g=3-4$ (in perpendicular mode) and $g=10-12$ (in perpendicular and parallel mode) that characterise the weak coupling at the active site that have been observed in cytochrome bo_3 oxidase from *E. coli* [65,66] and bovine heart C*c*O [67].

3.2. Electrochemical properties of the hemes

We have recently determined the reduction potentials of the five hemes present in cytochrome cbb_3 oxidase isolated from *P. stutzeri* (Table 2). In particular we were interested in the reduction potential of the *c*-type heme with bis-histidine ligation because cytochrome *c* coordinated in this fashion generally have a lower reduction potential ($E_m<100$ mV) than those with His/Met ligation due to a reduced ability to stabilise Fe(II) [52]. The reduction potentials that we determined for the three *c*-type hemes in the *P. stutzeri* enzyme (Table 2) are in good agreement with earlier studies [15]. Although the lowest potential *c*-type heme we observed in the *P. stutzeri* enzyme ($E^{\circ\prime}=+185$ mV) was somewhat higher than expected, it seems probable that this reduction potential is associated with the heme with bis-histidine ligation [64].

The potential that we determined for the low-spin *b*-type heme ($E^{\circ\prime}=+310$ mV) in the catalytic subunit of cytochrome cbb_3 oxidase is not dissimilar to that reported for the equivalent heme, heme *a*, of bacterial cytochrome aa_3 oxidase [68]. Moreover, the spacing of the potentials between heme *b* and heme b_3 ($\Delta E^{\circ\prime}=+85$ mV) in the present study is a little more that that found in aa_3-type oxidases (0 to +50 mV), [69] but rather less than the +200 mV we have measured for bacterial NOR [39].

The presence of three *c*-type hemes in the CcoNOP complex with very similar reduction potentials is puzzling. Most bacterial respiratory enzymes that use soluble periplasmic metalloproteins such as cytochrome *c* or azurin as an electron donor contain only one redox centre to receive electrons. For example, Cu_A ($E^{\circ\prime}=$ca. +240 mV) [70], serves as the immediate electron acceptor in cytochrome aa_3 oxidase of *P. denitrificans*. Whilst in bacterial NOR this role is fulfilled by the single *c*-type heme in NorC (+310 mV) [39,71]. Since it has been established that the CcoNO subcomplexes isolated from both *P. denitrificans* [13] and *B. japonicum* [53] are catalytically competent, it would appear that this role is fulfilled in cbb_3-type oxidases by the monoheme CcoO. However, the observed reduction potentials of the two *c*-type hemes in CcoP from *P. stutzeri* (Table 2) are consistent with electron transfer to the catalytic subunit (CcoN). Consequently the question arises under what circumstances does the cytochrome cbb_3 oxidase complex (CcoNOP), which cannot use quinol as an electron donor, require the diheme CcoP to transfer electrons to the catalytic subunit?

3.3. Dynamics of CO recombination to the cbb_3 oxidase complex

The kinetics of carbon monoxide (CO), an analogue of both O_2 and NO, recombination have been used to investigate the environments of the oxygen-binding hemes in globins [72], HCOs [1,73,74] and a number of heme based gas sensors. In all other classes (with a recent exception, see below) of HCO, CO binds only at the active site heme and provides a useful spectroscopic probe of the integrity and organisation of the dinuclear centre.

There are a number of reports that indicate that the binding of CO to fully reduced cytochrome cbb_3 oxidase is more complex than is the case for other HCOs. For example, the cytochrome cbb_3 oxidase purified from *B. japonicum* appears to bind CO to both the active site heme and a *c*-type cytochrome [31,51], whilst the published reduced-CO *minus* reduced absorption spectra of the enzyme purified from *R. capsulatus* [15], indicates CO binds only to a *c*-type cytochrome. The separately expressed periplasmic soluble domain of CcoP from *B. japonicum* expressed in *E. coli* can also bind CO [75]. However, there is no evidence of CO binding to a *c*-type heme in the enzyme purified from *P. denitrificans*, although this may be accounted for by close inspection of the reduced *minus* oxidised difference spectra [13,22] which shows the CcoP subunit to be lost during purification [13,53].

