



# The roles of *Rhodobacter sphaeroides* copper chaperones PCu<sub>A</sub>C and Sco (PrrC) in the assembly of the copper centers of the aa<sub>3</sub>-type and the cbb<sub>3</sub>-type cytochrome *c* oxidases<sup>☆</sup>

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## ARTICLE INFO

### Article history:

Received 1 November 2011

Received in revised form 30 December 2011

Accepted 2 January 2012

Available online 8 January 2012

### Keywords:

cbb<sub>3</sub>-type cytochrome *c* oxidase

aa<sub>3</sub>-type cytochrome *c* oxidase

Copper chaperone

Copper center assembly

Cu<sub>A</sub>

Sco protein

## ABSTRACT

The  $\alpha$  proteobacter *Rhodobacter sphaeroides* accumulates two cytochrome *c* oxidases (CcO) in its cytoplasmic membrane during aerobic growth: a mitochondrial-like aa<sub>3</sub>-type CcO containing a di-copper Cu<sub>A</sub> center and mono-copper Cu<sub>B</sub>, plus a cbb<sub>3</sub>-type CcO that contains Cu<sub>B</sub> but lacks Cu<sub>A</sub>. Three copper chaperones are located in the periplasm of *R. sphaeroides*, PCu<sub>A</sub>C, PrrC (Sco) and Cox11. Cox11 is required to assemble Cu<sub>B</sub> of the aa<sub>3</sub>-type but not the cbb<sub>3</sub>-type CcO. PrrC is homologous to mitochondrial Sco1; Sco proteins are implicated in Cu<sub>A</sub> assembly in mitochondria and bacteria, and with Cu<sub>B</sub> assembly of the cbb<sub>3</sub>-type CcO. PCu<sub>A</sub>C is present in many bacteria, but not mitochondria. PCu<sub>A</sub>C of *Thermus thermophilus* metallates a Cu<sub>A</sub> center *in vitro*, but its *in vivo* function has not been explored. Here, the extent of copper center assembly in the aa<sub>3</sub>- and cbb<sub>3</sub>-type CcOs of *R. sphaeroides* has been examined in strains lacking PCu<sub>A</sub>C, PrrC, or both. The absence of either chaperone strongly lowers the accumulation of both CcOs in the cells grown in low concentrations of Cu<sup>2+</sup>. The absence of PrrC has a greater effect than the absence of PCu<sub>A</sub>C and PCu<sub>A</sub>C appears to function upstream of PrrC. Analysis of purified aa<sub>3</sub>-type CcO shows that PrrC has a greater effect on the assembly of its Cu<sub>A</sub> than does PCu<sub>A</sub>C, and both chaperones have a lesser but significant effect on the assembly of its Cu<sub>B</sub> even though Cox11 is present. Scenarios for the cellular roles of PCu<sub>A</sub>C and PrrC are considered. The results are most consistent with a role for PrrC in the capture and delivery of copper to Cu<sub>A</sub> of the aa<sub>3</sub>-type CcO and to Cu<sub>B</sub> of the cbb<sub>3</sub>-type CcO, while the predominant role of PCu<sub>A</sub>C 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.

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## 1. Introduction

During aerobic growth in the laboratory the  $\alpha$  proteobacter *Rhodobacter sphaeroides* accumulates two cytochrome *c* oxidases (CcOs) in the cytoplasmic membrane. One is an aa<sub>3</sub>-type CcO with high similarity to mitochondrial CcO [1–5] while the other is a cbb<sub>3</sub>-type CcO, evolutionarily distant from the aa<sub>3</sub>-type CcO [6,7]. The

aa<sub>3</sub>-type CcO contains two heme A and two copper centers [4,5,8]. The di-copper Cu<sub>A</sub> 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  $\alpha$  proteobacteria, both soluble cytochromes *c* as well as membrane-anchored cytochrome *c*<sub>y</sub> transfer electrons to Cu<sub>A</sub>[9–14]. The two coppers of Cu<sub>A</sub> 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<sub>3</sub>-Cu<sub>B</sub> site in subunit I, where O<sub>2</sub> is reduced to water [15]. The mono-copper Cu<sub>B</sub> 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<sub>3</sub>-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<sub>2</sub> reduction site composed of five-coordinate heme b<sub>3</sub> plus Cu<sub>B</sub>[6,17,18]. However, the cbb<sub>3</sub>-type CcO lacks Cu<sub>A</sub>[6]. Instead of a subunit II like that of the aa<sub>3</sub>-type CcO, the cbb<sub>3</sub>-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*-chlorophenylhydrazine; CcO, cytochrome *c* oxidase; ICP-OES, inductively coupled plasma optical emission spectroscopy; TMPD, N,N,N',N'-tetramethyl-*p*-phenylenediamine

<sup>☆</sup> This article is part of a Special Issue entitled: Biogenesis/Assembly of Respiratory Enzyme Complexes.

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accept electrons from soluble cytochromes *c* or from membrane-bound cytochrome *c<sub>y</sub>* [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 *ccb<sub>3</sub>*-type CcO in other  $\alpha$  proteobacters [25–28]. The roles of three periplasmic copper chaperones – Sco, Cox11 and PCu<sub>A</sub>C – 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<sub>A</sub> 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<sub>A</sub>. The deletion of Sco from *B. subtilis* strongly reduces the expression of a *caa<sub>3</sub>*-type CcO containing Cu<sub>A</sub> but not the expression of a quinol oxidase that lacks Cu<sub>A</sub> [36]. Similarly, the deletion of the gene for Sco in *Bradyrhizobium japonicum* decreases the accumulation of its *aa<sub>3</sub>*-type CcO that contains Cu<sub>A</sub>, but not the *ccb<sub>3</sub>*-type CcO that lacks Cu<sub>A</sub> [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<sub>A</sub> assembly might be restricted to the reduction of the cysteines of the apo-Cu<sub>A</sub> center. In fact, a soluble form of Sco of *Thermus thermophilus* was found to reduce the disulfide of *Thermus* apo-Cu<sub>A</sub> *in vitro*, but it could not metallate the reduced apo-Cu<sub>A</sub> center [40].

