Molecular and Spectroscopic Analysis of the Cytochrome cbb₃ Oxidase from Pseudomonas stutzeri*

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Cytochrome cbb₃ oxidase, a member of the heme-copper oxidase superfamily, is characterized by its high affinity for oxygen while retaining the ability to pump protons. These attributes are central to its proposed role in the microaerobic metabolism of proteobacteria. We have completed the first detailed spectroscopic characterization of a cytochrome cbb₃ oxidase, the enzyme purified from Pseudomonas stutzeri. A combination of UV-visible and magnetic CD spectroscopies clearly identified four low-spin hemes and the high-spin heme of the active site. This heme complement is in good agreement with our analysis of the primary sequence of the ccoNOPQ operon and biochemical analysis of the complex. Near-IR magnetic CD spectroscopy revealed the unexpected presence of a low-spin bishistidine-coordinated c-type heme in the complex. This was shown to be one of two c-type hemes in the CcoP subunit by separately expressing the subunit in Escherichia coli. Separate expression of CcoP also allowed us to unambiguously assign each of the signals associated with low-spin ferric hemes present in the X-band EPR spectrum of the oxidized enzyme. This work both underpins future mechanistic studies on this distinctive class of bacterial oxidases and raises questions concerning the role of CcoP in electron delivery to the catalytic subunit.

The final step in the electron transport chain of mitochondria and aerobically respiring bacteria is the 4-electron reduction of dioxygen to water. This reaction is usually catalyzed by respiratory heme-copper oxidases, e.g. mitochondrial cytochrome c oxidase, integral membrane proteins that couple the free energy of oxygen reduction to the translocation of protons. Members of the superfamily of respiratory heme-copper oxidases are represented in all domains of life and are readily identified by a highly conserved catalytic subunit (subunit I) (1–3). The majority of heme-copper oxidases found in eubacteria fall into one of two classes according to their immediate electron donor. This can be a quinol, as in the case of cytochrome bo₃ from Escherichia coli, or a c-type cytochrome, e.g. cytochrome c₅₅₂, which is the electron donor for cytochrome aa₃ of Paracoccus denitrificans. Quinol oxidases lack the dinuclear copper center Cu₃, which is located in the hydrophilic domain of subunit II and which serves as the immediate electron acceptor in cytochrome c oxidases (4).

In recent years, a third highly diverged group of heme-copper oxidases, the cytochrome cbb₃ oxidases, have been described in proteobacteria (5). The cbb₃ oxidases are found only in proteobacteria and have been reported not only to have a very high affinity for oxygen (kₐ ≈ 7 nM) (6), but also to retain the ability to conserve the energy liberated from the oxygen reduction reaction (7–9). These properties give cbb₃ oxidases an essential role in the specialized energy metabolism that allows proteobacteria to colonize microaerobic environments. For instance, a cbb₃-type enzyme is the only energy-conserving terminal oxidase whose sequence is represented in the genomes of two important human pathogens, Helicobacter pylori (10) and Campylobacter jejuni (11). Moreover, expression of a cbb₃ oxidase is also necessary for symbiotic diazotrophs to fix dinitrogen. Here, the enzyme fulfills the dual roles of maintaining a very low oxygen tension, to protect the labile nitrogenase, and allowing aerobic respiration, to support the energetically demanding process of nitrogen fixation. Indeed, a cbb₃ oxidase was first identified in Bradyrhizobium japonicum and designated fixNOQP (ccoNOQP) because expression of this gene cluster is required to support symbiotic nitrogen fixation (12).

Subsequently, the fixNOQP (ccoNOQP) operon has been identified in other proteobacteria, where it is always close to a second gene cluster, fixGHIS (ccoGHIS), whose expression is required for the assembly of a functional cbb₃ oxidase (13). An analysis of the evolution of heme-copper oxidases based upon multiple sequence alignments of the catalytic subunits, represented in cbb₃ oxidases by CcoN (FixN), has recently been published (3). This suggests that the cbb₃ oxidases evolved independently of the functionally distinct, but structurally related bacterial nitric-oxide reductases (14) to fulfill a specialized role in microaerobic energy metabolism (5).

Cytochrome cbb₃ oxidases are predicted to contain four subunits, although in most preparations reported to date, there is only firm evidence for the presence of CcoN. Like subunit I in other heme-copper oxidases, CcoN has at least 12 transmembrane helices and contains the active site, a high-spin heme (heme b₃)¹ magnetically coupled to an adjacent copper ion.

¹To aid comparison with other heme-copper oxidases, we have adopted the following notation for the metal centers in CcoN, which is structurally related to cytochrome c oxidase subunit I. Heme b₃ is the low-spin bishistidine-coordinated heme in CcoN that is a homolog of heme a in cytochrome c oxidase. Heme b₃ is high spin in CcoN and equivalent to heme a₃ in cytochrome c oxidase and heme o₉ in E. coli cytochrome bo₃-quinol oxidase. In cytochrome c oxidase and quinol oxidases, this heme is magnetically coupled to a copper ion (Cu₃) to form a dinuclear center, which is the site of oxygen binding and reduction. Cu₃ is probably ligated by three conserved histidine residues, which also serve as ligands to Cu₃ in cytochrome c oxidase.

