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The roles of *Rhodobacter sphaeroides* copper chaperones PCu_AC and Sco (PrrC) in the assembly of the copper centers of the aa_3-type and the cbb_3-type cytochrome *c* oxidases ☆

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ABSTRACT

The α proteobacter *Rhodobacter sphaeroides* accumulates two cytochrome *c* oxidases (CcO) in its cytoplasmic membrane during aerobic growth: a mitochondrial-like aa_3-type CcO containing a di-copper Cu_A center and mono-copper Cu_B, plus a cbb_3-type CcO that contains Cu_B but lacks Cu_A. Three copper chaperones are located in the periplasm of *R. sphaeroides*, PCu_AC, PrrC (Sco) and Cox11. Cox11 is required to assemble Cu_B of the aa_3-type but not the cbb_3-type CcO. PrrC is homologous to mitochondrial Sco1; Sco proteins are implicated in Cu_A assembly in mitochondria and bacteria, and with Cu_B assembly of the cbb_3-type CcO. PCu_AC is present in many bacteria, but not mitochondria. PCu_AC of *Thermus thermophilus* metallates a Cu_A center *in vitro*, but its *in vivo* function has not been explored. Here, the extent of copper center assembly in the aa_3- and cbb_3-type CcOs of *R. sphaeroides* has been examined in strains lacking PCu_AC, PrrC, or both. The absence of either chaperone strongly lowers the accumulation of both CcOs in the cells grown in low concentrations of Cu^{2+}. The absence of PrrC has a greater effect than the absence of PCu_AC and PCu_AC appears to function upstream of PrrC. Analysis of purified aa_3-type CcO shows that PrrC has a greater effect on the assembly of its Cu_A than does PCu_AC, and both chaperones have a lesser but significant effect on the assembly of its Cu_B even though Cox11 is present. Scenarios for the cellular roles of PCu_AC and PrrC are considered. The results are most consistent with a role for PrrC in the capture and delivery of copper to Cu_A of the aa_3-type CcO and to Cu_B of the cbb_3-type CcO, while the predominant role of PCu_AC may be to capture and deliver copper to PrrC and Cox11. This article is part of a Special Issue entitled: Biogenesis/Assembly of Respiratory Enzyme Complexes.

1. Introduction

During aerobic growth in the laboratory the α proteobacter *Rhodobacter sphaeroides* accumulates two cytochrome *c* oxidases (CcOs) in the cytoplasmic membrane. One is an aa_3-type CcO with high similarity to mitochondrial CcO [1–5] while the other is a cbb_3-type CcO, evolutionarily distant from the aa_3-type CcO [6,7]. The aa_3-type CcO contains two heme A and two copper centers [4,5,8]. The di-copper Cu_A center in subunit II accepts electrons from cytochrome *c* and transfers them to low-spin heme *a* in subunit I. In *R. sphaeroides* and other α proteobacteria, both soluble cytochromes *c* as well as membrane-anchored cytochrome c_y transfer electrons to Cu_A[9–14]. The two coppers of Cu_A are bound by two copper-bridging cysteines, two histidines, one methionine and a backbone carbonyl group. From heme *a*, electrons flow to the buried heme a_3-Cu_B site in subunit I, where O_2 is reduced to water [15]. The mono-copper Cu_B center, ubiquitous and structurally conserved in the heme-Cu oxidase superfamily, is composed of three histidines that bind the single copper near the five-coordinate iron of the heme of the active site [4,5,16]. Cbb_3-type CcOs, widespread in proteobacteria, contain a subunit I with metal centers similar to all members of the heme-Cu oxidase superfamily, including an O_2 reduction site composed of five-coordinate heme b_3 plus Cu_B[6,17,18]. However, the cbb_3-type CcO lacks Cu_A[6]. Instead of a subunit II like that of the aa_3-type CcO, the cbb_3-type CcO contains two subunits with extra-membrane domains that bind *c*-type cytochromes and extend into the periplasm [17,19]. These two cytochrome *c* subunits function to

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; CcO, cytochrome *c* oxidase; ICP-OES, inductively coupled plasma optical emission spectroscopy; TMPD, N,N,N′,N′-tetramethyl-*p*-phenylenediamine

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accept electrons from soluble cytochromes *c* or from membrane-bound cytochrome c_y[20].

R. sphaeroides also contains several CcO-specific assembly proteins with homologs in other bacteria and/or mitochondria. Cox10 and Cox15 participate in the synthesis of heme A, and possibly its insertion, in bacteria and mitochondria [21,22]. Surf1 enhances the insertion of the active site heme in bacteria and mitochondria [22–24]. Proteins encoded by the ccoGHIS operon, including the copper transporter CcoI, have been implicated in the assembly of the cbb_3-type CcO in other α proteobacters [25–28]. The roles of three periplasmic copper chaperones – Sco, Cox11 and PCu_AC – are discussed below.

The Sco protein copper chaperones are present in mitochondria and widespread in bacterial species. Sco proteins are anchored in the membrane by a single transmembrane helix while a thioredoxin-like extramembrane domain extends into the inter-membrane space or the bacterial periplasm to bind a single copper, using two cysteines and one histidine [29,30]. Human and yeast mitochondria contain two Sco proteins [30–32]. Both Sco1 and Sco2 have been implicated in the assembly of Cu_A in mitochondria on the basis of CcO deficiency when one or both Sco proteins are deleted or mutated, plus demonstrations of interactions between Sco1, Sco2 and subunit II of CcO [30,33–35]. Bacterial Sco proteins have also been implicated in the assembly of Cu_A. The deletion of Sco from *B. subtilus* strongly reduces the expression of a caa_3-type CcO containing Cu_A but not the expression of a quinol oxidase that lacks Cu_A[36]. Similarly, the deletion of the gene for Sco in *Bradyrhizobium japonicum* decreases the accumulation of its aa_3-type CcO that contains Cu_A, but not the cbb_3-type CcO that lacks Cu_A[37]. The single Sco protein present in *R. sphaeroides* (termed PrrC) binds Cu(II) and Ni(II) [38], similar to human Sco1 [31,32]. PrrC has disulfide reductase activity [39], a finding which has stimulated discussion about whether the role of bacterial Sco proteins in Cu_A assembly might be restricted to the reduction of the cysteines of the apo-Cu_A center. In fact, a soluble form of Sco of *Thermus thermophilus* was found to reduce the disulfide of *Thermus* apo-Cu_A*in vitro*, but it could not metallate the reduced apo-Cu_A center [40].

Sco proteins have also been implicated in the assembly of Cu_B. The membrane-bound copper chaperone Cox11 is absolutely required for the assembly of the Cu_B center in the aa_3-type CcOs of α proteobacteria and mitochondria [30,41,42]. However, Cox11 is not required for the insertion of Cu_B into the cbb_3-type CcOs [37,41,42]. *Rhodobacter capsulatus* and *Pseudomonas aeruginosa* strains lacking their Sco proteins exhibit decreased accumulation of active cbb_3-type CcO, but increasing the concentration of exogenous copper restores the synthesis of active enzyme [43–45]. This indicates a role for Sco in the delivery of copper to the Cu_B center of the cbb_3-type CcO in these species. In contrast, the deletion of Sco from *B. japonicum* does not affect the accumulation of its cbb_3-type CcO [37].

Many bacteria contain a periplasmic copper chaperone of the PCu_AC family. In these proteins, a cupredoxin-like fold binds a single Cu(I) via methionine and histidine side chains [46]. Unlike Sco and Cox11, PCu_AC of *R. sphaeroides* and other α proteobacteria may not be tethered in the cytoplasmic membrane since computer analysis indicates that the predicted hydrophobic sequence at the N-terminus appears more like a signal sequence than a transmembrane helix. The hydrophilic domain of *R. sphaeroides* PCu_AC is 53–55% similar to its homologs in *Deinococcus radiodurans* and *Thermus thermophilus*, for which solution structures have been determined [40,46]. PCu_AC was originally suggested as a candidate for a functional analog of mitochondrial Cox17 in bacteria [46]; in mitochondria, Cox17 is soluble in the intermembrane space where it transfers copper to membrane-bound Sco and Cox11 [47]. Later, the same group performed NMR experiments of copper transfer and protein interaction to show that, *in vitro*, a recombinant form of *Thermus* PCu_AC inserted copper into the Cu_A site of soluble subunit II of the *Thermus* ba_3-type CcO [40]. One problem in extrapolating from this result to a universal mechanism for Cu_A assembly is that PCu_AC is not present in mitochondria.

It has also been suggested that PCu_AC may deliver copper to the Cu_B center of the cbb_3-type CcO of *B. japonicum*[37]. In a strain of *B. japonicum* lacking one of its two predicted PCu_AC proteins, total CcO activity decreased but the cbb_3-type CcO was not assayed independently [48].

With the demonstration that PCu_AC delivers copper to a Cu_A site *in vitro*[40], plus the suggestion that PCu_AC may be responsible for the assembly of Cu_B of a cbb_3-type CcO [37], there exists a need to explore the role of PCu_AC in the cell. For example, to what extent is PCu_AC required for the assembly of either the aa_3-type CcO or the cbb_3-type CcO? If both PCu_AC and Sco participate in the assembly of the copper centers of these two oxidases, are their functions unique or redundant? These questions and others have been examined in *R. sphaeroides*, a bacterium that has proven useful for elucidating functions of CcO assembly proteins also present in mitochondria, such as Cox11 and Surf1 [23,41]. This is partly due to the evolutionary relationship between *R. sphaeroides* and mitochondria [49,50] and also because this bacterium tends to accumulate partially assembled CcO forms that can be purified and analyzed [23,41,51,52]. The experiments presented here take advantage of the additional feature that *R. sphaeroides* accumulates both the aa_3-type and the cbb_3-type CcO during aerobic growth [1,6,20]. Therefore, the assembly of these two evolutionarily distant oxidases can be compared in the same cellular environment, *i.e.* with the same complement of copper chaperones and concentration of copper. The results show that PCu_AC has a significant effect on the assembly of Cu_A of the aa_3-type CcO and Cu_B of the cbb_3-type CcO. However, PrrC (*R. sphaeroides* Sco) has an even greater effect and PCu_AC appears to function upstream of PrrC rather than as a redundant copper delivery pathway.