Sco proteins have also been implicated in the assembly of Cu<sub>B</sub>. The membrane-bound copper chaperone Cox11 is absolutely required for the assembly of the Cu<sub>B</sub> center in the *aa<sub>3</sub>*-type CcOs of  $\alpha$  proteobacteria and mitochondria [30,41,42]. However, Cox11 is not required for the insertion of Cu<sub>B</sub> into the *ccb<sub>3</sub>*-type CcOs [37,41,42]. *Rhodobacter capsulatus* and *Pseudomonas aeruginosa* strains lacking their Sco proteins exhibit decreased accumulation of active *ccb<sub>3</sub>*-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<sub>B</sub> center of the *ccb<sub>3</sub>*-type CcO in these species. In contrast, the deletion of Sco from *B. japonicum* does not affect the accumulation of its *ccb<sub>3</sub>*-type CcO [37].

Many bacteria contain a periplasmic copper chaperone of the PCu<sub>A</sub>C 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<sub>A</sub>C of *R. sphaeroides* and other  $\alpha$  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<sub>A</sub>C is 53–55% similar to its homologs in *Deinococcus radiodurans* and *Thermus thermophilus*, for which solution structures have been determined [40,46]. PCu<sub>A</sub>C 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<sub>A</sub>C inserted copper into the Cu<sub>A</sub> site of soluble subunit II of the *Thermus* *ba<sub>3</sub>*-type CcO [40]. One problem in

extrapolating from this result to a universal mechanism for Cu<sub>A</sub> assembly is that PCu<sub>A</sub>C is not present in mitochondria.

It has also been suggested that PCu<sub>A</sub>C may deliver copper to the Cu<sub>B</sub> center of the *ccb<sub>3</sub>*-type CcO of *B. japonicum* [37]. In a strain of *B. japonicum* lacking one of its two predicted PCu<sub>A</sub>C proteins, total CcO activity decreased but the *ccb<sub>3</sub>*-type CcO was not assayed independently [48].

With the demonstration that PCu<sub>A</sub>C delivers copper to a Cu<sub>A</sub> site *in vitro* [40], plus the suggestion that PCu<sub>A</sub>C may be responsible for the assembly of Cu<sub>B</sub> of a *ccb<sub>3</sub>*-type CcO [37], there exists a need to explore the role of PCu<sub>A</sub>C in the cell. For example, to what extent is PCu<sub>A</sub>C required for the assembly of either the *aa<sub>3</sub>*-type CcO or the *ccb<sub>3</sub>*-type CcO? If both PCu<sub>A</sub>C 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<sub>3</sub>*-type and the *ccb<sub>3</sub>*-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<sub>A</sub>C has a significant effect on the assembly of Cu<sub>A</sub> of the *aa<sub>3</sub>*-type CcO and Cu<sub>B</sub> of the *ccb<sub>3</sub>*-type CcO. However, PrrC (*R. sphaeroides* Sco) has an even greater effect and PCu<sub>A</sub>C 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 Siström's media A [53] supplemented with 1  $\mu$ g/ml tetracycline, 50  $\mu$ g/ml spectinomycin and streptomycin and 25  $\mu$ g/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<sub>4</sub> from a stock solution. Glass and plastic containers used in the formulation and storage of concentrated media were bathed in 100  $\mu$ M 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<sub>4</sub> was added. For each growth, cells were taken from frozen stocks and grown on Siström'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 inoculate 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 21 baffled flasks for growth at 32 °C with rapid shaking. Cells were grown to late exponential phase (O.D.<sub>660 nm</sub> = 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<sub>A</sub>C

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

PCu<sub>A</sub>C under the control of the *R. sphaeroides* promoter for *coxI*, the gene for subunit I of the *aa*<sub>3</sub>-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<sub>A</sub>C gene (*Nde*I fwd 5'-GCCAAATCACACAGTCAGGAGAGACatATGACCCCG-3') and a *Sac*I restriction site in the 3' non-coding region of the gene (*Sac*I rev 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<sub>A</sub>C was inserted. The final product, named pPCu<sub>A</sub>C, contains the gene for PCu<sub>A</sub>C under the control of the *coxI* promoter; pPCu<sub>A</sub>C was transformed into *E. coli* S-17 for conjugation into *R. sphaeroides* by established procedures [56].

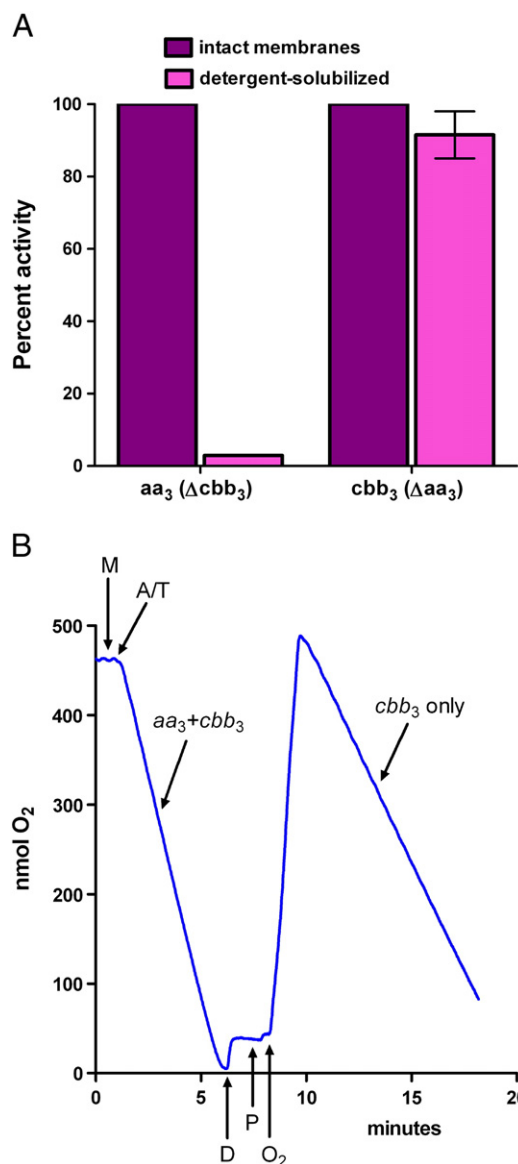
### 2.3. Inactivation of the genomic gene for PCu<sub>A</sub>C