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known as CuII to form a dinuclear center (15). A second heme (heme b), which serves to transfer electrons to the active site, is also contained within subunit I. Six absolutely conserved histidine residues (in helices II, VI, VII, and X) involved in ligating both heme iron as well as CuII are diagnostic of the entire superfamily (16). Rather unusually, CcoN contains only heme B, which lacks the hydroxyethylfarnesyl substituent of the porphyrin macrocycle, found in hemes O and A, the cofactors that characterize the dinuclear centers of most classical heme-copper oxidases.

The x-ray structures of a number of heme-copper oxidases reported over the past 5 years have proved useful in interpreting many years of biophysical experiments on mitochondrial cytochrome c oxidase (17–20). Unfortunately, few of the structural features that characterize the active site of typical heme-copper oxidases are conserved in the primary amino acid sequence in CcoN. The most notable difference is found in cytochrome c oxidase (17–20), where, in cytochrome b, which lacks the hydroxyethylfarnesyl substituent of the porphyrin macrocycle, found in hemes O and A, the cofactors that characterize the dinuclear centers of most classical heme-copper oxidases.

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To amplify the ccoP gene, forward and reverse primers were designed that contained artificial NdeI and SacI sites, respectively, which facilitated cloning into the expression vector pET21a (Novagen) to yield a new construct, pCooPall. DNA sequencing (MWG-BIOTECH AG) using standard T7 promoter/terminator primers or internal primers confirmed that the insert was identical in sequence to wild-type CcoP.

**EXPERIMENTAL PROCEDURES**

**Recombinant DNA Methods**

Initially, the ccoNOQP operon of *P. stutzeri* was sequenced by amplifying short internal regions of the operon using degenerate primers based on multiple sequence alignments of the ccoNOQP operons of nine other proteobacterial species. All PCRs contained genomic DNA as template, 50 pmol of each oligonucleotide primer (Genosys Biotechnologies, Inc.), and Extensor Hi-Fidelity PCR Master Mix (Abgene) in a total volume of 50 μl. Reactions were initiated by 2 min at 94 °C, followed by 25 cycles of denaturation (30 s at 94 °C), annealing (30 s at 55 °C), and extension (1 min/1 kb at 72 °C). After the final cycle, completion of polymerization was ensured by an additional incubation at 72 °C for 3 min. The PCR products were sequenced using an automated sequencer (ABI Prism 377) at the School of Biological Sciences, University of East Anglia (Norwich, UK).

Using this initial sequence as a template, primers were designed, and overlapping genomic DNA-derived PCR fragments were cloned into either pUC18 or pBluescript KS + (Table I). DNA cloning techniques were performed according to standard protocols (25). The inserts were then sequenced by MWG-BIOTECH AG (Ebersberg, Germany) using standard m13/pUC forward and reverse primers or appropriate internal primers.

**Bacterial Strains and Growth Conditions**

A single colony was used to inoculate 50 ml of LB medium, and the flask was incubated overnight with agitation (200 rpm) at 37 °C. This culture was then used to inoculate 15 liters of LB medium, which was grown in a BioFlow IV fermentor (New Brunswick Ltd.) at 30 °C for 28 h with constant stirring (135 rpm). Cells were harvested at 4 °C by centrifugation at 10,000 × g for 20 min and resuspended in cold 20 mM Tris-HCl (pH 7.5) before being rapidly frozen in liquid nitrogen and stored at −80 °C until required.

A single colony of *E. coli* JM109 (DE3) cells transformed with pEC86 (26) and pCooPall was selected on t-agar, supplemented with ampicillin (100 μg/ml) and chloramphenicol (35 μg/ml), and used to inoculate 50 ml of LB medium containing both antibiotics. From this culture, which was incubated overnight with shaking (200 rpm) at 37 °C, 1 ml was taken to inoculate 700 ml of TYP medium (supplemented with 100 μg/ml ampicillin and 35 μg/ml chloramphenicol) in a 2-liter flask. Typically, six 700-ml cultures were grown for 30 h with agitation (200 rpm) at 30 °C, yielding ~50 g of cells (wet weight). The cells were harvested at 4 °C by centrifugation at 10,000 × g for 20 min and resuspended in cold 20 mM sodium phosphate (pH 7.5) before spectroscopic assignments of each of the five heme groups, it was necessary to separately express in *E. coli* the diheme-containing subunit CcoP.