2. Material and methods

2.1. Bacterial growth

R. sphaeroides strains were grown in Sistrom's media A [53] supplemented with 1 μg/ml tetracycline, 50 μg/ml spectinomycin and streptomycin and 25 μg/ml kanamycin, when necessary. Batches of ten-fold concentrated media were prepared using Nanopure water, without the addition of copper. Copper was added to the media just before cell growth, when desired, by the addition of $CuSO_4$ from a stock solution. Glass and plastic containers used in the formulation and storage of concentrated media were bathed in 100 μM EDTA and rinsed with Nanopure water before use. Metal analysis of the media by inductively coupled plasma optical emission spectroscopy (ICP-OES) indicated that the final (1X) media contained <50 nM copper when no additional $CuSO_4$ was added. For each growth, cells were taken from frozen stocks and grown on Sistrom's agar, containing the final desired copper concentration, for three days at 30 °C. A heavy loop of cells from these plates was used to innoculate 100 ml of media in a 500 ml Erlenmeyer flask for overnight growth at 32 C with rapid shaking. 10 ml of these cultures were used to inoculate 600 ml of media in 2 l baffled flasks for growth at 32 °C with rapid shaking. Cells were grown to late exponential phase ($O.D._{660\ nm} = 1.0–1.2$), harvested by centrifugation at 4 °C and frozen at −80 °C. Flasks used for copper-deficient growth were kept separate. All of the flasks used for cell growth were prepared for re-use by rinsing them with Nanopure water after cell harvest followed by autoclaving. This procedure allows the previous cell culture to deplete the glass of copper.

2.2. Construction of a plasmid to express PCu_AC

A derivative of the broad host range vector PBBR1MCS-3 (tetracycline resistance) [54] was prepared with the gene for *R. sphaeroides*

PCu_AC under the control of the *R. sphaeroides* promoter for *coxI*, the gene for subunit I of the aa_3-type CcO, as follows. First, a 1010 bp fragment of genomic DNA was isolated from *R. sphaeroides* 2.4.1 by PCR using primers that created an *Nde*I restriction site at the ATG of the PCu_AC gene (NdeIfwd 5′-GCCAAATCACACAGTCAGGAGAGACAtATGACCCCG-3′) and a *Sac*I restriction site in the 3′ non-coding region of the gene (SacIrev 5′-GGCGGCTGCCAAGGGAGCtCGCGGGACCG-3′). After purification, this fragment was further restricted with *Nde*I and *Sac*I to remove the primer extensions. In order to prepare the host plasmid, a *Kpn*I–*Sac*I fragment containing the gene for subunit III (*coxIII*) 3′ to the *coxI* promoter was excised from pJG211, a derivative of PBBR1MCS-2 created previously [55]; the *Kpn*I–*Sac*I fragment was then cloned into the multiple cloning site of pBBR1MCS-3 [54]. This new derivative of pBBR1MCS-3 was restricted with *Nde*I and *Sac*I to release *coxIII* and the *Nde*I–*Sac*I DNA containing the gene for PCu_AC was inserted. The final product, named $pPCu_AC$, contains the gene for PCu_AC under the control of the *coxI* promoter; $pPCu_AC$ was transformed into *E. coli* S-17 for conjugation into *R. sphaeroides* by established procedures [56].

2.3. Inactivation of the genomic gene for PCuAC

Starting with $pPCu_AC$, a *Pst*I site and a *BamH*I site were introduced into the gene for PCu_AC using the QuikChange mutagenesis system (Agilent). A 300 bp DNA fragment completely internal to the gene was removed and cloned into the multiple cloning site of pKNOCK-Km [57] using its *Pst*I and *BamH*I restriction sites to create pJG245. pJG245 was transformed into *E. coli* S-17, conjugated into *R. sphaeroides* 2.4.1 and colonies resistant to 50 μg/ml kanamycin were selected. Genomic DNA was extracted from several of these colonies, PCR was performed using the primers presented in the previous section, and the resulting fragments were cloned into the TOPO 2.0 vector (Invitrogen). PCR analysis and DNA sequencing confirmed the interruption of the gene for PCu_AC by pJG245 and the absence of a normal gene in the genome. One of these strains was retained as *R. sphaeroides* ΔPCu_AC.

R. sphaeroides PRRC4, which contains a silent deletion of the gene for PrrC [58], was obtained from the laboratory of Prof. Sam Kaplan, U.T. Health Sciences, Houston. pJG245 was also used to inactivate the gene for PCu_AC in *R. sphaeroides* PRRC4 to create $\Delta PrrC–\Delta PCu_AC$.

2.4. Activity assays

The activity of purified aa_3-type CcO was measured as previously described [59]. Simultaneous measurements of the activity of the aa_3-type CcO and the cbb_3-type CcO in purified cytoplasmic membranes were performed as O_2 consumption assays using a Clark-type O_2 electrode (Yellow Springs) and a YSI 5300 dissolved O_2 monitor at 25 °C. The 1.7 ml reaction mixture contained 50 mM Tris–HCl, 75 mM KCl, pH 7.2, 5 μM CCCP, 0.8 μg/ml valinomycin and 0.1–0.3 mg intact, purified cytoplasmic membranes (measured as total membrane protein). O_2 consumption was initiated by the addition of ascorbic acid to 3 mM and TMPD to 0.3 mM. After the consumption of all of the O_2 in the reaction cuvette, dodecyl maltoside was added to a final concentration of 0.1% to solubilize the membranes. After 2 min, re-purified soybean phospholipids [60], sonicated into a stock solution of 40 mg/ml lipid in 10 mM Tris–HCl, pH 7.0 plus 1.0% dodecyl maltoside, were added to a final concentration of 0.5 mg/ml lipid. O_2 was returned to the reaction cuvette by blowing humidified 100% O_2 over the top of the solution until the O_2 concentration in the reaction cell equaled that of air-saturated buffer (*i.e.* the concentration of O_2 at the beginning of the experiment). The rate of ascorbate/TMPD-driven O_2 consumption was further measured until most of the O_2 in the cuvette was consumed. A representative O_2 electrode tracing of this assay is shown in Fig. 1B and discussed further in Results.

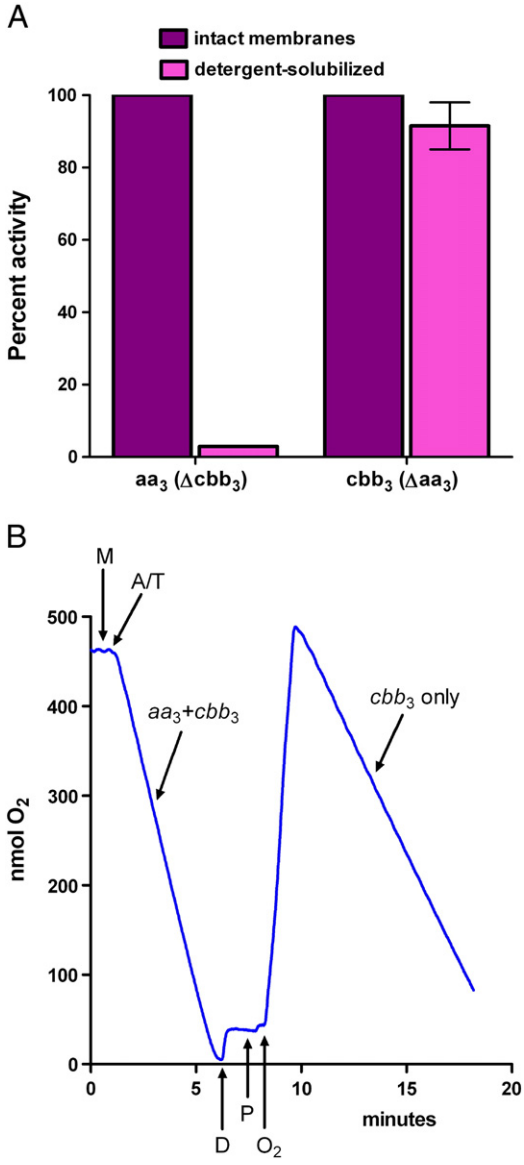

Fig. 1. A. The effect of dodecyl maltoside on the TMPD oxidase activity of the aa_3-type CcO and the cbb_3-type CcO in purified cytoplasmic membranes. The activity of the aa_3-type CcO was assayed in membranes isolated from *R. sphaeroides* CBB3Δ, which lacks the structural genes for the cbb_3-type CcO [77]. The activity of the cbb_3-type CcO was assayed in membranes isolated from *R. sphaeroides* YZ200, which lacks the coxII–III operon for the aa_3-type CcO [78]. The activities of the intact membranes are set to 100% and the assays were performed as described in Material and methods. The TMPD oxidase activity of the aa_3-type CcO is lost when detergent disrupts its interaction with membrane-bound cytochrome c_y, but the TMPD oxidase activity of the cbb_3-type CcO is retained. Error is standard deviation. B. A representative O_2 electrode tracing of the assay for the TMPD oxidase activity of the aa_3-type and cbb_3-type CcOs. See Material and methods and Results for details. Intact, purified cytoplasmic membranes (M) are added to the reaction cell and O_2 consumption is initiated by the addition of ascorbate and TMPD (A/T). The first register measures total CcO activity (aa_3 plus cbb_3). After all O_2 has been consumed, dodecyl maltoside (D) is added to solubilize the membranes and thereby disrupt the interaction of cytochrome c_y with the aa_3-type CcO. After 2 min, soybean phospholipids are added (P) followed by humidified O_2, which restores O_2 consumption activity. In the second register, only the activity of the cbb_3-type CcO is measured.

2.5. Copper content measurements

Purified CcO samples were incubated in 20 mM Tris–HCl, pH 7.4 (prepared in low-metal Nanopure water) containing 1.0 mM EDTA for 5 min and then the EDTA plus any other low molecular weight species were removed by a series of washes in 20 mM Tris–HCl, pH 7.4, in an ultrafiltration device with a 50 kDa cutoff membrane until

the EDTA concentration was calculated to be <0.01 μM. Samples containing 3.5 ml of 4 μM protein were injected into a Spectro Genesis ICP-OES spectrometer to simultaneously measure the concentrations of copper at 324.754 nm and sulfur at 180.731 nm. Each analysis yields the average of three successive determinations and each sample was analyzed two to three times. The element standards used to develop the regression lines were purchased from Inorganic Ventures. The concentration of CcO was obtained by dividing the sulfur concentration by the sum of cysteines and methionines in *R. sphaeroides* CcO (54).