Starting with pPCu<sub>A</sub>C, a *Pst*I site and a *Bam*HI site were introduced into the gene for PCu<sub>A</sub>C 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 *Bam*HI 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<sub>A</sub>C by pJG245 and the absence of a normal gene in the genome. One of these strains was retained as *R. sphaeroides* ΔPCu<sub>A</sub>C.

*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<sub>A</sub>C in *R. sphaeroides* PRRC4 to create ΔPrRC–ΔPCu<sub>A</sub>C.

### 2.4. Activity assays

The activity of purified *aa*<sub>3</sub>-type CcO was measured as previously described [59]. Simultaneous measurements of the activity of the *aa*<sub>3</sub>-type CcO and the *cbb*<sub>3</sub>-type CcO in purified cytoplasmic membranes were performed as O<sub>2</sub> consumption assays using a Clark-type O<sub>2</sub> electrode (Yellow Springs) and a YSI 5300 dissolved O<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> was returned to the reaction cuvette by blowing humidified 100% O<sub>2</sub> over the top of the solution until the O<sub>2</sub> concentration in the reaction cell equaled that of air-saturated buffer (*i.e.* the concentration of O<sub>2</sub> at the beginning of the experiment). The rate of ascorbate/TMPD-driven O<sub>2</sub> consumption was further measured until most of the O<sub>2</sub> in the cuvette was consumed. A representative O<sub>2</sub> 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*<sub>3</sub>-type CcO and the *cbb*<sub>3</sub>-type CcO in purified cytoplasmic membranes. The activity of the *aa*<sub>3</sub>-type CcO was assayed in membranes isolated from *R. sphaeroides* CBB3Δ, which lacks the structural genes for the *cbb*<sub>3</sub>-type CcO [77]. The activity of the *cbb*<sub>3</sub>-type CcO was assayed in membranes isolated from *R. sphaeroides* YZ200, which lacks the *coxII*–III operon for the *aa*<sub>3</sub>-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*<sub>3</sub>-type CcO is lost when detergent disrupts its interaction with membrane-bound cytochrome *c*<sub>v</sub>, but the TMPD oxidase activity of the *cbb*<sub>3</sub>-type CcO is retained. Error is standard deviation. B. A representative O<sub>2</sub> electrode tracing of the assay for the TMPD oxidase activity of the *aa*<sub>3</sub>-type and *cbb*<sub>3</sub>-type CcOs. See **Material and methods and Results** for details. Intact, purified cytoplasmic membranes (M) are added to the reaction cell and O<sub>2</sub> consumption is initiated by the addition of ascorbate and TMPD (A/T). The first register measures total CcO activity (*aa*<sub>3</sub> plus *cbb*<sub>3</sub>). After all O<sub>2</sub> has been consumed, dodecyl maltoside (D) is added to solubilize the membranes and thereby disrupt the interaction of cytochrome *c*<sub>v</sub> with the *aa*<sub>3</sub>-type CcO. After 2 min, soybean phospholipids are added (P) followed by humidified O<sub>2</sub>, which restores O<sub>2</sub> consumption activity. In the second register, only the activity of the *cbb*<sub>3</sub>-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  $<0.01 \mu\text{M}$ . Samples containing 3.5 ml of  $4 \mu\text{M}$  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

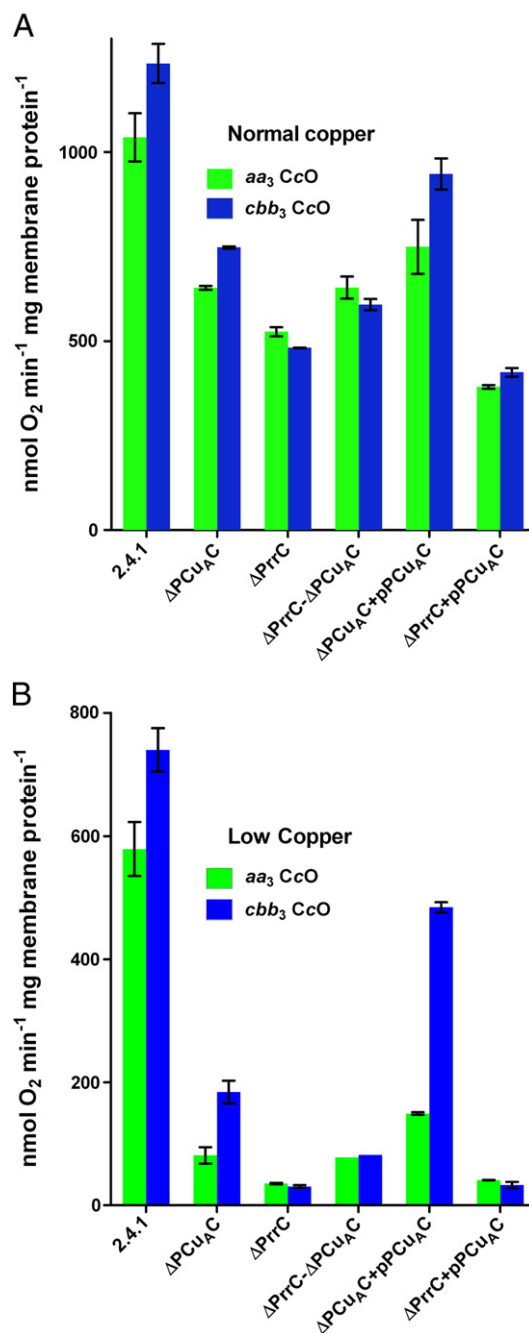
Cytoplasmic membranes were purified as in Hosler et al. [1]. The  $aa_3$ -type CcO was purified by Ni-affinity chromatography [41] followed by FPLC anion exchange chromatography on DEAE-5PW (Toso-Haas) [51]. The concentrations of total membrane protein were determined using the BioRad DC Protein Assay 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<sub>A</sub>C 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  $\alpha$  band absorbance  $\sim 605$  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  $\alpha$  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<sub>2</sub> 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<sub>y</sub>* is present [20]. The cytochrome *c<sub>y</sub>:aa<sub>3</sub>* 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<sub>2</sub> 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<sub>y</sub>:aa<sub>3</sub>* complex is fully dissociated. The amount of cytochrome *c<sub>y</sub>* 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<sub>y</sub>* 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