Table 2
Properties of the hemes in the cytochrome cbb_3 oxidase purified from *P. stutzeri*

Subunit	Redox centre	Heme axial ligands determined spectroscopically	Residues involved in heme-ligation from sequence analysis	Midpoint reduction potentials
CcoN	heme *b*	His/His	His-60, His-348	+310 mV, +265 mV
	heme b_3	n.d.	His-346	+225 mV
CcoO	heme *c*	His/Met	His-71	+245 mV, +215 mV
CcoP	heme *c*	His/Met	His-147, His-236	+245 mV, +205 mV
	heme *c*	His/His		+185 mV, +105 mV

The axial ligand sets to the low-spin ferric hemes in purified cytochrome cbb_3 oxidase were determined by room temperature NIR-MCD spectroscopy [37]. The residues serving as ligands to heme *b* and heme b_3 in CcoN were identified by multiple sequence alignments using derived amino acid sequence reported by Saraste and Castresana [37]. The proximal ligands to the three *c*-type hemes of CcoO and CcoP were identified in the derived amino acid sequences reported in Ref. [37] (GenBank AF521004) from the Cys–X–X–Cys–His motif that identifies the sites of covalent attachment of *c*-type hemes. Midpoint reduction potentials were determined in two separate experiments by mediated redox potentiometry of the cbb_3 oxidase monitored by UV/vis spectroscopy (R. Pitcher unpublished data).

Recent experiments in our laboratory on the purified cytochrome cbb_3 from *P. stutzeri* revealed evidence for CO binding not only to the oxygen binding heme b_3 in the active site, but also the bis-histidine liganded *c*-type heme in CcoP [76]. The interaction of CO with purified cytochrome cbb_3 oxidase appears to be quite specific to CcoP at relatively low CO concentrations ($K_d = 2 \times 10^{-6}$ M) with no interference from heme b_3. When we examined the CO binding properties of the separately expressed diheme CcoP subunit of the *P. stutzeri* enzyme we observed that it too will bind CO stoichiometrically, with the same equilibrium constant, but only after reduction with dithionite. The fact that CO does not bind after reduction with ascorbate implies that the *c*-type heme with the lowest reduction potential, that with bis-histidine ligation, must be reduced in order to bind CO (Fig. 3).

The rate of recombination of CO to the reduced active site heme of HCOs (7×10^4 M^{-1} s^{-1}) [73] is significantly less than to myoglobin (5×10^5 M^{-1} s^{-1}) [72] due to the photolysed CO interacting with reduced Cu_B. The transient ligation of CO at another site is reflected in saturating recombination kinetics at concentrations above 10 mM in bovine mitochondrial C*c*O [77] and cytochrome bo_3 oxidase from *E. coli* [78]. The intermediate species, Cu_B–CO has been directly observed by low-temperature FTIR spectroscopy, and is diagnostic of the heme-copper dinuclear centre [79]. Consequently, cytochrome bo_3, which lacks Cu_B in the dinuclear centre, shows a rate of CO recombination to this form that is higher (k_{on} 2×10^5 M^{-1} s^{-1}) than copper sufficient enzyme and proportional to the concentration of CO over the range of 0–20 mM [78]. In the closely related bacterial NOR, the rate of CO recombination to the reduced active site heme is four orders of magnitude greater (1.7×10^8 M^{-1} s^{-1}) than that seen in other HCOs [74]. This is presumably because after photolysis, CO does not interact with non-heme iron that replaces Cu_B in the dinuclear centre of NOR [80].

The binding of CO to the reduced active site heme b_3 in cytochrome cbb_3 oxidase is appreciably weaker ($K_d = 3 \times 10^{-5}$ M) either than to the *c*-type heme in CcoP [76] or the dinuclear centre heme of other HCOs, in which the dissociation constant for CO binding is in