2.6. Other

Cytoplasmic membranes were purified as in Hosler et al. [1]. The aa_3-type CcO was purified by Ni-affinity chromatography [41] followed by FPLC anion exchange chromatography on DEAE-5PW (Toso-Haas) [51]. The concentrations of total membrane protein were determined using the BioRad DC Protein Assay system.

3. Results

3.1. Measuring the accumulation of fully assembled aa_3 and cbb_3 CcOs in the cytoplasmic membranes of R. sphaeroides cells

In order to determine the effect of PrrC and PCu_AC on the assembly of the aa_3-type and cbb_3-type CcOs of *R. sphaeroides* it is necessary to assess the accumulation of the active, and therefore fully assembled, protein complexes in the cytoplasmic membranes of various strains. Visible spectroscopy has often been used to measure the accumulation of the aa_3-type CcO due to its unique α band absorbance ~605 nm. However, several studies have demonstrated the ability of *R. sphaeroides* cells to insert incompletely assembled, inactive aa_3-type CcO complexes into the membrane [23,41,51,52]. Many of these partially assembled forms contain heme *a* and thereby absorb in the α band region. Thus, in a population of the aa_3-type CcO containing partially and fully assembled forms it is difficult to parse the fraction of fully assembled CcO by visible spectroscopy. Visible spectroscopy of the cbb_3-type CcO in the intact membrane is confounded by absorbance signals arising from other *b*- and *c*-type cytochromes.

In this study, ascorbate/TMPD-driven O_2 consumption has been used to assess the accumulation of fully assembled aa_3-type and cbb_3-type CcO in purified cytoplasmic membranes. Both CcO types efficiently oxidize TMPD as long as a cytochrome *c* is present [61,62]. Soluble cytochromes *c* are removed during the purification of the cytoplasmic membranes, but membrane-bound cytochrome c_y is present [20]. The cytochrome c_y:aa_3 complex in the intact membrane catalyzes rapid TMPD oxidation, but disruption of the membrane with low levels of dodecyl maltoside stops electron flow from ascorbate/TMPD (Fig. 1A). In contrast, the cbb_3-type CcO contains extramembrane *c*-type cytochromes that allow this enzyme to oxidize TMPD in both its membrane-bound and detergent-solubilized forms (Fig. 1A). An oxygen electrode tracing of the assay, as described in Material and methods, is shown in Fig. 1B. Ascorbate/TMPD-driven O_2 consumption by the intact membrane reflects the activity of both CcOs, while after dissolution of the membrane by dodecyl maltoside only the cbb_3-type CcO is capable of oxidizing TMPD. The activity of the aa_3-type oxidase is obtained by subtraction. The detergent to membrane protein ratio is maintained within experimentally determined limits to be sure that the cytochrome c_y:aa_3 complex is fully dissociated. The amount of cytochrome c_y is sufficient to catalyze high rates of TMPD oxidation by the aa_3-type CcO in membranes isolated from wild-type cells grown in copper-sufficient media (Fig. 2A). Therefore, the content of cytochrome c_y is unlikely to be rate limiting for samples where the rates of TMPD oxidation are slower. Because the purified intact membranes may contain sealed vesicles, uncoupler (CCCP plus valinomycin) is added to prevent the formation of any membrane potential that could slow CcO activity. The membrane permeability of TMPD ensures its access to both sides of a vesicle and the use of saturating concentrations of ascorbate and TMPD avoids the partitioning of electrons when both CcOs are active.

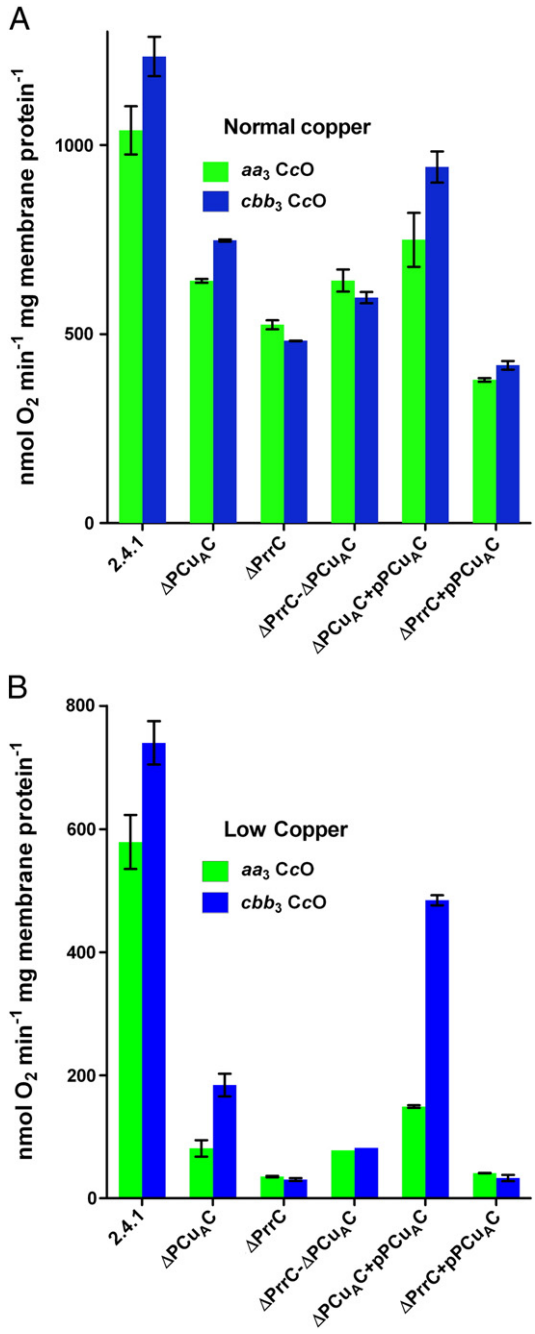

Fig. 2. The activities of the aa_3-type and cbb_3-type CcOs in cytoplasmic membranes purified from wild-type *R. sphaeroides* cells and from strains containing different amounts of the copper chaperones PrrC (Sco) and PCu_AC. Assays were performed as described in Material and methods and Results. A. Oxidase activities in membranes isolated from cells grown in 1.6 μM Cu^{2+} B. Oxidase activities in membranes isolated from cells grown in <50 nM Cu^{2+}. Error is standard deviation.

The ability of *R. sphaeroides* to express multiple terminal oxidases [63] also assists assays of the accumulation of the two cytochrome *c* oxidases. A 94–96% reduction in the content of the cytochrome *c* oxidases (shown below) has little effect on the growth rate of the cells (data not shown), even though aerobic growth of *R. sphaeroides* is dependent upon a functional terminal oxidase. The likely reason is the expression of a quinol oxidase with homology to the heme *bb*-type

(non-copper) quinol oxidase of *Pseudomonas aeruginosa*[64]. Carbon-monoxide difference spectra (not shown) of membranes isolated from *R. sphaeroides* cells lacking the cbb_3-type C*c*O [65] are consistent with expression of the heme *bb*-type quinol oxidase during aerobic growth. Characterization of this enzyme from *Pseudomonas nautica* indicates that it has no TMPD oxidase activity [66], consistent with our observations.

3.2. The effect of PrrC and PCu_AC deletions on C*c*O accumulation in the membrane

The activity measurements using purified cytoplasmic membranes show that wild-type *R. sphaeroides* cells (strain 2.4.1), containing normal levels of PrrC and PCu_AC, accumulate both the aa_3-type and the cbb_3-type oxidases during aerobic growth (Fig. 2A). A derivative of *R. sphaeroides* 2.4.1 (wild-type) that lacks PrrC (PRRC4 or ΔPrrC) was obtained from the laboratory of Sam Kaplan, UT Health Sciences, Houston [58]. *R. sphaeroides* ΔPCu_AC was created by inactivating the gene for PCu_AC in *R. sphaeroides* 2.4.1 using a pKNOCK construct as described in Material and methods. A strain lacking both chaperones (ΔPrrC–ΔPCu_AC) was created by using the same pKNOCK construct to inactivate the gene for PCu_AC in *R. sphaeroides* PRRC4. In cells grown aerobically in media containing 1.6 μM Cu^{2+}, the absence of PrrC leads to a 50% decrease in the content of fully assembled aa_3-type C*c*O and a 60% loss of fully assembled cbb_3-type C*c*O (Fig. 2A). The absence of PCu_AC leads to a 40% decrease in both oxidases. These decreases imply that both PrrC and PCu_AC play a role in the assembly of the aa_3-type C*c*O and the cbb_3-type C*c*O. However, the continued accumulation of significant amounts of both oxidases in the absence of either or both chaperones indicates the existence of multiple pathways for copper delivery.

The *R. sphaeroides* strains lacking PrrC and PCu_AC were then grown in copper-deficient media, as described in Material and methods. With a concentration of <50 nM Cu^{2+} in the media, the amount of free copper in the periplasmic space of each cell is vanishingly small. Nevertheless, *R. sphaeroides* 2.4.1 grows as rapidly in media containing <50 nM Cu^{2+} as it does in media containing 1.6 μM Cu^{2+} (data not shown). In wild-type cells grown in low copper, the amounts of fully assembled aa_3-type C*c*O and cbb_3-type C*c*O in the cell membrane each decrease by ~40% (Fig. 2A, B). Assuming an initial Cu^{2+} concentration of 50 nM, simple calculations indicate that by the time of cell harvest essentially all of the copper initially present in the media has been captured and incorporated into aa_3-type and the cbb_3-type heme-Cu oxidases. In order to accomplish this, the cells must be using systems with a high affinity for copper and with a high efficiency for the transfer of captured copper to the three copper binding sites in the two heme-Cu oxidases. Therefore, the extent of copper center assembly in the copper chaperone mutants grown in copper-deficient media should best reveal the capability of each chaperone to contribute to the assembly of each center.