**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<sub>A</sub>C. Assays were performed as described in Material and methods and Results. A. Oxidase activities in membranes isolated from cells grown in  $1.6 \mu\text{M Cu}^{2+}$ . B. Oxidase activities in membranes isolated from cells grown in  $<50 \text{ nM Cu}^{2+}$ . Error is standard deviation.

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.

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*<sub>3</sub>-type CcO [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<sub>A</sub>C deletions on CcO 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<sub>A</sub>C, accumulate both the *aa*<sub>3</sub>-type and the *cbb*<sub>3</sub>-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<sub>A</sub>C was created by inactivating the gene for PCu<sub>A</sub>C in *R. sphaeroides* 2.4.1 using a pKNOCK construct as described in Material and methods. A strain lacking both chaperones (ΔPrrC–ΔPCu<sub>A</sub>C) was created by using the same pKNOCK construct to inactivate the gene for PCu<sub>A</sub>C in *R. sphaeroides* PRRC4. In cells grown aerobically in media containing 1.6 μM Cu<sup>2+</sup>, the absence of PrrC leads to a 50% decrease in the content of fully assembled *aa*<sub>3</sub>-type CcO and a 60% loss of fully assembled *cbb*<sub>3</sub>-type CcO (Fig. 2A). The absence of PCu<sub>A</sub>C leads to a 40% decrease in both oxidases. These decreases imply that both PrrC and PCu<sub>A</sub>C play a role in the assembly of the *aa*<sub>3</sub>-type CcO and the *cbb*<sub>3</sub>-type CcO. 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<sub>A</sub>C were then grown in copper-deficient media, as described in Material and methods. With a concentration of <50 nM Cu<sup>2+</sup> 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<sup>2+</sup> as it does in media containing 1.6 μM Cu<sup>2+</sup> (data not shown). In wild-type cells grown in low copper, the amounts of fully assembled *aa*<sub>3</sub>-type CcO and *cbb*<sub>3</sub>-type CcO in the cell membrane each decrease by ~40% (Fig. 2A, B). Assuming an initial Cu<sup>2+</sup> 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*<sub>3</sub>-type and the *cbb*<sub>3</sub>-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*<sub>3</sub>-type and *cbb*<sub>3</sub>-type CcO to only 6% and 4%, respectively, of their levels in normal cells grown in low copper (Fig. 2B). The absence of PCu<sub>A</sub>C has a significant but less detrimental effect on oxidase assembly. Accumulation of the fully assembled *aa*<sub>3</sub>-type CcO in ΔPCu<sub>A</sub>C cells is 14% that of wild-type cells grown in low copper media while the accumulation of active *cbb*<sub>3</sub>-type CcO is 25% that of wild-type (Fig. 2B). While the reduced accumulations of active *aa*<sub>3</sub>-type CcO could be due to decreased Cu<sub>A</sub> or Cu<sub>B</sub> assembly, or both, the decreases in active *cbb*<sub>3</sub>-type CcO should only reflect impaired assembly of its Cu<sub>B</sub> center.

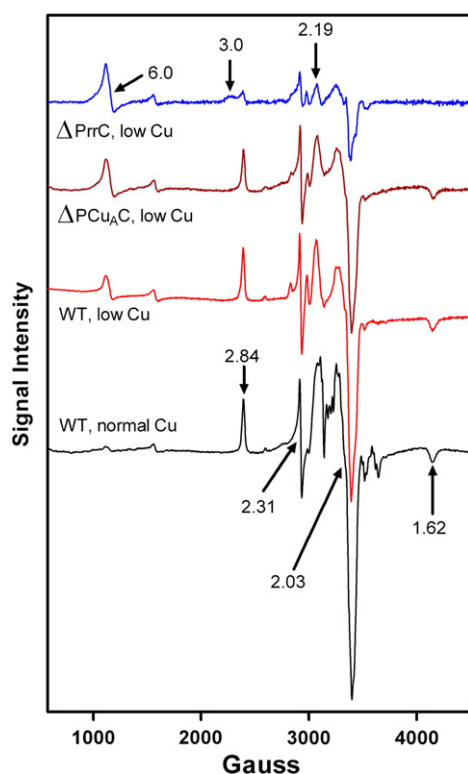
A significant question is whether PrrC and PCu<sub>A</sub>C have parallel, redundant roles in the assembly of the copper centers or more separate functions. Two tests were performed to explore this question. First, a strain lacking both PrrC and PCu<sub>A</sub>C was prepared. If the role of PCu<sub>A</sub>C