**Table I**

| Strain or plasmid | Description | Ref. or source |
|-------------------|-------------|---------------|
| *E. coli* DH5α    | supE44 Δ(lacU169)ø(a80 lacZM15) recA1 endA1 gyrA96 thi-1 hsdR17 relA1 | Invitrogen |
| *E. coli* JM109 (DE3) | supE44 recA1 endA1 gyrA96 hsdR17 relA1 thi Δ(lac-proAB)λ(DE3) | Invitrogen |
| *P. stutzeri* ZoBell | (ATCC 14405) | Ref. 24 |
| **Plasmid**       |             |               |
| pUC18             | Cloning vector, Amp’ | Amersham Biosciences |
| pBluescript II KS + | Cloning vector, Amp’ | Stratagene |
| pET21a            | Expression vector with lac operator | Novagen |
| pET22b            | Expression vector with pelB | Novagen |
| pEC86             | Plasmid containing *cem* gene cluster | Ref. 26 |
| pCooPall          | pET21a containing ccoP gene from *P. stutzeri* | This work |
| pCooPsol          | pET22b containing soluble region of ccoP from *P. stutzeri* (missing 80 N-terminal residues) | This work |
| pCcoO             | pET21a containing entire *ccoO* gene from *P. stutzeri* | This work |
| pCcoNO            | pUC18 containing *ccoNO* genes and 135 bp upstream of *ccoN* | This work |
| pCco1             | pUC18 containing 1.9-kb fragment from *P. stutzeri* *ccoNO* operon | This work |
| pCco2             | pBluescript II KS + containing 1.5-kb fragment from *P. stutzeri* *ccoNO* operon | This work |

**Cytochrome cbb₃ from *P. stutzeri***

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being rapidly frozen in liquid nitrogen and stored at ~80°C until needed.

**Purification of Cytochrome cbb3 Oxidase**

Previously frozen *P. stutzeri* cells were thawed, and the following additions were made (final concentrations given): 2 mM MgCl₂, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 1 mM leupeptin, 1 mM pepstatin, and 5 µg/ml DNase. The cells were broken by a single passage through a French pressure cell (1200 p.s.i.). Approximately 15 min after the cells were disrupted, EDTA (5 mM final concentration) was added to the cell-free extract, which was then centrifuged at 10,000 × g for 20 min at 4°C to remove any unbroken cells. The resulting supernatant was centrifuged at 160,000 × g for 2 h at 4°C, and the pellet, which contained the cytoplasmic membranes, was resuspended in 40 ml of 20 mM Tris-HCl and 2.5 mM EDTA, and 0.08% (w/v) sodium deoxycholate (pH 7.5). To remove any peripheral membrane proteins, 100 ml of 20 mM Tris-HCl, 500 mM NaCl, 2.5 mM EDTA, and 0.08% (w/v) sodium deoxycholate (pH 7.5) was added dropwise with stirring. After stirring on ice for an additional 5 min, the washed membrane fraction was sedimented by centrifugation at 125,000 × g for 90 min at 4°C. Solubilization of the integral membrane proteins and subsequent purification were essentially as described by Urbani et al. (24). Purified cytochrome *cbb₃* oxidase was analyzed by SDS-PAGE and both UV-visible and EPR spectroscopies prior to storage at ~80°C until needed.

**Purification of CcOP**

Previously frozen *E. coli* cells containing heterologously expressed CcOP were thawed, and the following additions were made (final concentrations given): 2 mM MgCl₂, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 1 µM leupeptin, 1 µM pepstatin, and 5 µg/ml DNase. The cells were broken by a single passage through a French pressure cell (1200 p.s.i.). Approximately 15 min after breaking, EDTA (5 mM final concentration) was added to the cell-free extract, which was then centrifuged at 10,000 × g for 20 min at 4°C to remove any unbroken cells. The resulting supernatant was centrifuged at 31,000 × g for 4 h at 4°C. The pellet (which was enriched in cytoplasmic membranes containing CcOP) was resuspended in 20 mM sodium phosphate and 50 mM EDTA (pH 7.5), mixed with an equal volume of 10 mM urea to remove any peripheral membrane proteins, and centrifuged at 100,000 × g for 90 min at 4°C. The cytoplasmic membranes were washed with ~200 ml of 20 mM sodium phosphate and 50 mM EDTA (pH 7.5), followed by centrifugation at 100,000 × g for 90 min at 4°C.

The total amount of protein in the washed membranes was estimated by the BCA method (Pierce) using bovine serum albumin (1 mg/ml) as a standard. Solubilization of the membrane proteins was accomplished by stirring a suspension of the cytoplasmic membranes for 30 min at 4°C in 20 mM sodium phosphate and 50 mM EDTA (pH 7.5), to which dodecyl-β-D-maltoside (DM)² had been added (2.5 g of detergent/g total protein). The extracted membranes were sedimented at 160,000 × g for 60 min, and the supernatant (which contained CcOP) was loaded onto a pre-equilibrated 75-ml DEAE fast flow column (2.6 cm × 100 cm). The column was washed with 150 ml of 20 mM sodium phosphate, 50 mM EDTA, and 0.02% (w/v) DM (pH 7.5) before eluting the bound CcOP with a stepwise salt gradient formed in the same buffer (140–260 mM NaCl in 20 mM increments). At each step in the gradient, the column was washed with 2 column volumes of buffer containing the appropriate salt concentration. After analysis by SDS-PAGE and both UV-visible and EPR spectroscopies, purified CcOP was stored at ~80°C until needed.