In cells grown in low copper media, the absence of PrrC (ΔPrrC) has a strong effect on the assembly of both heme-Cu oxidases. ΔPrrC accumulates fully assembled aa_3-type and cbb_3-type C*c*O to only 6% and 4%, respectively, of their levels in normal cells grown in low copper (Fig. 2B). The absence of PCu_AC has a significant but less detrimental effect on oxidase assembly. Accumulation of the fully assembled aa_3-type C*c*O in ΔPCu_AC cells is 14% that of wild-type cells grown in low copper media while the accumulation of active cbb_3-type C*c*O is 25% that of wild-type (Fig. 2B). While the reduced accumulations of active aa_3-type C*c*O could be due to decreased Cu_A or Cu_B assembly, or both, the decreases in active cbb_3-type C*c*O should only reflect impaired assembly of its Cu_B center.

A significant question is whether PrrC and PCu_AC have parallel, redundant roles in the assembly of the copper centers or more separate functions. Two tests were performed to explore this question. First, a strain lacking both PrrC and PCu_AC was prepared. If the role of PCuAC in the assembly of either oxidase is redundant to that of PrrC it would be expected that removing both chaperones would have a greater effect on the accumulation of the fully assembled oxidase than removing PrrC or PCu_AC individually. In fact, the assembly and accumulation of the aa_3-type C*c*O and the cbb_3-type C*c*O in cells lacking both PrrC and PCu_AC is no less than in cells lacking only PrrC or only PCu_AC (Fig. 2). This suggests separate functions for the two chaperones. However, for unknown reasons the double deletion strain accumulates slightly greater amounts of both oxidases under both normal copper and low copper growth conditions. This clouds the interpretation of the result. As a second test, an expression plasmid for PCu_AC was created (pPCu_AC; see Material and methods) in order to vary the *in vivo* ratio of PCu_AC to PrrC. The introduction of pPCu_AC into the copper-limited ΔPCu_AC strain increases the accumulation of the aa_3-type C*c*O from 14% to 26%, compared to wild-type, and the synthesis of the cbb_3-type C*c*O from 25% of wild-type to 65% (Fig. 2B). The incomplete restoration of C*c*O assembly suggests that the plasmid-borne expression of PCu_AC is less efficient than the expression of PCu_AC from the genome. Nonetheless, the introduction of pPCu_AC has an obvious effect. Therefore, it is significant that using pPCu_AC to increase the amount of PCu_AC in cells lacking PrrC fails to enhance the assembly of the aa_3-type C*c*O and yields but a slight increase in the accumulation of the cbb_3-type C*c*O, from 4% to 8% (Fig. 2B). The results suggest that PCu_AC cannot compensate for the absence of PrrC in the synthesis of the aa_3-type C*c*O or the cbb_3-type C*c*O, *i.e.* the *in vivo* role of the two chaperones is not redundant. The results obtained using the same strains grown in normal copper (Fig. 2A) support this conclusion.

3.3. The effect of PCu_AC and PrrC deletions on the Cu_A and Cu_B centers of the aa_3-type C*c*O

Unlike the cbb_3-type C*c*O which contains a single copper as Cu_B, decreased accumulation of active aa_3-type C*c*O in the chaperone deletion strains could result from impaired assembly of Cu_B or the di-copper Cu_A center, or both. In order to explore these possibilities, the aa_3-type C*c*O was purified from wild-type *R. sphaeroides* cells, ΔPCuAC and ΔPrrC and examined by X-band EPR spectroscopy and metal analysis. Fig. 3 shows the EPR spectra of four purified C*c*O samples, all normalized using the content of six-coordinate heme *a* as reported by α-band absorbance at 604–605 nm. The presence of heme *a* is a constant feature in both fully assembled and partially assembled aa_3-type C*c*O forms in *R. sphaeroides*, except for the apo-oxidase complex [23,41,51,52]. As shown in previous studies [23,41,51], relative changes in the content of Cu_A can be assessed using the peak-to-trough amplitude of the $g = 2.03$ signal for Cu_A in X-band EPR spectra of oxidized C*c*O. The population of the aa_3-type C*c*O purified from wild-type cells grown in copper-depleted media shows ~30% less Cu_A than C*c*O isolated from wild-type cells grown in copper-sufficient media. This indicates that the lower content of active aa_3-type C*c*O in membranes of wild-type cells grown in low copper (Fig. 2) is mostly due to the decreased assembly of Cu_A. The result also confirms that copper is a limiting reagent for *R. sphaeroides* cells grown in low copper media. The absence of PCu_AC further lowers the Cu_A content of purified aa_3-type C*c*O by approximately 15%. Assembly of the aa_3-type C*c*O in the presence of PCu_AC but the absence of PrrC has a more pronounced effect; the Cu_A content is reduced to ~20% of that present in the aa_3-type C*c*O isolated from wild-type cells grown in 1.6 μM Cu^{2+} or ~30% of the Cu_A content of wild-type cells grown in low copper. Protein gels (not shown) indicate that the loss of Cu_A is not accompanied by a decrease in the content of subunit II.

Unlike Cu_A, there are no direct signals for Cu_B in the X-band EPR spectrum. There are, however, two heme-specific transitions that are qualitatively characteristic for C*c*O lacking Cu_B, as described previously for C*c*O that assembles in the absence of functional Cox11

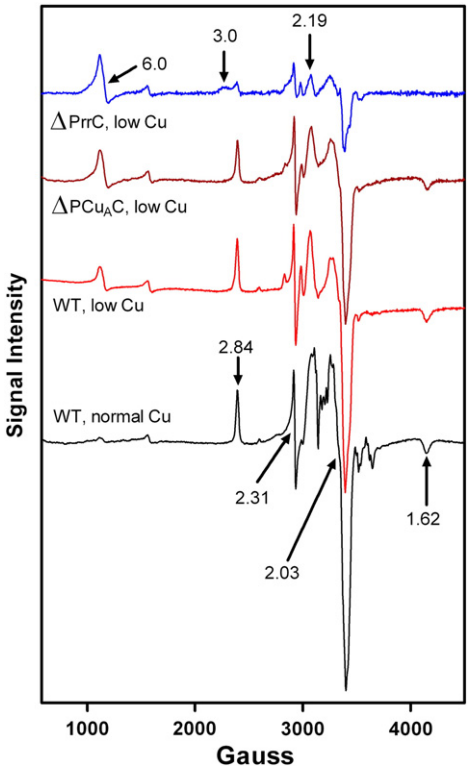

Fig. 3. EPR spectra of purified aa_3-type CcO assembled in wild-type cells grown in 1.6 μM Cu^{2+} or <50 nM Cu^{2+} (low Cu), and in cells lacking PCu_AC or PrrC and grown in <50 nM Cu^{2+}. The spectra were recorded at X band using a Bruker (Billerica, MA) EMX spectrometer. Each spectrum is an average of ten scans taken at 10 K using 25–50 μM CcO. The spectra were recorded using a microwave power of 2 mW at 9.38 GHz. The sweep time was 160 s, and the time constant was 83 ms. The amplitudes of the spectra were normalized by the heme *a* content of the samples as described in Results.

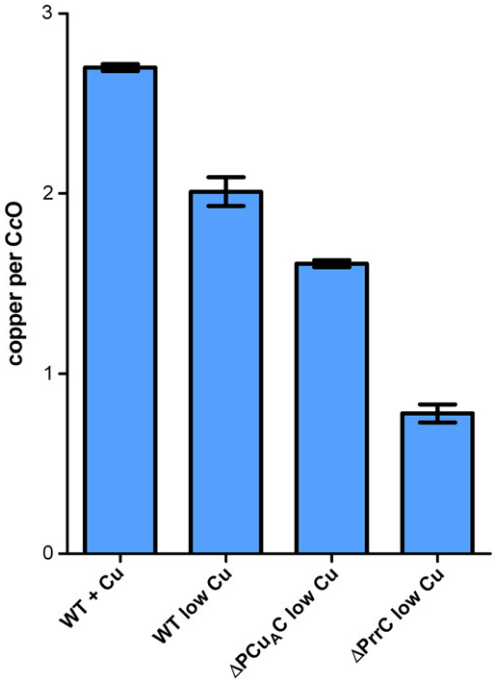

Fig. 4. Copper content per CcO of the aa_3-type CcO samples used in Fig. 3. The number of coppers per CcO was determined by ICP-OES as described in Material and methods. Error is standard deviation.

[41,42]. In normal, oxidized CcO, heme a_3 and Cu_B are strongly spin-coupled and therefore EPR silent [67]. When Cu_B is reduced to Cu^{1+} spin coupling is lost and an axial signal indicative of high-spin heme appears at $g \sim 6$ [68]. The same is true for the aa_3-type CcO of *R. sphaeroides* that lacks Cu_B but contains all of the other metal centers (termed ΔCox11 or ΔCu_B) [41]. However, in ΔCu_B the intensity of the high-spin heme signal at $g \sim 6$ is always less than an equivalent concentration of a high-spin heme standard and the shape of the signal can vary (data not shown). Recent resonance Raman spectra of an *R. sphaeroides* CcO form lacking Cu_B shows the presence of significant amounts of low-spin, six-coordinate heme a_3 (Rousseau, unpublished data). From this result it follows that structural heterogeneity of heme a_3 in CcO molecules lacking Cu_B apparently leads to a mixture of low-spin and high-spin heme a_3, which accounts for the observed variability in the $g \sim 6$ signal. Thus, the increasing amplitudes of the $g \sim 6$ signal in the EPR spectra of ΔPCu_AC and ΔPrrC (Fig. 3) reflect the loss of Cu_B but it is difficult to use this signal to quantify the extent of the loss.

Another Cu_B-related signal arises from low-spin heme *a*, which is close to heme a_3 in subunit I. Heme *a* of normal CcO shows signals at $g = 2.84$, 2.31 and 1.62. In the absence of Cu_B, the signals are altered, *e.g.* the $g = 2.84$ signal shifts to $g = 3$ and becomes more broad, probably due to multiple orientations of heme *a*. The broad $g = 3.0$ signal is most evident in the EPR spectrum of CcO isolated from ΔPrrC cells (Fig. 3) indicating that this protein contains less Cu_B than CcO isolated from ΔPCu_AC or wild-type cells.