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<sub>A</sub>C individually. In fact, the assembly and accumulation of the *aa*<sub>3</sub>-type CcO and the *cbb*<sub>3</sub>-type CcO in cells lacking both PrrC and PCu<sub>A</sub>C is no less than in cells lacking only PrrC or only PCu<sub>A</sub>C (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<sub>A</sub>C was created (pPCu<sub>A</sub>C; see Material and methods) in order to vary the *in vivo* ratio of PCu<sub>A</sub>C to PrrC. The introduction of pPCu<sub>A</sub>C into the copper-limited ΔPCu<sub>A</sub>C strain increases the accumulation of the *aa*<sub>3</sub>-type CcO from 14% to 26%, compared to wild-type, and the synthesis of the *cbb*<sub>3</sub>-type CcO from 25% of wild-type to 65% (Fig. 2B). The incomplete restoration of CcO assembly suggests that the plasmid-borne expression of PCu<sub>A</sub>C is less efficient than the expression of PCu<sub>A</sub>C from the genome. Nonetheless, the introduction of pPCu<sub>A</sub>C has an obvious effect. Therefore, it is significant that using pPCu<sub>A</sub>C to increase the amount of PCu<sub>A</sub>C in cells lacking PrrC fails to enhance the assembly of the *aa*<sub>3</sub>-type CcO and yields but a slight increase in the accumulation of the *cbb*<sub>3</sub>-type CcO, from 4% to 8% (Fig. 2B). The results suggest that PCu<sub>A</sub>C cannot compensate for the absence of PrrC in the synthesis of the *aa*<sub>3</sub>-type CcO or the *cbb*<sub>3</sub>-type CcO, *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<sub>A</sub>C and PrrC deletions on the Cu<sub>A</sub> and Cu<sub>B</sub> centers of the *aa*<sub>3</sub>-type CcO

Unlike the *cbb*<sub>3</sub>-type CcO which contains a single copper as Cu<sub>B</sub>, decreased accumulation of active *aa*<sub>3</sub>-type CcO in the chaperone deletion strains could result from impaired assembly of Cu<sub>B</sub> or the di-copper Cu<sub>A</sub> center, or both. In order to explore these possibilities, the *aa*<sub>3</sub>-type CcO was purified from wild-type *R. sphaeroides* cells, ΔPCu<sub>A</sub>C and ΔPrrC and examined by X-band EPR spectroscopy and metal analysis. Fig. 3 shows the EPR spectra of four purified CcO 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*<sub>3</sub>-type CcO 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<sub>A</sub> can be assessed using the peak-to-trough amplitude of the *g* = 2.03 signal for Cu<sub>A</sub> in X-band EPR spectra of oxidized CcO. The population of the *aa*<sub>3</sub>-type CcO purified from wild-type cells grown in copper-depleted media shows ~30% less Cu<sub>A</sub> than CcO isolated from wild-type cells grown in copper-sufficient media. This indicates that the lower content of active *aa*<sub>3</sub>-type CcO in membranes of wild-type cells grown in low copper (Fig. 2) is mostly due to the decreased assembly of Cu<sub>A</sub>. The result also confirms that copper is a limiting reagent for *R. sphaeroides* cells grown in low copper media. The absence of PCu<sub>A</sub>C further lowers the Cu<sub>A</sub> content of purified *aa*<sub>3</sub>-type CcO by approximately 15%. Assembly of the *aa*<sub>3</sub>-type CcO in the presence of PCu<sub>A</sub>C but the absence of PrrC has a more pronounced effect; the Cu<sub>A</sub> content is reduced to ~20% of that present in the *aa*<sub>3</sub>-type CcO isolated from wild-type cells grown in 1.6 μM Cu<sup>2+</sup> or ~30% of the Cu<sub>A</sub> content of wild-type cells grown in low copper. Protein gels (not shown) indicate that the loss of Cu<sub>A</sub> is not accompanied by a decrease in the content of subunit II.

Unlike Cu<sub>A</sub>, there are no direct signals for Cu<sub>B</sub> in the X-band EPR spectrum. There are, however, two heme-specific transitions that are qualitatively characteristic for CcO lacking Cu<sub>B</sub>, as described previously for CcO that assembles in the absence of functional Cox11

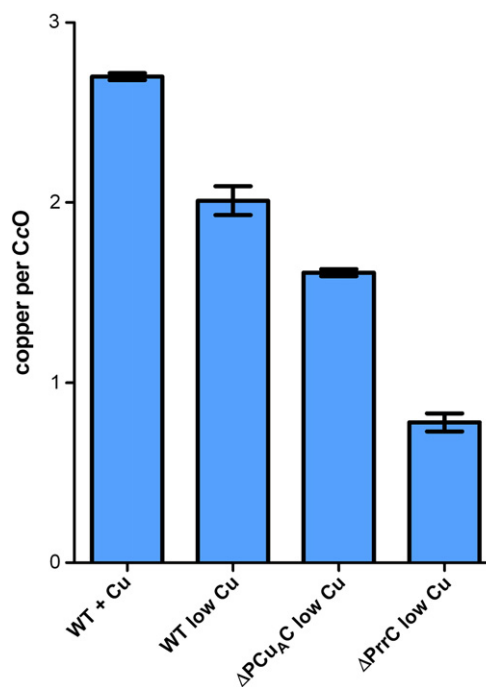


**Fig. 3.** EPR spectra of purified  $aa_3$ -type CcO assembled in wild-type cells grown in  $1.6 \mu\text{M Cu}^{2+}$  or  $<50 \text{ nM Cu}^{2+}$  (low Cu), and in cells lacking PCu<sub>A</sub>C or PrrC and grown in  $<50 \text{ nM Cu}^{2+}$ . The spectra were recorded at X band using a Bruker (Billerica, MA) EMX spectrometer. Each spectrum is an average of ten scans taken at 10 K using 25–50  $\mu\text{M CcO}$ . 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.