**Analytical Methods**

**Gel Electrophoresis**—Protein was analyzed on 15%-SDS-polyacrylamide gels essentially as described by Laemmli (27). Gels were stained to indicate the presence of c-type cytochromes (28) or with Coomassie Brilliant Blue R-250. The following buffer was used for sample preparation irrespective of the method of protein visualization: 6% urea, 5% (w/v) SDS, 0.1% (w/v) glycerol, and 0.05% (w/v) bromphenol blue. Samples were incubated at 50°C for 5 min directly before loading onto the gels.

**Mass Spectrometry**—Masses of the individual subunits were determined in protein samples (~5 µM) using a surface-enhanced laser desorption/ionization (SELDI) mass spectrometer (Ciphergen) with H₄ hydrophobic surface chips according to the manufacturer's protocol. Calibrations using horse heart myoglobin and horseradish peroxidase were done prior to each set of experiments, and the masses obtained are considered to be accurate to within 0.2%.

**Oxygen Uptake Measurements**—The standard reaction medium (10 ml) consisted of air-saturated 20 mM sodium phosphate, 50 µM EDTA, and 0.02% DM (pH 7.5). Oxygen consumption was measured polarographically at 25°C with a Clark-type electrode (EDT Instruments, Kent, UK) using 1 mM sodium ascorbate and 0.1 mM TMDP as the artificial electron donors.

**Heme Quantification**—The amount of heme was determined by the pyridine hemochromogen method using an inverse matrix for simultaneous determination of concentrations of hemes A, B, and C as described by Berry and Trumppower (29).

**Spectroscopy**—UV-visible electronic absorption spectra were recorded on a Hitachi U3100 spectrophotometer. Because cytochrome *cbb₃* oxidase has a very high affinity for oxygen, totally anaerobic conditions are essential for spectroscopic work that requires the analysis of either partly or fully reduced enzyme. These conditions were obtained by allowing protein samples (prepared in a 3.5-ml five-sided cuvette (Hellma)) to stir overnight in an anaerobic (<1 ppm O₂) cabinet (Belle Technology, Portesham, UK). The next morning, the magnetic follower was removed, and the cuvette was made light-tight by fitting rubber suba-seal covered by Parafilm. All subsequent additions were made using gas-tight syringes. β-D-Glucose was added to a final concentration of 10 mM, followed by glucose oxidase/catalase (2 and 25 units/ml, respectively). The contents of the cuvette were mixed by inversion and left for ~30 min to allow for the removal of any traces of oxygen. Reduction of cytochrome *cbb₃* oxidase was achieved by the addition of small aliquots of either dithionite (~50 µM final concentration) or 2 mM NADH plus 0.1 µM phenazine methosulfate (respective final concentrations).

EPR spectra were recorded using an ER-200D X-band spectrometer (Spectrospin, Bruker) equipped with a liquid helium flow cryostat (ESR-9, Oxford Instruments) and interfaced to an ESP1600 computer. Spectra at 9.66 GHz were collected using 2.0-milliwatt (mW) microwave power with a modulation frequency and amplitude of 100 kHz and 1 millitesla, respectively.

**RESULTS**

**DNA Sequence of the P. stutzeri ccoNOPQ Operon**

The first requirement of this study was to obtain an authentic DNA sequence of the *ccoVOQP* operon from *P. stutzeri*. The derived amino acid sequence was expected to inform as to the number of c-type hemes in the complex and their potential hydrophobic surface chips according to the manufacturer's protocol. To this end, a series of degenerate primers based on multiple sequence conserved heme ligands in the catalytic subunit (CcoN). To this end, a series of degenerate primers based on multiple sequence conserved heme ligands in the catalytic subunit (CcoN). The PCR product that we obtained clearly contained both the 3'-end of *ccoP* and some 300 bp of *ccoG*.
The sequence of the 5' end of the ccoNOQP operon was obtained by taking account of the similar organization of the upstream region in both P. stutzeri and P. aeruginosa (Fig. 1) (30, 31). The aerotaxis receptor gene (aer) is located upstream of ccoNOQP in P. aeruginosa. A degenerate primer based on a multiple sequence alignment of four aer sequences was designed, which allowed amplification of both the 5' end of ccoN and a 100 bp region immediately upstream. The size of the PCR product and the sequence data (Fig. 1) suggest that unlike P. aeruginosa, the ccoNOQP operon in P. stutzeri is not tandemly repeated.

In total, a continuous region of 3579 bp was sequenced (data not shown). This region spans four open reading frames in close proximity (ccoN, ccoO, ccoQ, and ccoP) that together account for 3155 bp of sequence. The amino termini of CcoN, CcoO, and CcoP, respectively, that diagnose the covalent binding of heme to the enzyme complex after purification. The discrepancy between the apparent molecular mass of CcoN determined by SDS-PAGE and that predicted from its DNA sequence is probably caused by the absence of the signal transduction domain.