Quantitative estimates of the relative amounts of Cu_A and Cu_B present in the samples of purified aa_3-type CcO are possible if the total amount of copper is also known. The total copper content of the samples was determined using ICP-OES (Fig. 4); the concentration of CcO was simultaneously determined from the sulfur content. The copper per CcO value for the complex isolated from wild-type cells grown in copper-sufficient media is 2.7, within 10% of the value of 3.0 expected for absolutely pure, fully assembled CcO. Protein gels (not shown) indicate that the error can be attributed to additional sulfur from small amounts of contaminating protein, and that the level of protein contamination is similar in all of the samples. The total copper content of the aa_3-type CcO purified from ΔPCu_AC is ~60% that of the copper present in wild-type cells grown in 1.6 μM Cu^{2+}, while the aa_3-type CcO isolated from ΔPrrC contains 29% of the normal amount of copper (Fig. 4). The fractional amounts of Cu_A (Fig. 5) present in the aa_3-type CcOs isolated from wild-type *R. sphaeroides*, ΔPCu_AC and ΔPrrC are estimated from the $g = 2.03$ signals of the EPR spectra of Fig. 3, as discussed above. In order to estimate the amount of Cu_B, the amount of copper present as Cu_A is subtracted from the total amount of copper given by the data of Fig. 4. The calculations assume two coppers per Cu_A because studies of the reconstitution of Cu_A sites show that the normal set of Cu_A ligands will bind two coppers, but not one [69–71]. The results compiled in Fig. 5 show that the absence of PCu_AC or PrrC affects the assembly of both Cu_A and Cu_B, but the absence of either chaperone has a greater effect on the assembly of Cu_A than Cu_B. Also, the absence of PrrC has a greater effect on the assembly of Cu_A and Cu_B than does the absence of PCu_AC.

The decreased content of Cu_B in the aa_3-type CcOs of ΔPrrC and ΔPCu_AC could be an indirect consequence of impaired assembly of Cu_A if the Cu_B center is destabilized by the absence of Cu_A. In other words, the loss of Cu_B may only occur in those CcO molecules that already lack Cu_A. However, previous work shows that the reverse interaction does not take place, *i.e.* the absence of Cu_B does not destabilize Cu_A[41]. Moreover, the presence of a stable CcO form that lacks Cu_A but not Cu_B can be deduced from the observation that the CcO sample purified from wild-type *R. sphaeroides* cells grown in low copper shows a 30% loss of Cu_A but only a 10% loss of Cu_B (Fig. 5). The decreased contents of Cu_B (Fig. 5) could also arise from the formation of ΔCu_B, *i.e.* CcO lacking only Cu_B, in the absence of PCu_AC or PrrC. Measurements of the O_2 reduction activities of the CcO forms (Fig. 5) support the latter hypothesis. For the CcOs isolated from

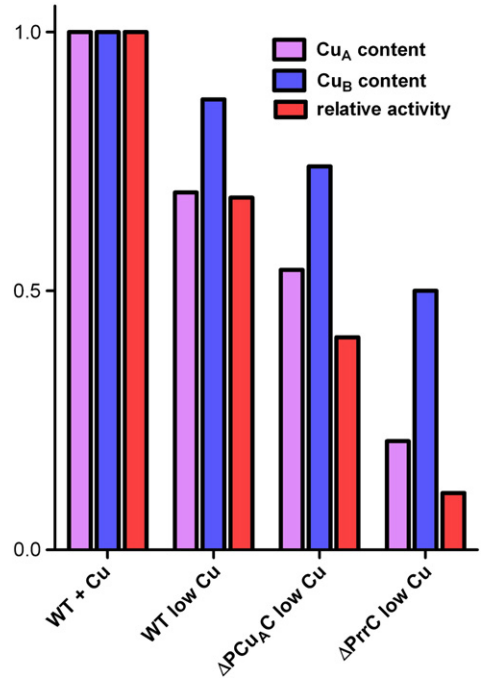

Fig. 5. The relative Cu_A content, Cu_B content and O_2 reduction activity of purified aa_3-type CcO assembled in wild-type cells grown in 1.6 μM Cu^{2+} or <50 nM Cu^{2+} (low Cu), and in cells lacking PCu_AC or PrrC and grown in <50 nM Cu^{2+}. The fractional content of Cu_A, relative to wild-type cells grown in 1.6 mM Cu^{2+}, is taken from the amplitudes of the $g=2.03$ signal in Fig. 3. The fractional content of Cu_B is estimated as described in Results. The relative activities are taken from the TN_{max} values for cytochrome *c*-driven O_2 reduction measured at pH 6.5 as described in Varanasi and Hosler [59]. The TN_{max} for the wild-type aa_3-type CcO grown in 1.6 mM Cu^{2+} (100%) is $1717 \pm 41\ e^{-}s^{-1}$.

ΔPCu$_A$C and ΔPrrC, the activities of the purified CcO populations indicate the presence of greater amounts of inactive CcO than would be predicted if Cu_B is absent only from those CcO molecules that already lack Cu_A. In fact, for CcO isolated from ΔPrrC, the measured activity fits well to that predicted assuming separate populations of CcO that are inactive because they lack either Cu_A or Cu_B.

4. Discussion

Both PCu_AC and PrrC enhance the assembly of the aa_3-type CcO, particularly when exogenous copper levels are low (<50 nM Cu^{2+}), a situation in which the cell's more efficient pathways for copper capture and delivery should predominate. Under low copper conditions, the absence of PrrC decreases the accumulation of fully assembled aa_3-type CcO in the bacterial membrane by up to 94% while the absence of PCu_AC decreases its accumulation by up to 86% (Fig. 2B). The effect of deleting PrrC is similar to results obtained using strains of *Bacillus subtilis*[36] and *Bradyrhizobium japonicum*[37] that lack their Sco proteins, as well as the losses of mitochondrial CcO in eukaryotic cells lacking a functional Sco1 [30]. Increasing the exogenous copper level from <50 nM to 1.6 μM partially restores the assembly of the aa_3-type CcO in the absence of PrrC in *R. sphaeroides* (Fig. 2A); similar results have been reported for strains of *B. subtilus* and *B. japonicum* lacking Sco1 [36,37], and in human cells lacking fully functional Sco2 [72,73]. The chaperone associated with Cu_B assembly in the aa_3-type CcO, Cox11, is present in *R. sphaeroides* ΔPrrC and ΔPCu$_A$C. Accordingly, normal assembly of Cu_B takes place in ΔPrrC and ΔPCu$_A$C when they are grown in copper-sufficient media (data not shown). As for Cu_A assembly, the greater concentration of Cu^{2+} may facilitate its self-assembly or it may drive the metallation of Cu_A by a chaperone with a low efficiency of copper delivery to Cu_A. The presence of sufficient exogenous copper may be responsible for the finding that the deletion of PCu_AC in *B. japonicum* has no effect on the CcO activity of aerobically-grown cells [48].

PrrC and PCu_AC also enhance the assembly of the cbb_3-type CcO, especially in cells grown in limited amounts of copper. In the absence of PrrC, the accumulation of active cbb_3-type CcO is reduced by 96% while in the absence of PCu_AC its accumulation decreases by 75% (Fig. 2B). Once again, increasing the exogenous copper concentration ~thirty-fold partially compensates for the absence of these chaperones. The results obtained for PrrC agree with earlier reports showing that the deletion of the Sco proteins of *R. capsulatus* and *Pseudomonas aeruginosa* lowers the accumulation of active cbb_3-type CcO in these bacteria [43–45]. In apparent contradiction, the deletion of Sco1 of *B. japonicum* has little effect on the accumulation of its cbb_3-type CcO [37], but it is not clear if exogenous copper concentrations were limiting. A previous study of *R. sphaeroides* PRRC4 (ΔPrrC) reported no large loss of total CcO activity (aa_3 plus cbb_3) in this strain. This is actually consistent with the results reported here as the cells of the previous study were grown in the presence of 1.6 μM Cu^{2+}[58]. Examination of the requirement for a copper chaperone under conditions where the metal is a limiting reagent appears necessary for concluding whether or not the chaperone contributes to metal center assembly.

4.1. Cellular roles of PCu_AC and PrrC in the assembly of the Cu_B center of the aa_3-type CcO

In studies in *B. subtilus*, *B. japonicum* and mitochondria, the decreased accumulation of the aa_3-type CcO in the absence of Sco has been attributed to impaired assembly of the Cu_A center [30,36,37]. The results of this study show that the absence of PrrC, and to a lesser extent PCu_AC, impairs the assembly of *both* Cu_A and Cu_B of the aa_3-type CcO (Fig. 5). The role of PrrC, in particular, can be described as specific since in the absence of PrrC some CcO assembles with Cu_A but without Cu_B. Nonetheless, Cu_B assembly requires Cox11, which is present in all of the strains used in these experiments, and neither PrrC nor PCu_AC can assemble Cu_B in the absence of Cox11. One possible explanation of these findings is that PrrC delivers copper to Cox11 more efficiently than Cox11 self-metallates in the presence of low exogenous Cu^{2+} concentrations.

4.2. Cellular roles of PCu_AC and PrrC in the assembly of Cu_A of the aa_3-type CcO

The question of how Sco and PCu_AC participate in the assembly of Cu_A has previously been addressed using an *in vitro* system by Abriata et al. [40]. A soluble form of PCu_AC of *Thermus thermophilus* delivered both coppers to the ligands of Cu_A in a soluble form of subunit II of the ba_3-type CcO of *T. thermophilus*. In the same study, a soluble form of Sco of *T. thermophilus* was incapable of delivering copper into apo-Cu_A but it could provide the disulfide reductase activity necessary to reduce the cysteines of the Cu_A center prior to copper delivery.

In this study, the analyses of purified aa_3-type oxidase show that the absence of PrrC or PCu_AC primarily impairs the assembly of Cu_A, with PrrC having the greater effect. The assembly of Cu_B is also affected, as discussed above, but to a lesser extent. In addition to the analyses of purified CcO, the measurements of the accumulation of active aa_3-type CcO in the cytoplasmic membranes of different strains of *R. sphaeroides* (Fig. 2) can be used to explore how Sco (PrrC) and PCu_AC participate in the assembly of Cu_A in the cell. This is possible because 1) the activity of the aa_3-type CcO depends upon the successful assembly of both Cu_B and Cu_A, and 2) the major effect of deleting either chaperone is impairment of the assembly of Cu_A.