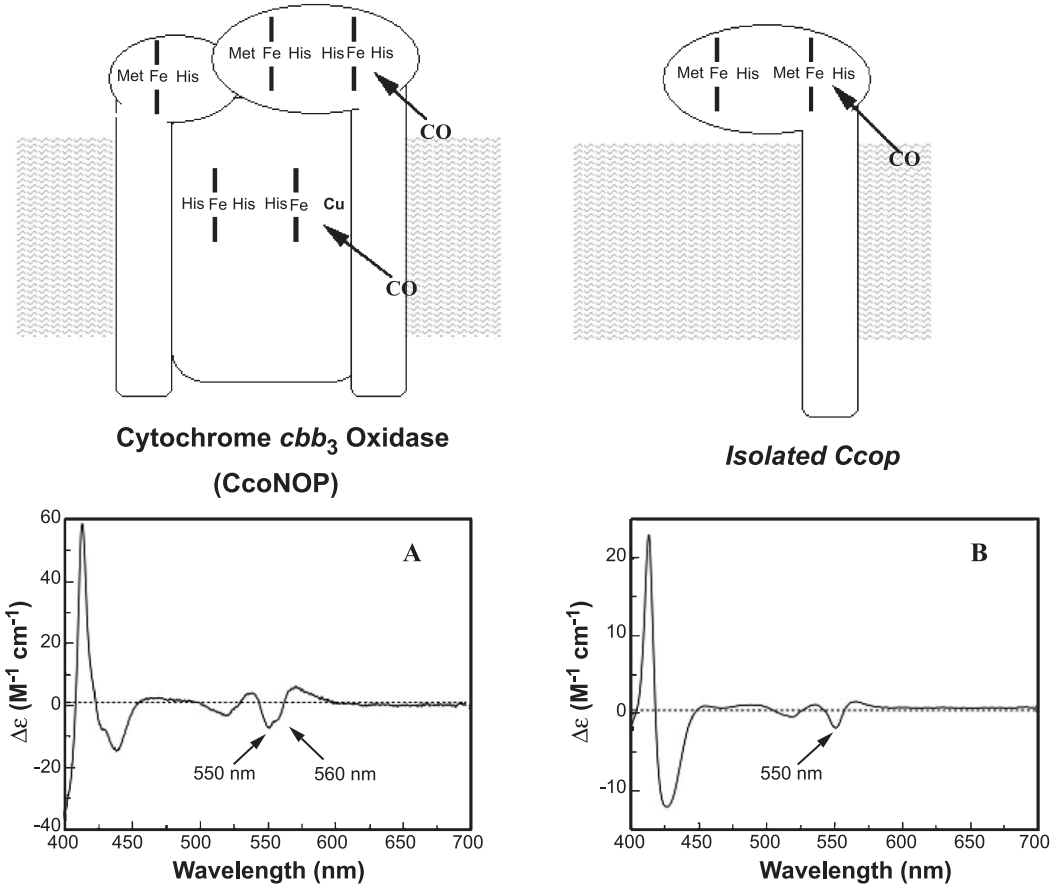

Fig. 3. Binding of CO to the reduced heme centres in cytochrome cbb_3 oxidase purified from *P. stutzeri*. In the isolated enzyme complex (CcoNOP) CO can bind to both ferrous heme b_3 in the reduced dinuclear centre and to the bis-histidine ligated *c*-type heme in CcoP giving rise to troughs in the static reduced-CO *minus* reduced difference spectrum at 560 and 550 nm, respectively (Panel A). In isolated CcoP, CO only binds to the ferrous bis-histidine ligated *c*-type heme giving rise to a single feature at 550 nm (Panel B).

the order of 1 μM [73,81,82]. Moreover, in cytochrome cbb_3 oxidase, the kinetics of CO recombination with the dinuclear centre after photolysis are quite different to those reported for all other HCOs. In the cbb_3-type oxidase from *P. stutzeri* CO recombination is rapid (k_{obs} = ca. 800 s^{-1}), but apparently independent of the CO concentration (between 0.025 and 1 mM) in the bulk phase [76]. Over the same concentration range the observed rate constants that describe CO binding to CcO [73] and cytochrome bo_3 [81] appears to be linearly dependent on CO concentration. However, if the range of CO concentrations examined is extended to >10 mM it is clear that this reaction shows saturation kinetics with the observed rate constant the rate of recombination being limited (k_{obs} = 190 s^{-1}) by the participation of the Cu(I)–CO intermediate (K_{eq} = 2.4 mM) in the ligand-binding process [78]. Hence it is possible that CO binding to reduced heme b_3 in cytochrome cbb_3 oxidase also exhibits saturation kinetics (k_{obs} = ca. 800 s^{-1}), but that the equilibrium constant for the Cu(I)–CO intermediate is significantly smaller.

These atypical CO recombination kinetics are also consistent with the recent time-resolved FTIR experiments that show, in contrast to other HCOs, that the transient decay of the Cu_B–CO complex in cytochrome cbb_3 oxidase of *P. stutzeri* is concurrent with the formation of the Fe(II)–CO complex [56]. What is clear is that these data argue for a very differently organized, perhaps more open, dinuclear centre in which gas exchange between Cu_B and heme b_3 is significantly enhanced. This may reflect an important determinant of the proposed high affinity for substrate of cbb_3-type oxidases.