Multiple sequence alignments indicated the six conserved histidine residues in CcoN responsible for ligating heme a (His460 and His484), heme b (His278 and His363), and CcoO (His278 and His305). There are one and two conserved CXXCH motifs in CcoN and CcoO, respectively, that diagnose the covalent binding of c-type hemes (Fig. 2). It was originally assumed that all three c-type hemes would have a methionine as a second axial ligand to the heme iron (12). However, the emergence of more ccoNOQP sequences from diverse proteobacteria has made it apparent that CcoO and CcoP also contain a single conserved histidine residue that in principle could also fulfill this role (Fig. 2).
features in the visible region between 530 and 550 nm (Fig. 3). In all preparations that we have studied so far, there is an additional weaker feature centered at 645 nm. This was assigned to one of a pair of ligand-to-metal charge-transfer bands of the high-spin ferric heme b3 (see below and Refs. 34 and 35). The intensity of this feature varied somewhat between preparations, probably due to some degree of heterogeneity in the dinuclear center, which is a phenomenon well described in other heme-copper oxidases (36). Upon complete reduction of the anaerobic enzyme with excess dithionite, the Soret peak shifted to 417 nm with a distinct shoulder at 420 nm. Also two features intensified in the visible region at 551 and 521 nm with distinct shoulders at 559 and 528 nm, respectively. The peaks at 417, 521, and 551 nm are characteristic of ferrous c-type hemes, whereas the shoulders at 420, 528, and 559 nm are indicative of ferrous b-type hemes.

RT-MCD Spectroscopy—The UV-visible MCD spectrum informs as to the number and spin state of the ferric hemes present in an enzyme, whereas the NIR-MCD spectrum diagnoses the axial ligands of low-spin ferric hemes. The UV-visible region of the RT-MCD spectrum of fully oxidized cytochrome cbb₃ oxidase as isolated is dominated by a derivative-shaped feature in the Soret region of the spectrum (Fig. 4). The form and intensity of this feature are consistent with the presence of four low-spin ferric hemes, as is the intensity of the trough at 569 nm. We suppose these features

![Fig. 2. Multiple sequence alignments of CcoO and CcoP.](image-url)
arise from the three c-type hemes in CcoO and CcoP and the magnetically isolated low-spin ferric heme b in CcoN.

The relatively weak contribution of a single high-spin ferric heme, arising from the active-site heme b3 of CcoN, to the UV-visible MCD spectrum would be difficult to see against a background of four low-spin ferric hemes. However, we have previously described a feature in the MCD spectrum of other heme-copper oxidases that corresponds to the ligand-to-metal charge-transfer band seen in the UV-visible electronic absorption spectrum. This feature, known as CT2, is one of a pair of ligand-to-metal charge-transfer bands that indicate the presence of high-spin ferric b- or o-type hemes at the active sites of cytochrome bo3 (34, 35) and bacterial nitric-oxide reductase (37). The energy and intensity of CT2 depend on the nature of the distal (exogenous) ligand bound to the heme (35, 37). A feature corresponding to CT2 is clearly resolved in the RT-MCD spectrum of cytochrome cbb3 oxidase (Fig. 4A) and has a minimum at ~635 nm that we take to indicate the presence of water as the sixth ligand.

Thus, the UV-visible MCD spectroscopic analysis of the oxidized enzyme complex is consistent with the presence of five hemes, four low-spin and one high-spin, which is in good agreement both with our analysis of the derived amino acid sequence of the ccoNOPQ operon and the analysis of the heme content of the purified enzyme. To obtain information about the axial ligands of the four low-spin hemes, we recorded the NIR-MCD spectrum of oxidized cytochrome cbb3 oxidase at room temperature. The spectrum contains two prominent, but overlapping features with peaks at 1580 and 1790 nm (Fig. 4B), characteristic of the porphyrin-to-ferric charge-transfer transitions of low-spin ferric hemes. The energies of such transitions report the heme axial ligands (38, 39). In the case of oxidized cytochrome cbb3, the maxima at 1580 and 1790 nm arise from low-spin ferric hemes that have bishistidine (His/His) and His/Met axial ligands, respectively. This confirms the presence of both His/His-coordinated hemes (heme b) and His/Met (c-type hemes) as predicted by our analysis of the derived amino acid sequence. However, the relative intensities of the two features indicate the presence of His/His- and His/Met-coordinated low-spin ferric hemes at a ratio of 2:2, and not 1:3 as anticipated. Therefore, of the three low-spin c-type hemes present in CcoO and CcoP, rather unexpectedly, one must have His/His axial ligation.