Several scenarios of the cellular roles of the two copper chaperones in the process of Cu_A assembly can be envisioned for discussion (Fig. 6). Only one of these, Scenario E, is fully consistent with the results of this study. PrrC has been shown to have disulfide reductase

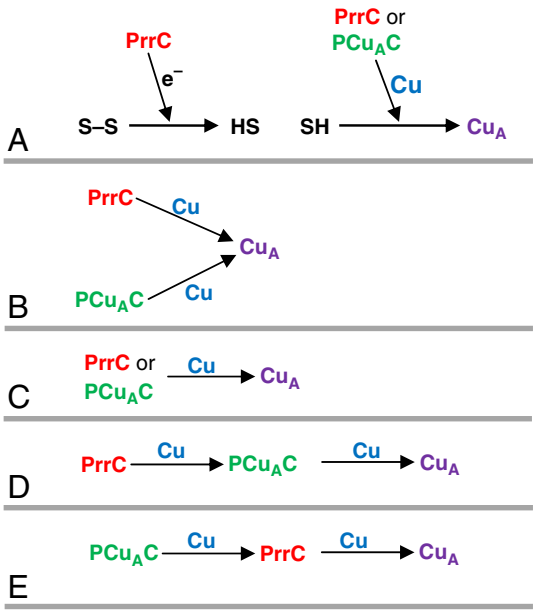

Fig. 6. Possible schemes for the functions of PCu_AC and PrrC in the assembly of Cu_A of the aa_3-type CcO. Explanations are provided in the text. In Scheme A the sulfurs (S) are those of the two cysteine ligands of Cu_A.

activity [39]. Extrapolating from the results of Abriata et al. [40], Scenario A of Fig. 6 explains the strong effect of the deletion of PrrC by positing that PrrC does not deliver copper but rather functions as the predominant disulfide reductase in the periplasm that prepares the cysteines of the nascent Cu_A center for copper binding. However, Scenario A is not consistent with results presented here and elsewhere. First, PrrC is not required for increased levels of Cu_A assembly in ΔPrrC cells grown in higher copper, even though the requirement for PrrC as the predominant disulfide reductase should remain. Second, *B. subtilus* and *B. japonicum* also assemble Cu_A without Sco, given sufficient copper [36,37]. Third, both *B. japonicum* and *R. sphaeroides* contain a separate membrane-bound disulfide reductase (tlpA) that has been implicated in the assembly of the aa_3-type CcO [74]. Scenario B of Fig. 6 posits that PCu_AC and PrrC each deliver one copper to Cu_A. In this case, the removal of either chaperone from the cell should have the same effect on the accumulation of active enzyme. However, the observation is that the removal of PrrC has a significantly greater effect than the removal of PCu_AC. In Scenario C, PrrC and PCu_AC are independently capable of delivering both coppers to Cu_A. The greater effect of PrrC on Cu_A assembly does not rule out this possibility, *e.g.* PrrC could be present in significantly greater amounts than PCu_AC. However, Scenario C does predict that removing both PrrC and PCu_AC should impair the assembly of the aa_3-type oxidase more than removing either chaperone alone. This is not observed; the double deletion of PrrC and PCuAC shows no greater loss of the aa_3-type CcO than either of the separate deletions. Moreover, increasing the PCu_AC population in the cell membrane fails to enhance the assembly of Cu_A if PrrC is absent (ΔPrrC + $pPCu_AC$; Fig. 2B). This is inconsistent with a significant role for PCu_AC in directly delivering both coppers to Cu_A. The failure of increased expression of PCu_AC to enhance CcO assembly in cells lacking PrrC also disfavors Scenario D, in which PCu_AC delivers both coppers to Cu_A while the (observed) stronger requirement for PrrC is postulated to derive from its ability to drive the assembly process by efficiently metallating PCu_AC. Scenario D is further disfavored by the evolutionary relationship between *R. sphaeroides* and mitochondria. Mitochondria do not contain a homolog of PCu_AC, but PrrC is homologous to mitochondrial Sco1 (the primary sequence of the extramembrane domain of PrrC is >50% similar to that of human Sco1). The catalytic cores of the aa_3-type CcO of *R. sphaeroides* and mitochondrial CcO are highly similar, and mitochondria contain homologs of other bacterial assembly proteins required for assembly of the catalytic core (Cox11, Surf1, Cox10, Cox15). These homologies make it likely that the assembly process for Cu_A in *R. sphaeroides* will be fundamentally similar to that of mitochondria. Scenario E, in which PrrC (Sco) delivers both coppers to Cu_A, is consistent with the all of the data of this study, as well as with previous observations in *B. subtilus*, *B. japonicum* and mitochondria [30,36,37]. PCu_AC is posited to supply copper to PrrC, based on the finding that its presence does enhance the assembly of Cu_A. This assignment is equivalent to stating that PCu_AC plays a role in copper homeostasis, with the explicit recognition that copper transfer at low copper concentrations must require protein–protein interaction. In Scenario E, the role of PCu_AC is similar to that of Cox17 of mitochondria, as previously proposed [46]. A homolog of Cox17 is not present in bacteria.

As yet, there exists no obvious way to reconcile Scenario E of Fig. 6 with the *in vitro* Cu_A assembly study of Abriata et al. [40] in which *T. thermophilus* Sco does not deliver copper to soluble *T. thermophilus* Cu_A. Interestingly, *T. thermophilus* lacks Cox11, indicating that its system for the assembly of Cu_B of its ba_3-type CcO differs from those bacteria that synthesize a mitochondrial-like aa_3-type CcO. Thus, *T. thermophilus* may also have different requirements for the assembly of Cu_A of the ba_3-type CcO. As stated above, the similarities between the aa_3-type CcOs of *R. sphaeroides* and mitochondria, along with the retention of Cox11 and Sco in both, make it likely that their Cu_A and Cu_B assembly pathways share basic commonality.

4.3. The roles of PrrC and PCu_AC in the assembly of Cu_B of the cbb_3-type CcO

The roles of PCu_AC and PrrC in the assembly of the Cu_B center of the cbb_3-type CcO appear remarkably similar to their roles in the assembly of the Cu_A center of the aa_3-type CcO. The removal of either PrrC or PCu_AC decreases the assembly of Cu_B in the cbb_3-type CcO, using the amount of active oxidase as a measure of the extent of Cu_B assembly. The removal of PrrC has a greater effect than the removal of PCu_AC and increasing the amount of PCu_AC in the cell membrane enhances the assembly of Cu_B only if PrrC is present. Once again, the most straightforward conclusion is that PrrC delivers copper to the Cu_B center and that this method of assembly predominates when exogenous copper levels are low. PCu_AC enhances the assembly of Cu_B, most likely by facilitating the delivery of copper to PrrC as proposed above. This proposed role for PrrC in *R. sphaeroides* is consistent with the previous proposals for the roles of the Sco proteins of *R. capsulatus* and *P. aeruginosa* in the assembly of their cbb_3-type CcOs [43,44]. Moreover, Ekici et al. [28] report physical interactions between *R. capsulatus* Sco (SenC) and subunits of its cbb_3-type CcO. Further, a putative Cu-ATPase, CcoI, has been shown to be required for the assembly of the cbb_3-type CcO in several *a* proteobacteria [28]. It seems likely that CcoI and the bacterial Sco proteins, including PrrC, cooperate in the assembly of Cu_B of this oxidase.

4.4. Gene regulation by PrrC

Sco proteins have been argued to play a role in gene regulation [43,58] leading to the consideration that the reduced accumulation of the aa_3-type and cbb_3-type oxidases observed in *R. sphaeroides* strains lacking PrrC could result from down-regulated expression of the apo-proteins of the terminal oxidases. Direct gene expression experiments are not included. However, the isolation and analysis of partially assembled forms of the aa_3-type CcO from ΔPrrC cells grown in low copper shows directly that the loss of active CcO is due to decreased assembly of Cu_A and Cu_B (Fig. 5), and not to decreased expression of the apo-proteins. Consistent with this, the finding that increased levels of copper restore significant levels of both oxidases even in the absence of PrrC (Fig. 2A and ref. [58]) argues

that the absence of PrrC is not responsible for the low accumulation of both oxidases in cells grown in low copper. The deletion of the Sco homolog (SenC) of *R. capsulatus* does cause a two-fold decrease in the expression of subunit I of its cbb_3-type C*c*O [43]. However, this decrease in expression was shown to be a secondary *result* of a larger decrease in C*c*O accumulation due to impaired assembly of Cu_B in the absence of SenC, since the expression of the cbb_3-type C*c*O in *R. capsulatus* is partly controlled by a signal transduction system that responds to the level of its activity [75,76].