4. Outstanding issues in cytochrome cbb_3 oxidase biochemistry

In spite of recent progress in characterising the cytochrome cbb_3 oxidase from *P. stutzeri* there are a number of outstanding issues concerning the biochemistry of this class of HCOs that urgently need to be addressed. The cytochrome cbb_3 oxidases are proposed, on the basis of the apparent K_m that has been measured for the enzyme from *B. japonicum* to be high affinity oxidases, which is certainly commensurate with their role in microaerobic metabolism. The kinetic trapping of O_2 as peroxide by HCOs has been described in terms of three rate constants: k_{et} (the rate of internal electron transfer), K_{eq} (the equilibrium constant for O_2 binding), and k_{in} (the rate of electron input). These can be used to determine an apparent K_m under turnover conditions [83]. It will be of considerable interest, given the unusual CO binding properties if such a model is valid for the cbb_3-type enzymes. Moreover, given the different routes and possibly the different rates, of electron input to the catalytic subunit via CcoO and CcoP it is conceivable that the apparent K_m for dioxygen might be varied according to the needs of the organism.

4.1. Can the reaction cycle of cytochrome cbb_3 oxidase employ radical based chemistry?

There is emerging evidence from spectroscopic and ligand-binding experiments for differences in the organisation of the dinuclear centre of cbb_3-type oxidases compared with other HCOs. Consequently, there is a need to address the issue of whether the reaction cycle of cytochrome cbb_3 oxidases is distinct from that of other HCOs. The product of the reaction of the two-electron reduced (mixed valence) form of these typical HCOs with dioxygen is an oxyferryl intermediate, P_M in which the substrate dioxygen molecule has been cleaved [84]. Alternatively, P_M is readily formed in the reaction of the fully oxidised (fast) enzyme in its reaction with H_2O_2 [85,86]. In each case, formation of P_M is associated with the oxidation of the covalently linked His/Tyr cross-linked structure in the active site [49,87,88]. Since this tyrosine residue is not conserved in cbb_3 oxidases, the only oxygen-reducing HCO in which this is the case, the question arises as to whether these oxidases can utilise radical chemistry in dioxygen reduction.

Although there are not yet any reports of an attempt to generate the P_M intermediate in the cbb_3-type oxidases, it is of interest to note that CcoN contains a conserved tyrosine, Tyr-260 (the numbering her corresponds to the *P. stutzeri* sequence), in close proximity to a Cu_B histidine ligand. In addition *P. stutzeri* CcoN also contains a conserved glycine residue (Gly-254) close to the dinuclear centre. A glycine residue is responsible for stabilising a radical in formate H-lyase [89]. It is also possible that a radical is generated on another amino acid, for example a tryptophan residue as in yeast cytochrome *c* peroxidase [90] or by the oxidation of the porphyrin macrocycle itself as is the case in catalase and horseradish peroxidase [91].

4.2. Do cytochrome cbb_3 oxidases represent a fundamentally different design of proton pump?

In the absence of either a three-dimensional structure of a cbb_3-type oxidase or experiments that inform on the roles of conserved residues in CcoN, it is not yet possible to conclude whether cytochrome cbb_3 oxidases have independently evolved a distinctive method for coupling oxygen reduction to proton translocation. Alternatively, the mechanism of energy transduction might be functionally conserved in the cbb_3-type oxidases, even though key residues of the D- and K-channels are apparently missing. This appears to be the case in both the caa_3-type oxidase of *R. marinus* [34] and the ba_3-type oxidase of *T. thermophilus* [92,93]. Moreover, the loss of activity in *P. denitrificans* cytochrome aa_3 that is associated with the substitution of a conserved glutamate (Glu-286), that lies at the top of the D-channel, with any residue other than aspartate can be compensated for by replacing a nearby phenylalanine residue with a tyrosine [94]. The effect of these multiple substitutions is to produce an enzyme with a D-channel that closely resembles that of

the cytochrome caa_3 oxidase from *R. marinus* and which functions both as an oxygen reductase and a proton pump.