X-band EPR Spectroscopy—Having established both the number of hemes in the cytochrome cbb3 complex and axial ligands of the four low-spin hemes, we wished to apply this information to assign the signals in the X-band EPR spectrum of fully oxidized cytochrome cbb3 oxidase (Fig. 5). In fact, this

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**Fig. 3.** UV-visible spectra of P. stutzeri cytochrome cbb3 oxidase. The enzyme concentration was 2.5 mM in 20 mM sodium phosphate, 50 mM EDTA, and 0.02% (w/v) DM (pH 7.5). The spectra were recorded at room temperature. The solid trace represents the air-oxidized enzyme as purified. The dashed trace depicts the spectrum of the fully reduced enzyme after the addition of excess dithionite. The inset shows a close-up of the α- and β-regions.

**Fig. 4.** RT-MCD spectrum of oxidized cytochrome cbb3 oxidase from P. stutzeri. The enzyme concentration was 130 μM in 20 mM HEPES, 50 mM EDTA, and 0.02% (w/v) DM (pH 7.5). The spectrum was recorded at room temperature.
The complexity of the EPR signals present in the X-band spectrum of cytochrome cbb3 oxidase complex made assigning any given signal to a particular heme very difficult. In particular, we wished to identify the EPR signature of the His/His-ligated c-type hemes that we had identified in our observation that the X-band EPR spectrum of one preparation of cytochrome cbb3 contained a much larger high-spin ferric heme signal (data not shown). Oxygen uptake assays done on the same sample revealed it to have one-third of the catalytic activity of the other samples. These results suggest a failure to incorporate Cuu into the catalytic heme subunit of all heme-copper oxidases examined and for the subunit to be weakly coupled to the nearby Cu(II) ion (40). However, a signal at $g = 6.02$ must arise from a small amount of uncoupled high-spin ferric heme b3. This can be explained in terms of a small proportion of the preparation being damaged or lacking CuII, something that we have previously observed for cytochrome bo$_3$ from E. coli (41). This view is consistent with our observation that the X-band EPR spectrum of one preparation of cytochrome cbb3 contained a much larger high-spin ferric heme signal (data not shown). Oxygen uptake assays done on the same sample revealed it to have one-third of the catalytic activity of the other samples. These results suggest a failure to incorporate Cuu into the catalytic sample, which would account for both the magnetically isolated high-spin heme and the low activity. Signals at $g = 4.3$ and 2.06 represent minor levels of adventitious Fe(III) and Cu(II), respectively.

The spectra in the X-band region are rather complex and clearly contain signals that arise from both high- and low-spin ferric hemes. By analogy to other heme-copper oxidases, the high-spin ferric heme in the dinuclear center (heme b3) is expected to be EPR-silent due to its being weakly coupled to the nearby Cu(II) ion (40). However, a signal at $g = 6.02$ must arise from a small amount of uncoupled high-spin ferric heme b3. This can be explained in terms of a small proportion of the preparation being damaged or lacking CuII, something that we have previously observed for cytochrome bo$_3$ from E. coli (41). This view is consistent with our observation that the X-band EPR spectrum of one preparation of cytochrome cbb3 contained a much larger high-spin ferric heme signal (data not shown). Oxygen uptake assays done on the same sample revealed it to have one-third of the catalytic activity of the other samples. These results suggest a failure to incorporate Cuu into the catalytic sample, which would account for both the magnetically isolated high-spin heme and the low activity. Signals at $g = 4.3$ and 2.06 represent minor levels of adventitious Fe(III) and Cu(II), respectively.

The features at $g = 2.99, 2.24$, and 1.53 are immediately recognizable as the spectrum of a bishistidine-ligated heme, in which the ligand planes are oriented approximately parallel. This is typical for the isolated low-spin heme in the major subunit of all heme-copper oxidases examined and for the equivalent heme in nitric-oxide reductase. This was therefore assigned to the low-spin heme of CooN. There are clearly at least two additional features overlapping with the $g = 2.99$ peak (indicated by arrows in Fig. 5), which are undoubtedly the $g_5$ signals of two of the other low-spin hemes identified in the X-band EPR spectra under similar conditions.

The only contaminant present in the membrane fraction that contributed to the UV-visible spectrum was cytochrome bd oxidase. Cytochrome bd is readily identified in the reduced spectrum by peaks at 560 and 635 nm. Initially, the presence of cytochrome bd in the membranes was problematic because it eluted from the anion-exchange column at $\sim 195$ mN NaCl, just before CooP, which eluted at $\sim 220$ mN NaCl. Consequently, further purification of CooP sometimes required rechromatography on a second anion-exchange column with the same shallow stepwise gradient as the first. CooP obtained by this method, although often partially reduced, was pure as judged by SDS-PAGE analysis (Fig. 6).

Analysis of the heme content of CooP using the pyridine hemochromogen method confirmed the presence of two c-type hemes and established a molar extinction coefficient for oxidized CooP: $e_{405} = 2.7 \times 10^{5}$ M$^{-1}$ cm$^{-1}$. Analysis of the samples used for EPR spectroscopy by the pyridine hemochromogen method was used to confirm that no spectroscopic contaminants (in particular, cytochrome bd), were present.