Acknowledgements

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References

[1] J.P. Hosler, J. Fetter, M.M. Tecklenburg, M. Espe, C. Lerma, S. Ferguson-Miller, Cytochrome aa_3 of *Rhodobacter sphaeroides* as a model for mitochondrial cytochrome *c* oxidase. Purification, kinetics, proton pumping, and spectral analysis, J. Biol. Chem. 267 (1992) 24264–24272.
[2] J. Cao, J. Hosler, J. Shapleigh, A. Revzin, S. Ferguson-Miller, Cytochrome aa_3 of *Rhodobacter sphaeroides* as a model for mitochondrial cytochrome *c* oxidase. The coxII/coxIII operon codes for structural and assembly proteins homologous to those in yeast, J. Biol. Chem. 267 (1992) 24273–24278.
[3] J.P. Shapleigh, R.B. Gennis, Cloning, sequencing, and deletion from the chromosome of the gene encoding subunit I of the aa_3-type cytochrome *c* oxidase of *Rhodobacter sphaeroides*, Mol. Microbiol. 6 (1992) 635–642.
[4] M. Svensson-Ek, J. Abramson, G. Larsson, S. Tornroth, P. Brzezinski, S. Iwata, The X-ray crystal structures of wild-type and EQ(I-286) mutant cytochrome *c* oxidases from *Rhodobacter sphaeroides*, J. Mol. Biol. 321 (2002) 329–339.
[5] L. Qin, C. Hiser, A. Mulichak, R.M. Garavito, S. Ferguson-Miller, Identification of conserved lipid/detergent-binding sites in a high-resolution structure of the membrane protein cytochrome *c* oxidase, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 16117–16122.
[6] J.A. Garcia-Horsman, E. Berry, J.P. Shapleigh, J.O. Alben, R.B. Gennis, A novel cytochrome *c* oxidase from *Rhodobacter sphaeroides* that lacks Cu_A, Biochemistry 33 (1994) 3113–3119.
[7] J. Hemp, D.E. Robinson, K.B. Ganesan, T.J. Martinez, N.L. Kelleher, R.B. Gennis, Evolutionary migration of a post-translationally modified active-site residue in the proton-pumping heme-copper oxygen reductases, Biochemistry 45 (2006) 15405–15410.
[8] J.P. Hosler, S. Ferguson-Miller, M.W. Calhoun, J.W. Thomas, J. Hill, L. Lemieux, J. Ma, C. Georgiou, J. Fetter, J. Shapleigh, M.J. Tecklenburg, G.T. Babcock, R.B. Gennis, Insight into the active-site structure and function of cytochrome oxidase by analysis of site-directed mutants of bacterial cytochrome aa_3 and cytochrome *bo*, J. Bioenerg. Biomembr. 25 (1993) 121–136.
[9] Y. Zhen, C.W. Hoganson, G.T. Babcock, S. Ferguson-Miller, Definition of the interaction domain for cytochrome *c* on cytochrome *c* oxidase. I. Biochemical, spectral, and kinetic characterization of surface mutants in subunit II of *Rhodobacter sphaeroides* cytochrome aa_3, J. Biol. Chem. 274 (1999) 38032–38041.
[10] K. Wang, Y. Zhen, R. Sadoski, S. Grinnell, L. Geren, S. Ferguson-Miller, B. Durham, F. Millett, Definition of the interaction domain for cytochrome *c* on cytochrome c oxidase. II. Rapid kinetic analysis of electron transfer from cytochrome *c* to *Rhodobacter sphaeroides* cytochrome oxidase surface mutants, J. Biol. Chem. 274 (1999) 38042–38050.
[11] M.F. Otten, J. van der Oost, W.N. Reijnders, H.V. Westerhoff, B. Ludwig, R.J. Van Spanning, Cytochromes c_{550}, c_{552}, and c_1 in the electron transport network of *Paracoccus denitrificans*: redundant or subtly different in function? J. Bacteriol. 183 (2001) 7017–7026.
[12] C. Rios-Velazquez, R.L. Cox, T.J. Donohue, Characterization of *Rhodobacter sphaeroides* cytochrome c_2 proteins with altered heme attachment sites, Arch. Biochem. Biophys. 389 (2001) 234–244.
[13] H. Myllykallio, F.E. Jenney Jr., C.R. Moomaw, C.A. Slaughter, F. Daldal, Cytochrome c_y of *Rhodobacter capsulatus* is attached to the cytoplasmic membrane by an uncleaved signal sequence-like anchor, J. Bacteriol. 179 (1997) 2623–2631.
[14] V. Drosou, F. Malatesta, B. Ludwig, Mutations in the docking site for cytochrome *c* on the *Paracoccus* heme aa_3 oxidase, Eur. J. Biochem. 269 (2002) 2980–2988.
[15] P. Brzezinski, J. Reimann, P. Adelroth, Molecular architecture of the proton diode of cytochrome *c* oxidase, Biochem. Soc. Trans. 36 (2008) 1169–1174.
[16] V.R. Kaila, M.P. Johansson, D. Sundholm, L. Laakkonen, M. Wikstrom, The chemistry of the Cu_B site in cytochrome *c* oxidase and the importance of its unique His–Tyr bond, Biochim. Biophys. Acta 1787 (2009) 221–233.
[17] V. Sharma, A. Puustinen, M. Wikstrom, L. Laakkonen, Sequence analysis of the cbb_3 oxidases and an atomic model for the *Rhodobacter sphaeroides* enzyme, Biochemistry 45 (2006) 5754–5765.
[18] J. Hemp, C. Christian, B. Barquera, R.B. Gennis, T.J. Martinez, Helix switching of a key active-site residue in the cytochrome cbb_3 oxidases, Biochemistry 44 (2005) 10766–10775.
[19] S. Buschmann, E. Warkentin, H. Xie, J.D. Langer, U. Ermler, H. Michel, The structure of cbb_3 cytochrome oxidase provides insights into proton pumping, Science 329 (2010) 327–330.
[20] F. Daldal, S. Mandaci, C. Winterstein, H. Myllykallio, K. Duyck, D. Zannoni, Mobile cytochrome c_2 and membrane-anchored cytochrome c_y are both efficient electron donors to the cbb_3- and aa_3-type cytochrome *c* oxidases during respiratory growth of *Rhodobacter sphaeroides*, J. Bacteriol. 183 (2001) 2013–2024.
[21] B.M. Brown, Z. Wang, K.R. Brown, J.A. Cricco, E.L. Hegg, Heme O synthase and heme A synthase from *Bacillus subtilis* and *Rhodobacter sphaeroides* interact in *Escherichia coli*, Biochemistry 43 (2004) 13541–13548.
[22] I.C. Soto, F. Fontanesi, J. Liu, A. Barrientos, Biogenesis and assembly of eukaryotic cytochrome *c* oxidase catalytic core, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbabio.2011.09.005.
[23] D. Smith, J. Gray, L. Mitchell, W.E. Antholine, J.P. Hosler, Assembly of cytochrome *c* oxidase in the absence of assembly protein Surf1p leads to loss of the active site heme, J. Biol. Chem. 280 (2005) 17652–17656.
[24] A. Hannappel, F.A. Bundschuh, B. Ludwig, Role of Surf1 in heme recruitment for bacterial COX biogenesis, Biochim. Biophys. Acta (in press), doi:10.1016/j.bbabio.2011.09.007.
[25] C. Kulajta, J.O. Thumfart, S. Haid, F. Daldal, H.G. Koch, Multi-step assembly pathway of the cbb_3-type cytochrome *c* oxidase complex, J. Mol. Biol. 355 (2006) 989–1004.
[26] G. Pawlik, C. Kulajta, I. Sachelaru, S. Schroder, B. Waidner, P. Hellwig, F. Daldal, H.G. Koch, The putative assembly factor CcoH is stably associated with the cbb_3-type cytochrome oxidase, J. Bacteriol. 192 (2010) 6378–6389.
[27] O. Preisig, R. Zufferey, H. Hennecke, The *Bradyrhizobium japonicum* fixGHIS genes are required for the formation of the high-affinity cbb_3-type cytochrome oxidase, Arch. Microbiol. 165 (1996) 297–305.
[28] S. Ekici, G. Pawlik, E. Lohmeyer, H.G. Koch, F. Daldal, Biogenesis of cbb_3-type cytochrome *c* oxidase in *Rhodobacter capsulatus*, Biochim. Biophys. Acta (in press), doi:10.1016/j.bbabio.2011.09.007.
[29] L. Banci, I. Bertini, G. Cavallaro, S. Ciofi-Baffoni, Seeking the determinants of the elusive functions of Sco proteins, FEBS J. 278 (2011) 2244–2262.
[30] P.A. Cobine, F. Pierrel, D.R. Winge, Copper trafficking to the mitochondrion and assembly of copper metalloenzymes, Biochim. Biophys. Acta 1763 (2006) 759–772.
[31] L. Banci, I. Bertini, V. Calderone, S. Ciofi-Baffoni, S. Mangani, M. Martinelli, P. Palumaa, S. Wang, A hint for the function of human Sco1 from different structures, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 8595–8600.
[32] Y.C. Horng, S.C. Leary, P.A. Cobine, F.B. Young, G.N. George, E.A. Shoubridge, D.R. Winge, Human Sco1 and Sco2 function as copper-binding proteins, J. Biol. Chem. 280 (2005) 34113–34122.
[33] S.C. Leary, F. Sasarman, T. Nishimura, E.A. Shoubridge, Human SCO2 is required for the synthesis of CO II and as a thiol-disulphide oxidoreductase for SCO1, Hum. Mol. Genet. 18 (2009) 2230–2240.
[34] A. Lode, M. Kuschel, C. Paret, G. Rodel, Mitochondrial copper metabolism in yeast: interaction between Sco1p and Cox2p, FEBS Lett. 485 (2000) 19–24.
[35] A. Lode, C. Paret, G. Rodel, Molecular characterization of *Saccharomyces cerevisiae* Sco2p reveals a high degree of redundancy with Sco1p, Yeast 19 (2002) 909–922.
[36] N.R. Mattatall, J. Jazairi, B.C. Hill, Characterization of YpmQ, an accessory protein required for the expression of cytochrome *c* oxidase in *Bacillus subtilis*, J. Biol. Chem. 275 (2000) 28802–28809.
[37] D. Buhler, R. Rossmann, S. Landolt, S. Balsiger, H.M. Fischer, H. Hennecke, Disparate pathways for the biogenesis of cytochrome oxidases in *Bradyrhizobium japonicum*, J. Biol. Chem. 285 (2010) 15704–15713.
[38] A.G. McEwan, A. Lewin, S.L. Davy, R. Boetzel, A. Leech, D. Walker, T. Wood, G.R. Moore, PrrC from *Rhodobacter sphaeroides*, a homologue of eukaryotic Sco proteins, is a copper-binding protein and may have a thiol-disulfide oxidoreductase activity, FEBS Lett. 518 (2002) 10–16.
[39] A.C. Badrick, A.J. Hamilton, P.V. Bernhardt, C.E. Jones, U. Kappler, M.P. Jennings, A.G. McEwan, PrrC, a Sco homologue from *Rhodobacter sphaeroides*, possesses thiol-disulfide oxidoreductase activity, FEBS Lett. 581 (2007) 4663–4667.
[40] L.A. Abriata, L. Banci, I. Bertini, S. Ciofi-Baffoni, P. Gkazonis, G.A. Spyroulias, A.J. Vila, S. Wang, Mechanism of Cu_A assembly, Nat. Chem. Biol. 4 (2008) 599–601.
[41] L. Hiser, M. Di Valentin, A.G. Hamer, J.P. Hosler, Cox11p is required for stable formation of the Cu_B and magnesium centers of cytochrome *c* oxidase, J. Biol. Chem. 275 (2000) 619–623.
[42] A.K. Thompson, D. Smith, J. Gray, H.S. Carr, A. Liu, D.R. Winge, J.P. Hosler, Mutagenic analysis of Cox11 of *Rhodobacter sphaeroides*: insights into the assembly of Cu_B of cytochrome *c* oxidase, Biochemistry 49 (2010) 5651–5661.
[43] D.L. Swem, L.R. Swem, A. Setterdahl, C.E. Bauer, Involvement of SenC in assembly of cytochrome *c* oxidase in *Rhodobacter capsulatus*, J. Bacteriol. 187 (2005) 8081–8087.
[44] E. Frangipani, D. Haas, Copper acquisition by the SenC protein regulates aerobic respiration in *Pseudomonas aeruginosa* PAO1, FEMS Microbiol. Lett. 298 (2009) 234–240.
[45] F. Borsetti, V. Tremaroli, F. Michelacci, R. Borghese, C. Winterstein, F. Daldal, D. Zannoni, Tellurite effects on *Rhodobacter capsulatus* cell viability and superoxide dismutase activity under oxidative stress conditions, Res. Microbiol. 156 (2005) 807–813.
[46] L. Banci, I. Bertini, S. Ciofi-Baffoni, E. Katsari, N. Katsaros, K. Kubicek, S. Mangani, A copper(I) protein possibly involved in the assembly of Cu_A center of bacterial cytochrome *c* oxidase, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 3994–3999.
[47] Y.C. Horng, P.A. Cobine, A.B. Maxfield, H.S. Carr, D.R. Winge, Specific copper transfer from the Cox17 metallochaperone to both Sco1 and Cox11 in the assembly of yeast cytochrome *c* oxidase, J. Biol. Chem. 279 (2004) 35334–35340.