[41,42]. In normal, oxidized CcO, heme  $a_3$  and Cu<sub>B</sub> are strongly spin-coupled and therefore EPR silent [67]. When Cu<sub>B</sub> is reduced to Cu<sup>1+</sup> 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<sub>B</sub> but contains all of the other metal centers (termed  $\Delta\text{Cox11}$  or  $\Delta\text{Cu}_B$ ) [41]. However, in  $\Delta\text{Cu}_B$  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<sub>B</sub> 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<sub>B</sub> 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  $\Delta\text{PCu}_A\text{C}$  and  $\Delta\text{PrrC}$  (Fig. 3) reflect the loss of Cu<sub>B</sub> but it is difficult to use this signal to quantify the extent of the loss.

Another Cu<sub>B</sub>-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<sub>B</sub>, 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  $\Delta\text{PrrC}$  cells (Fig. 3) indicating that this protein contains less Cu<sub>B</sub> than CcO isolated from  $\Delta\text{PCu}_A\text{C}$  or wild-type cells.

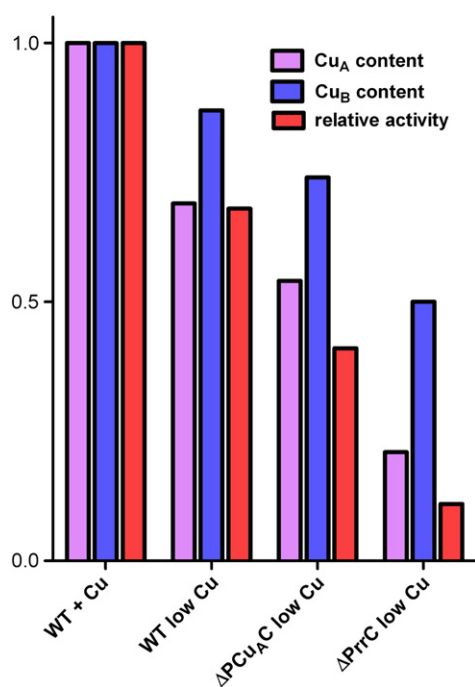
Quantitative estimates of the relative amounts of Cu<sub>A</sub> and Cu<sub>B</sub> present in the samples of purified  $aa_3$ -type CcO are possible if the total amount of copper is also known. The total copper content of the samples was determined using ICP-OES (Fig. 4); the concentration of CcO



**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.

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  $\Delta\text{PCu}_A\text{C}$  is  $\sim 60\%$  that of the copper present in wild-type cells grown in  $1.6 \mu\text{M Cu}^{2+}$ , while the  $aa_3$ -type CcO isolated from  $\Delta\text{PrrC}$  contains 29% of the normal amount of copper (Fig. 4). The fractional amounts of Cu<sub>A</sub> (Fig. 5) present in the  $aa_3$ -type CcOs isolated from wild-type *R. sphaeroides*,  $\Delta\text{PCu}_A\text{C}$  and  $\Delta\text{PrrC}$  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<sub>B</sub>, the amount of copper present as Cu<sub>A</sub> is subtracted from the total amount of copper given by the data of Fig. 4. The calculations assume two coppers per Cu<sub>A</sub> because studies of the reconstitution of Cu<sub>A</sub> sites show that the normal set of Cu<sub>A</sub> ligands will bind two coppers, but not one [69–71]. The results compiled in Fig. 5 show that the absence of PCu<sub>A</sub>C or PrrC affects the assembly of both Cu<sub>A</sub> and Cu<sub>B</sub>, but the absence of either chaperone has a greater effect on the assembly of Cu<sub>A</sub> than Cu<sub>B</sub>. Also, the absence of PrrC has a greater effect on the assembly of Cu<sub>A</sub> and Cu<sub>B</sub> than does the absence of PCu<sub>A</sub>C.

The decreased content of Cu<sub>B</sub> in the  $aa_3$ -type CcOs of  $\Delta\text{PrrC}$  and  $\Delta\text{PCu}_A\text{C}$  could be an indirect consequence of impaired assembly of Cu<sub>A</sub> if the Cu<sub>B</sub> center is destabilized by the absence of Cu<sub>A</sub>. In other words, the loss of Cu<sub>B</sub> may only occur in those CcO molecules that already lack Cu<sub>A</sub>. However, previous work shows that the reverse interaction does not take place, i.e. the absence of Cu<sub>B</sub> does not destabilize Cu<sub>A</sub>[41]. Moreover, the presence of a stable CcO form that lacks Cu<sub>A</sub> but not Cu<sub>B</sub> 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<sub>A</sub> but only a 10% loss of Cu<sub>B</sub> (Fig. 5). The decreased contents of Cu<sub>B</sub> (Fig. 5) could also arise from the formation of  $\Delta\text{Cu}_B$ , i.e. CcO lacking only Cu<sub>B</sub>, in the absence of PCu<sub>A</sub>C or PrrC. Measurements of the O<sub>2</sub> reduction activities of the CcO forms (Fig. 5) support the latter hypothesis. For the CcOs isolated from



**Fig. 5.** The relative Cu<sub>A</sub> content, Cu<sub>B</sub> content and O<sub>2</sub> reduction activity of purified *aa*<sub>3</sub>-type CcO assembled in wild-type cells grown in 1.6 μM Cu<sup>2+</sup> or <50 nM Cu<sup>2+</sup> (low Cu), and in cells lacking PCu<sub>A</sub>C or PrrC and grown in <50 nM Cu<sup>2+</sup>. The fractional content of Cu<sub>A</sub>, relative to wild-type cells grown in 1.6 mM Cu<sup>2+</sup>, is taken from the amplitudes of the *g* = 2.03 signal in Fig. 3. The fractional content of Cu<sub>B</sub> is estimated as described in Results. The relative activities are taken from the TN<sub>max</sub> values for cytochrome *c*-driven O<sub>2</sub> reduction measured at pH 6.5 as described in Varanasi and Hosler [59]. The TN<sub>max</sub> for the wild-type *aa*<sub>3</sub>-type CcO grown in 1.6 mM Cu<sup>2+</sup> (100%) is 1717 ± 41 e<sup>-</sup>s<sup>-1</sup>.