Spectroscopic Analysis of Recombinant CooP

UV-visible Spectroscopy—The electronic absorption spectrum of the fully oxidized CooP subunit contains a Soret maximum at 408 nm and two broad, but resolved peaks in the
visible region (Fig. 7). There was no feature beyond 551 nm that would indicate the presence of a high-spin ferric heme. Partial reduction of CcoP with ascorbate resulted in a shift in the Soret peak to 414 nm and a decrease in its intensity. It also led to a change in the visible region with the appearance of highly resolved bands at 521 and 551 nm, whose intensity indicated that one of the two c-type cytochromes had become reduced. Subsequent complete reduction with excess dithionite caused the Soret peak to intensify and to move to 416 nm. It also caused an increase in the intensity of the features in the visible region at 521 and 551 nm, consistent with the reduction of a second c-type cytochrome.

**X-band EPR Spectroscopy**—The X-band EPR spectrum of oxidized CcoP contains signals arising from two low-spin hemes (Fig. 8). The features at $g_z = 2.98$, 2.25, and 1.53 are due to a low-spin ferric heme with rhombic symmetry. However, a broader signal is clearly resolved as a shoulder on the left of $g_z = 2.98$. When the microwave power was increased from 2 to 20 mW, the $g_z$ feature could be clearly resolved into two signals ($g = 3.19$ and 2.98) corresponding to two different hemes. The ability to separate them in this manner is a consequence of different spin-relaxation states of the two hemes. The feature at $g_z = 2.47$ arises from a heme with His/OH ligation. When the sample was incompletely reduced with ascorbate, $g_z = 2.98$ remained. When the sample was then fully reduced with excess dithionite, this feature disappeared (data not shown).

**NIR-MCD Spectroscopy**—Our EPR measurements indicated that of the two low-spin c-type hemes in CcoP, only one was readily reduced by NADH in the presence of the mediator phenazine methosulfate (reduction potential ($E_m$) ~ +80 mV) (Fig. 8). This implies considerable spacing of the reduction potential of the two hemes. Typically, His/Met-ligated c-type hemes have a midpoint reduction potential of over +150 mV and are readily reduced by ascorbate. This suggested that the second heme must have a reduction potential of <50 mV, a value that would be commensurate with His/His ligation. Confirmation of this came from the NIR-MCD spectrum of recombinant CcoP (data not shown). This reveals the presence of both His/Met- and His/His-coordinated low-spin hemes. Consequently, we can be certain that a His/His-ligated c-type heme is associated with CcoP.

**DISCUSSION**

Since they were first recognized as a novel and distinctive class of heme-copper oxidases in *B. japonicum*, cytochrome cbb$_3$ oxidases have been purified from several species of proteobacteria, including *P. denitrificans* (7), *R. sphaeroides* (48), *R. capsulatus* (49), *B. japonicum* (6), and *P. stutzeri* (24). Rather poor yields and a tendency for the complex to dissociate have made
detailed biochemical characterization difficult, and a structure is not yet available, although crystallization conditions have been reported (24). However, there is evidence that cytochrome cbb₃ oxidases are characterized by an unusually high affinity for oxygen (kₐₖ ~ 7 nM) (6) combined with an ability to pump protons (9). The present study, using the purified enzyme from P. stutzeri, represents the first detailed spectroscopic characterization of this little understood class of heme-copper oxidases and provides some interesting insights into the organization of the enzyme complex as well as raises some questions concerning the role of CcoP.

To ensure that the enzyme from P. stutzeri really represents a prototypic cytochrome cbb₃ oxidase, we sequenced the gene cluster (ccoNOPQ) that encodes the four structural genes together with the immediate flanking regions of the genome. The derived amino acid sequence of CcoNOP is ~80% identical to that of P. aeruginosa enzyme, an observation that suggests that the P. stutzeri enzyme is indeed a typical cytochrome cbb₃ oxidase. The hydrodynamic properties of the purified P. stutzeri cytochrome cbb₃ oxidase, prepared in identical fashion to the enzyme used for the spectroscopic studies described here, have recently been reported (24). Analytical centrifugation combined with mass spectroscopy showed that each complex contained CcoN, CcoP, and CcoO at a ratio of 1:1:1. Biochemical analysis of the enzyme purified for this work is consistent with these findings. Moreover, comparison of the experimentally determined N termini (24) and molecular masses (this study) of CcoN, CcoO, and CcoP is in good agreement with the derived amino acid sequences reported here. These derived amino acid sequences also indicated the presence of three c-type hemes; in common with other cytochrome cbb₃ oxidases, the enzyme isolated from P. stutzeri contains two membrane-anchored subunits that contain c-type cytochromes. These are the monoheme CcoO (23 kDa) and the diheme CcoP (35 kDa), one or both of which may serve to transfer electrons derived from the bc₁ complex to the catalytic subunit. Despite the presence of a single heme c-binding site, CcoO exhibits minimal similarity to known c-type cytochromes (50), with the notable exception of NorC, a membrane-anchored cytochrome c subunit of nitric-oxide reductase (51). Equilibrium redox titrations (49) have identified two electrochemically distinct c-type cytochromes with Eₚₚ = -265 and +320 mV and Eₚₚ = +385 mV for heme b. Further titrations of a membrane fraction derived from a mutant (M7) that incorporates only CcoO into the enzyme complex allowed the assignment of the +320-mV reduction potential to the c-type heme in this subunit. Mutagenesis studies on the assembly and function of the individual subunits are also consistent with these assignments (23). These results led to the proposal that electrons are passed from the electron donor to heme b via CcoO and CcoC in this order, although the requirement for such a degenerate electron transfer chain is far from clear.