[48] H. Arunothayanan, M. Nomura, R. Hamaguchi, M. Itakura, K. Minamisawa, S. Tajima, Copper metallochaperones are required for the assembly of bacteroid cytochrome *c* oxidase which is functioning for nitrogen fixation in soybean nodules, Plant Cell Physiol. 51 (2010) 1242–1246.
[49] D. Yang, Y. Oyaizu, H. Oyaizu, G.J. Olsen, C.R. Woese, Mitochrondrial origins, Proc. Natl. Acad. Sci. U. S. A. 82 (1985) 4443–4447.
[50] F.R. Whatley, The establishment of mitochondria: *Paracoccus* and *Rhodopseudomonas*, Ann. N. Y. Acad. Sci. 361 (1981) 330–340.
[51] M.R. Bratton, L. Hiser, W.E. Antholine, C. Hoganson, J.P. Hosler, Identification of the structural subunits required for formation of the metal centers in subunit I of cytochrome *c* oxidase of *Rhodobacter sphaeroides*, Biochemistry 39 (2000) 12989–12995.
[52] L. Hiser, J.P. Hosler, Heme A is not essential for assembly of the subunits of cytochrome *c* oxidase of *Rhodobacter sphaeroides*, J. Biol. Chem. 276 (2001) 45403–45407.
[53] W.R. Sistrom, A requirement for sodium in the growth of *Rhodopseudomonas spheroides*, J. Gen. Microbiol. 22 (1960) 778–785.
[54] M.E. Kovach, R.W. Phillips, P.H. Elzer, R.M. Roop II, K.M. Peterson, pBBR1MCS: a broad-host-range cloning vector, Biotechniques 16 (1994) 800–802.
[55] L. Varanasi, D. Mills, A. Murphree, J. Gray, C. Purser, R. Baker, J. Hosler, Altering conserved lipid binding sites in cytochrome *c* oxidase of *Rhodobacter sphaeroides* perturbs the interaction between subunits I and III and promotes suicide inactivation of the enzyme, Biochemistry 45 (2006) 14896–14907.
[56] T. Donohue, S. Kaplan, Genetic techniques in *Rhodospirillaceae*, Methods Enzymol. 204 (1991) 459–485.
[57] M.F. Alexeyev, The pKNOCK series of broad-host-range mobilizable suicide vectors for gene knockout and targeted DNA insertion into the chromosome of gram-negative bacteria, Biotechniques 26 (1999) 824–826 828.
[58] J.M. Eraso, S. Kaplan, From redox flow to gene regulation: role of the PrrC protein of *Rhodobacter sphaeroides* 2.4.1, Biochemistry 39 (2000) 2052–2062.
[59] L. Varanasi, J. Hosler, Alternative initial proton acceptors for the D pathway of *Rhodobacter sphaeroides* cytochrome *c* oxidase, Biochemistry 50 (2011) 2820–2828.
[60] N. Sone, M. Yoshida, H. Hirata, Y. Kagawa, Asolectin purification procedure, J. Biochem. (Tokyo) 81 (1977) 519–528.
[61] P. Nicholls, V. Hildebrandt, B.C. Hill, F. Nicholls, J.M. Wrigglesworth, Pathways of cytochrome *c* oxidation by soluble and membrane-bound cytochrome aa_3, Can. J. Biochem. 58 (1980) 969–977.
[62] O. Preisig, R. Zufferey, L. Thony-Meyer, C.A. Appleby, H. Hennecke, A high-affinity cbb_3-type cytochrome oxidase terminates the symbiosis-specific respiratory chain of *Bradyrhizobium japonicum*, J. Bacteriol. 178 (1996) 1532–1538.
[63] C. Mackenzie, M. Choudhary, F.W. Larimer, P.F. Predki, S. Stilwagen, J.P. Armitage, R.D. Barber, T.J. Donohue, J.P. Hosler, J.E. Newman, J.P. Shapleigh, R.E. Sockett, J. Zeilstra-Ryalls, S. Kaplan, The home stretch, a first analysis of the nearly completed genome of *Rhodobacter sphaeroides* 2.4.1, Photosynth. Res. 70 (2001) 19–41.
[64] N.J. Mouncey, E. Gak, M. Choudhary, J. Oh, S. Kaplan, Respiratory pathways of *Rhodobacter sphaeroides* 2.4.1(T): identification and characterization of genes encoding quinol oxidases, FEMS Microbiol. Lett. 192 (2000) 205–210.
[65] J.I. Oh, S. Kaplan, The cbb_3 terminal oxidase of *Rhodobacter sphaeroides* 2.4.1: structural and functional implications for the regulation of spectral complex formation, Biochemistry 38 (1999) 2688–2696.
[66] S. Arnaud, F. Malatesta, B. Guigliarelli, J.P. Gayda, P. Bertrand, R. Miraglio, M. Denis, Purification and characterization of the oxidase from the marine bacterium *Pseudomonas nautica* 617, Eur. J. Biochem. 198 (1991) 349–356.
[67] D.J. Hunter, A.J. Moody, P.R. Rich, W.J. Ingledew, EPR spectroscopy of *Escherichia coli* cytochrome *bo* which lacks Cu_B, FEBS Lett. 412 (1997) 43–47.
[68] R. Aasa, P.J. Albracht, K.E. Falk, B. Lanne, T. Vanngard, EPR signals from cytochrome *c* oxidase, Biochim. Biophys. Acta 422 (1976) 260–272.
[69] P. Lappalainen, R. Aasa, B.G. Malmstrom, M. Saraste, Soluble Cu_A-binding domain from the *Paracoccus* cytochrome *c* oxidase, J. Biol. Chem. 268 (1993) 26416–26421.
[70] J. van der Oost, P. Lappalainen, A. Musacchio, A. Warne, L. Lemieux, J. Rumbley, R.B. Gennis, R. Aasa, T. Pascher, B.G. Malmstrom, et al., Restoration of a lost metal-binding site: construction of two different copper sites into a subunit of the *E. coli* cytochrome *o* quinol oxidase complex, EMBO J. 11 (1992) 3209–3217.
[71] V. Zickermann, A. Wittershagen, B.O. Kolbesen, B. Ludwig, Transformation of the Cu_A redox site in cytochrome *c* oxidase into a mononuclear copper center, Biochemistry 36 (1997) 3232–3236.
[72] M. Jaksch, C. Paret, R. Stucka, N. Horn, J. Muller-Hocker, R. Horvath, N. Trepesch, G. Stecker, P. Freisinger, C. Thirion, J. Muller, R. Lunkwitz, G. Rodel, E.A. Shoubridge, H. Lochmuller, Cytochrome *c* oxidase deficiency due to mutations in SCO2, encoding a mitochondrial copper-binding protein, is rescued by copper in human myoblasts, Hum. Mol. Genet. 10 (2001) 3025–3035.
[73] L. Salviati, E. Hernandez-Rosa, W.F. Walker, S. Sacconi, S. DiMauro, E.A. Schon, M.M. Davidson, Copper supplementation restores cytochrome *c* oxidase activity in cultured cells from patients with SCO2 mutations, Biochem. J. 363 (2002) 321–327.
[74] H. Loferer, M. Bott, H. Hennecke, *Bradyrhizobium japonicum* TlpA, a novel membrane-anchored thioredoxin-like protein involved in the biogenesis of cytochrome aa_3 and development of symbiosis, EMBO J. 12 (1993) 3373–3383.
[75] S. Elsen, L.R. Swem, D.L. Swem, C.E. Bauer, RegB/RegA, a highly conserved redox-responding global two-component regulatory system, Microbiol. Mol. Biol. Rev. 68 (2004) 263–279.
[76] Y.J. Kim, I.J. Ko, J.M. Lee, H.Y. Kang, Y.M. Kim, S. Kaplan, J.I. Oh, Dominant role of the cbb3 oxidase in regulation of photosynthesis gene expression through the PrrBA system in *Rhodobacter sphaeroides* 2.4.1, J. Bacteriol. 189 (2007) 5617–5625.
[77] J.I. Oh, S. Kaplan, Oxygen adaptation. The role of the CcoQ subunit of the cbb_3 cytochrome *c* oxidase of *Rhodobacter sphaeroides* 2.4.1, J. Biol. Chem. 277 (2002) 16220–16228.
[78] Y. Zhen, J. Qian, K. Follmann, T. Hayward, T. Nilsson, M. Dahn, Y. Hilmi, A.G. Hamer, J.P. Hosler, S. Ferguson-Miller, Overexpression and purification of cytochrome *c* oxidase from *Rhodobacter sphaeroides*, Protein Expr. Purif. 13 (1998) 326–336.