ΔPCu<sub>A</sub>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<sub>B</sub> is absent only from those CcO molecules that already lack Cu<sub>A</sub>. 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<sub>A</sub> or Cu<sub>B</sub>.

#### 4. Discussion

Both PCu<sub>A</sub>C and PrrC enhance the assembly of the *aa*<sub>3</sub>-type CcO, particularly when exogenous copper levels are low (<50 nM Cu<sup>2+</sup>), 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*<sub>3</sub>-type CcO in the bacterial membrane by up to 94% while the absence of PCu<sub>A</sub>C 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*<sub>3</sub>-type CcO in the absence of PrrC in *R. sphaeroides* (Fig. 2A); similar results have been reported for strains of *B. subtilis* and *B. japonicum* lacking Sco1 [36,37], and in human cells lacking fully functional Sco2 [72,73]. The chaperone associated with Cu<sub>B</sub> assembly in the *aa*<sub>3</sub>-type CcO, Cox11, is present in *R. sphaeroides* ΔPrrC and ΔPCu<sub>A</sub>C. Accordingly, normal assembly of Cu<sub>B</sub> takes place in ΔPrrC and ΔPCu<sub>A</sub>C when they are grown in copper-sufficient media (data not shown). As for Cu<sub>A</sub> assembly, the greater concentration of Cu<sup>2+</sup> may facilitate its self-assembly or it may drive the metallation of Cu<sub>A</sub> by a chaperone with a low efficiency of copper delivery to Cu<sub>A</sub>. The presence of sufficient exogenous copper may be responsible for the finding that the

deletion of PCu<sub>A</sub>C in *B. japonicum* has no effect on the CcO activity of aerobically-grown cells [48].

PrrC and PCu<sub>A</sub>C also enhance the assembly of the *cbb*<sub>3</sub>-type CcO, especially in cells grown in limited amounts of copper. In the absence of PrrC, the accumulation of active *cbb*<sub>3</sub>-type CcO is reduced by 96% while in the absence of PCu<sub>A</sub>C 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*<sub>3</sub>-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*<sub>3</sub>-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*<sub>3</sub> plus *cbb*<sub>3</sub>) 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<sup>2+</sup> [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<sub>A</sub>C and PrrC in the assembly of the Cu<sub>B</sub> center of the *aa*<sub>3</sub>-type CcO

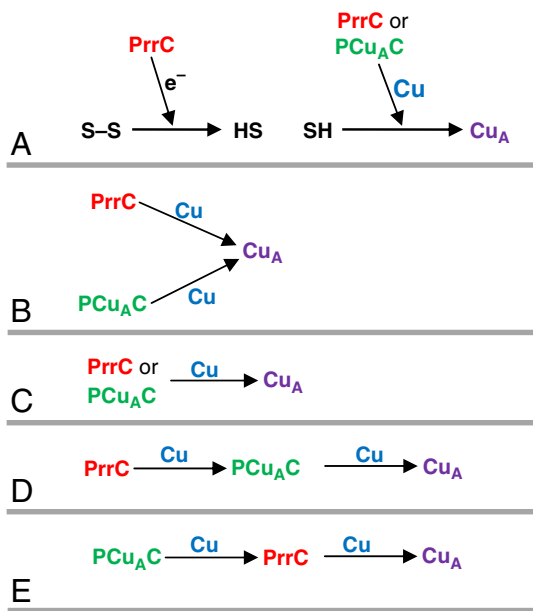
In studies in *B. subtilis*, *B. japonicum* and mitochondria, the decreased accumulation of the *aa*<sub>3</sub>-type CcO in the absence of Sco has been attributed to impaired assembly of the Cu<sub>A</sub> center [30,36,37]. The results of this study show that the absence of PrrC, and to a lesser extent PCu<sub>A</sub>C, impairs the assembly of both Cu<sub>A</sub> and Cu<sub>B</sub> of the *aa*<sub>3</sub>-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<sub>A</sub> but without Cu<sub>B</sub>. Nonetheless, Cu<sub>B</sub> assembly requires Cox11, which is present in all of the strains used in these experiments, and neither PrrC nor PCu<sub>A</sub>C can assemble Cu<sub>B</sub> 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<sup>2+</sup> concentrations.

#### 4.2. Cellular roles of PCu<sub>A</sub>C and PrrC in the assembly of Cu<sub>A</sub> of the *aa*<sub>3</sub>-type CcO

The question of how Sco and PCu<sub>A</sub>C participate in the assembly of Cu<sub>A</sub> has previously been addressed using an *in vitro* system by Abriata et al. [40]. A soluble form of PCu<sub>A</sub>C of *Thermus thermophilus* delivered both coppers to the ligands of Cu<sub>A</sub> in a soluble form of subunit II of the *ba*<sub>3</sub>-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<sub>A</sub> but it could provide the disulfide reductase activity necessary to reduce the cysteines of the Cu<sub>A</sub> center prior to copper delivery.

In this study, the analyses of purified *aa*<sub>3</sub>-type oxidase show that the absence of PrrC or PCu<sub>A</sub>C primarily impairs the assembly of Cu<sub>A</sub>, with PrrC having the greater effect. The assembly of Cu<sub>B</sub> 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*<sub>3</sub>-type CcO in the cytoplasmic membranes of different strains of *R. sphaeroides* (Fig. 2) can be used to explore how Sco (PrrC) and PCu<sub>A</sub>C participate in the assembly of Cu<sub>A</sub> in the cell. This is possible because 1) the activity of the *aa*<sub>3</sub>-type CcO depends upon the successful assembly of both Cu<sub>B</sub> and Cu<sub>A</sub>, and 2) the major effect of deleting either chaperone is impairment of the assembly of Cu<sub>A</sub>.

Several scenarios of the cellular roles of the two copper chaperones in the process of Cu<sub>A</sub> 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<sub>A</sub>C and PrrC in the assembly of Cu<sub>A</sub> of the aa<sub>3</sub>-type CcO. Explanations are provided in the text. In Scheme A the sulfurs (S) are those of the two cysteine ligands of Cu<sub>A</sub>.