However, there is a further difference between the cytochrome cbb_3 oxidases and all other HCOs, including the enzymes found in *R. marinus* and *T. thermophilus*, that might suggest that the mechanism of energy transduction evolved independently in cytochrome cbb_3. The cbb_3-type oxidases contain a *b*-type heme rather than an *a*-type or *o*-type heme in the dinuclear centre which is not only the site of oxygen reduction but is also proposed to be the site of proton-pumping in the aa_3-type oxidases. Analysis of the bacterial [47,92] and mitochondrial [46] C*c*O structures implicates the hydroxyl group of the heme a_3 hydroxyethylfarnesyl (hydroxyethylgeranylgeranyl in the case of cytochrome ba_3 oxidase of *T. thermophilus*) side chain in a pathway that may be involved in conducting protons to the dinuclear centre. The same hydroxyl group is also the favoured source of one of the protons involved in O—O bond splitting in a model based on hybrid density functional theory calculations [95]. In this respect, it is of interest that activity is completely abolished by the substitution of a *b*-type heme for the active site high-spin a_3 heme of *P. denitrificans* cytochrome ba_3 [96] or the active site heme o_3 of *E. coli* cytochrome bo_3 [4].

4.3. The role of the diheme CcoP in the cbb_3 oxidase complex

The requirement for the diheme subunit CcoP subunit in the cbb_3 oxidase complex is not entirely clear. In principle, it could simply provide an alternate route for electron transfer into the complex, but as already mentioned its presence is not essential for catalytic activity [13,53]. Our recent studies of the enzyme from *P. stutzeri* have yielded evidence that in the oxidized state one of the *c*-type hemes in this subunit is a six-coordinate (bis-histidine) species [41], that when reduced can bind CO [76]. Transient illumination of this His–Fe(II)–CO species, either in the cytochrome cbb_3 complex or in isolated recombinant CcoP, leads to photolysis of the Fe(II)–CO resulting in penta-coordinate species that can undergo competing reactions. It can either react with what we presume to be the endogenous histidine ligand to yield bis-histidine ligated ferrous heme or it can recombine with CO. The kinetics of this process are similar to those displayed by a recently characterised hexacoordinate rice nonsymbiotic hemoglobin [97].

Note that CO binding to a *c*-type cytochrome has also been reported in the *cbo*-type oxidase of *Methylobacillus flagellatus*, a member of the HCO superfamily that contains a *c*-type heme and heme O–Cu_B dinuclear centre [98], but it is probably not a general characteristic of this family of enzymes since there is no indication of CO ligation to the reduced cytochrome *c* of the cytochrome caa_3 oxidase of *Bacillus subtilis* [82].

A number of lines of circumstantial evidence suggest that CcoP might serve as a gas-sensing rather than electron-receiving domain. First of all, in purple photosynthetic bacteria, CcoQ has been proposed to transduce an unknown signal, possibly related to electron flow through cytochrome cbb_3 oxidase complex, and repress the expression of gene involved in photosynthesis [54,99,100]. Since CcoQ does not contain any redox active cofactors that could sense electron flow directly it would have to respond to a change in environment that was sensed elsewhere in the cytochrome cbb_3 oxidase complex. Secondly, the kinetics of CO-recombination displayed by some plant hemoglobins, which contain a six-coordinate, bis-histidine, heme and are involved in physiological stress responses such as hypoxia, are very similar to those we have recently reported for CcoP [76]. Finally, it has been suggested that in the absence of oxidative stress regulators like OxyR, SoxR or SoxS in *H. pylori* that cytochrome cbb_3, which is the only terminal oxidase in that organism, might both directly sense environmental changes encountered by this bacterium and transduce the signal. However, it remains to be demonstrated whether or not CcoP is able to bind dioxygen and fulfill this role.

5. Concluding remarks

It was clear even from the initial studies of the *fixNOQP* operon in *B. japonicum* that the cytochrome cbb_3 oxidases represented a distinctive class of HCO. The cbb_3-type oxidases are usually found in Proteobacteria that express these oxidases in response to microaerobic conditions to permit the colonisation of oxygen-limited environments. The identification of the *P. stutzeri* enzyme as a model system has allowed considerable progress in terms of the comparative biochemistry of the cytochrome cbb_3 oxidases [36,41,48,56,76]. This approach has already uncovered a wealth of new information that strongly suggests that the molecular architecture of the dinuclear centre of the cbb_3-type oxidases is quite different to that found in the structurally defined and intensely studied cytochrome aa_3 oxidases [56,76]. The consequences of these differences which may affect not only the affinity for dioxygen of the cbb_3-type oxidases, but also how they operate as proton pumps will only be fully understood when a high-resolution three-dimensional structure emerges. Currently only the *P. stutzeri* cytochrome cbb_3 oxidase has been reported to yield crystals, but these only diffract to a maximum resolution of 5 Å [48]. A refined structure is urgently needed if the mechanism and role(s) in bacterial anaerobic metabolism of this, thus far, rather neglected class of heme-copper oxidases is to be fully understood.

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