The cytochrome cbb₃ oxidase operon includes a fourth gene, ccoQ, which is predicted to encode a small membrane-bound polypeptide. The presence of CcoQ in a purified complex has so far been demonstrated immunologically only in B. japonicum (52), and its function remains unclear. In-frame deletion mutants of ccoQ constructed in B. japonicum (23) and R. sphaeroides (53) have no apparent effect upon the assembly or activity of cytochrome cbb₃ oxidase, although the amount of cytochrome c appears to be somewhat decreased in the ΔccoQ mutant of B. japonicum (23). 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 cytochrome cbb₃ 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. sphaeroides and R. capsulatus, but that are not present in non-photosynthetic species (53, 54).

The combination of UV-visible and RT-MCD spectroscopies confirmed the presence of five different hemes in the oxidized enzyme. Of these, four are low-spin species, and the fifth is the high-spin heme b₃ in the active site. Because there is no evidence of a significant amount of either high-spin heme b₃ or Cu₉ in the X-band EPR spectrum of the oxidized enzyme, we conclude that these two species must be magnetically coupled to form an EPR-silent species. Even at higher microwave powers and lower temperatures (4 K), we could find no evidence of the kind of broad EPR features that diagnose weak coupling between heme o₃ and Cu₉ in the dinuclear center of E. coli cytochrome bo₃ (40, 55).

An additional conclusion from our spectroscopic analysis is that all three c-type hemes must be low-spin six-coordinate species. This in itself was not surprising, except that the NIR-MCD spectroscopic data indicated that one of these hemes must have His/His coordination. Isolation of heterologously expressed CcoP confirmed that the His/His-ligated c-type heme is associated with this subunit rather than CcoO, which, as noted earlier, is a distant relative of NorC. Further evidence for the His/His-ligated heme comes from the X-band EPR and UV-visible electronic absorption spectra of CcoP after partial reduction with ascorbate (Eₚₚ = +50 mV). In general, His/His-ligated c-type hemes have reduction potentials that are ~200 mV lower than those of His/Met-ligated species due to the relative stabilization of the ferrous state (50).

Thus far, there are only two published EPR spectra of cytochrome cbb₃ oxidases in the literature, of preparations of the enzyme from R. capsulatus (49) and R. sphaeroides (48), both of which were recorded to seek evidence of a Cu₉ site. Although rather dilute, both samples generated spectra clearly demonstrating the absence of a dinuclear Cu₉ center and also indicating a coupled active site along with the presence of low-spin ferric heme. The X-band EPR spectrum of oxidized cytochrome cbb₃ oxidase in this report is rather more complex, with signals arising from at least four low-spin ferric hemes. To interpret this spectrum, we must consider also the MCD data and the X-band EPR spectrum of CcoP. Together, these data provide three important pieces of information. First, the large ∆θₐₚ signal in the EPR spectrum of the complex, which resembles a similar signal in the structurally related NorC, almost certainly arises from the single His/Met-coordinated c-type heme in CcoO. Second, the intense well resolved signal that dominates the complex rhombic trio (gₑ = 2.99, gₓ = 2.23, and gₒ = 1.50) is absent from the EPR spectrum of CcoP and was consequently assigned to heme b of CcoO. Finally, the two other contributors to this rhombic trio (gₑ = 3.19 and 2.98) that appear as shoulders on the main heme b signal are both present in the EPR spectrum of CcoP. The gₒ = 3.19 signal disappeared upon reduction with ascorbate and was consequently assigned to the His/Met-ligated species, leaving the gₒ = 2.98 component, which was assigned to the His/His-ligated c-type heme.

It is well established that oxidases, which terminate aerobic respiratory chains, accept relatively high potential electrons from soluble electron donors or directly from quinols. For example, Cu₉, which serves as the immediate electron acceptor for c-type cytochromes in aa₃-type oxidases, has a reduction potential of approximately +240 mV (56). In bacterial nitric-oxide reductase, a close relative of the heme-copper oxidases, the role of electron acceptor is fulfilled by NorC, a membrane-bound protein containing a single c-type heme (Eₚₚ = +310 mV) (43). Consequently, the question arises as to why cytochrome...
Cytochrome cbb₃ from P. stutzeri

Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

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