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<sub>A</sub> 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<sub>A</sub> assembly in ΔPrrC cells grown in higher copper, even though the requirement for PrrC as the predominant disulfide reductase should remain. Second, *B. subtilis* and *B. japonicum* also assemble Cu<sub>A</sub> 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<sub>3</sub>-type CcO [74]. Scenario B of Fig. 6 posits that PCu<sub>A</sub>C and PrrC each deliver one copper to Cu<sub>A</sub>. 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<sub>A</sub>C. In Scenario C, PrrC and PCu<sub>A</sub>C are independently capable of delivering both coppers to Cu<sub>A</sub>. The greater effect of PrrC on Cu<sub>A</sub> assembly does not rule out this possibility, e.g. PrrC could be present in significantly greater amounts than PCu<sub>A</sub>C. However, Scenario C does predict that removing both PrrC and PCu<sub>A</sub>C should impair the assembly of the aa<sub>3</sub>-type oxidase more than removing either chaperone alone. This is not observed; the double deletion of PrrC and PCu<sub>A</sub>C shows no greater loss of the aa<sub>3</sub>-type CcO than either of the separate deletions. Moreover, increasing the PCu<sub>A</sub>C population in the cell membrane fails to enhance the assembly of Cu<sub>A</sub> if PrrC is absent (ΔPrrC + pPCu<sub>A</sub>C; Fig. 2B). This is inconsistent with a significant role for PCu<sub>A</sub>C in directly delivering both coppers to Cu<sub>A</sub>. The failure of increased expression of PCu<sub>A</sub>C to enhance CcO assembly in cells lacking PrrC also disfavors Scenario D, in which PCu<sub>A</sub>C delivers both coppers to Cu<sub>A</sub> while the (observed) stronger requirement for PrrC is postulated to derive from its ability to drive the assembly process by efficiently metallating PCu<sub>A</sub>C. Scenario D is further disfavored by the evolutionary relationship between *R. sphaeroides* and mitochondria. Mitochondria do not contain a homolog of PCu<sub>A</sub>C, 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<sub>3</sub>-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<sub>A</sub> in *R. sphaeroides* will be fundamentally similar to that of mitochondria. Scenario E, in which PrrC (Sco) delivers both coppers to Cu<sub>A</sub>, is consistent with all of the data of this study, as well as with previous observations in *B. subtilis*, *B. japonicum* and mitochondria [30,36,37]. PCu<sub>A</sub>C is posited to supply copper to PrrC, based on the finding that its presence does enhance the assembly of Cu<sub>A</sub>. This assignment is equivalent to stating that PCu<sub>A</sub>C 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<sub>A</sub>C 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<sub>A</sub> assembly study of Abriata et al. [40] in which *T. thermophilus* Sco does not deliver copper to soluble *T. thermophilus* Cu<sub>A</sub>. Interestingly, *T. thermophilus* lacks Cox11, indicating that its system for the assembly of Cu<sub>B</sub> of its ba<sub>3</sub>-type CcO differs from those bacteria that synthesize a mitochondrial-like aa<sub>3</sub>-type CcO. Thus, *T. thermophilus* may also have different requirements for the assembly of Cu<sub>A</sub> of the ba<sub>3</sub>-type CcO. As stated above, the similarities between the aa<sub>3</sub>-type CcOs of *R. sphaeroides* and mitochondria, along with the retention of Cox11 and Sco in both, make it likely that their Cu<sub>A</sub> and Cu<sub>B</sub> assembly pathways share basic commonality.

#### 4.3. The roles of PrrC and PCu<sub>A</sub>C in the assembly of Cu<sub>B</sub> of the cbb<sub>3</sub>-type CcO

The roles of PCu<sub>A</sub>C and PrrC in the assembly of the Cu<sub>B</sub> center of the cbb<sub>3</sub>-type CcO appear remarkably similar to their roles in the assembly of the Cu<sub>A</sub> center of the aa<sub>3</sub>-type CcO. The removal of either PrrC or PCu<sub>A</sub>C decreases the assembly of Cu<sub>B</sub> in the cbb<sub>3</sub>-type CcO, using the amount of active oxidase as a measure of the extent of Cu<sub>B</sub> assembly. The removal of PrrC has a greater effect than the removal of PCu<sub>A</sub>C and increasing the amount of PCu<sub>A</sub>C in the cell membrane enhances the assembly of Cu<sub>B</sub> only if PrrC is present. Once again, the most straightforward conclusion is that PrrC delivers copper to the Cu<sub>B</sub> center and that this method of assembly predominates when exogenous copper levels are low. PCu<sub>A</sub>C enhances the assembly of Cu<sub>B</sub>, 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<sub>3</sub>-type CcOs [43,44]. Moreover, Ekici et al. [28] report physical interactions between *R. capsulatus* Sco (SenC) and subunits of its cbb<sub>3</sub>-type CcO. Further, a putative Cu-ATPase, CcoI, has been shown to be required for the assembly of the cbb<sub>3</sub>-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<sub>B</sub> 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<sub>3</sub>-type and cbb<sub>3</sub>-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<sub>3</sub>-type CcO from ΔPrrC cells grown in low copper shows directly that the loss of active CcO is due to decreased assembly of Cu<sub>A</sub> and Cu<sub>B</sub> (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*<sub>3</sub>-type CcO [43]. However, this decrease in expression was shown to be a secondary result of a larger decrease in CcO accumulation due to impaired assembly of Cu<sub>B</sub> in the absence of SenC, since the expression of the *cbb*<sub>3</sub>-type CcO in *R. capsulatus* is partly controlled by a signal transduction system that responds to the level of its activity [75,76].

## Acknowledgements

Supported by National Institutes of Health Grant GM 56824 (J.P.H.) and National Science Foundation MCB-0843537 (A.L.).

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