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Protein chaperones mediating copper insertion into the Cu_A site of the aa_3-type cytochrome *c* oxidase of *Paracoccus denitrificans*

Banaja Priyadarshini Dash, Melanie Alles [1], Freya Alena Bundschuh, Oliver-M.H. Richter, Bernd Ludwig *

Institute of Biochemistry, Molecular Genetics, Goethe University, D-60438 Frankfurt, Germany

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ABSTRACT

The biogenesis of the mitochondrial cytochrome *c* oxidase is a complex process involving the stepwise assembly of its multiple subunits encoded by two genetic systems. Moreover, several chaperones are required to recruit and insert the redox-active metal centers into subunits I and II, two *a*-type hemes and a total of three copper ions, two of which form the Cu_A center located in a hydrophilic domain of subunit II. The copper-binding Sco protein(s) have been implicated with the metallation of this site in various model organisms.
Here we analyze the role of the two Sco homologues termed ScoA and ScoB, along with two other copper chaperones, on the biogenesis of the cytochrome *c* oxidase in the bacterium *Paracoccus denitrificans* by deleting each of the four genes individually or pairwise, followed by assessing the functionality of the assembled oxidase both in intact membranes and in the purified enzyme complex. Copper starvation leads to a drastic decrease of oxidase activity in membranes from strains involving the *scoB* deletion. This loss is shown to be of dual origin, (i) a severe drop in steady-state oxidase levels in membranes, and (ii) a diminished enzymatic activity of the remaining oxidase complex, traced back to a lower copper content, specifically in the Cu_A site of the enzyme. Neither of the other proteins addressed here, ScoA or the two PCu proteins, exhibit a direct effect on the metallation of the Cu_A site in *P. denitrificans*, but are discussed as potential interaction partners of ScoB.

1. Introduction

Cytochrome *c* oxidase (Cox; complex IV) is the terminal enzyme of the respiratory electron transport chain of mitochondria and many bacteria, and belongs to the super family of heme/copper oxidases. The enzyme catalyzes the reduction of molecular oxygen to water linking this process to proton translocation across the membrane that eventually drives ATP production. The mitochondrial enzyme consists of up to 13 different subunits, with only the core subunits I–III encoded by the mitochondrial genome. These subunits are highly conserved among different organisms and represent the key components of bacterial oxidases as well [1–7]. Subunit I houses two heme *a* moieties (*a* and a_3) and a copper ion (Cu_B), the latter two forming the binuclear center catalyzing oxygen reduction. Subunit II carries two copper ions in its Cu_A center, a hydrophilic domain oriented towards the intermembrane space (or the periplasm, resp.). During the stepwise reduction of Cox, cytochrome *c* docks to this domain and transfers one electron at a time to the Cu_A center.

Mitochondrial Cox biogenesis is a highly complex process involving the coordinated expression of both the mitochondrial and the nuclear genes, the protein import into and subunit assembly within the inner membrane of the organelle, as well as the insertion of the redox-active metal centers, altogether requiring the assistance of a large number of accessory proteins [8–11]. Focusing on the redox-active metal insertion into mitochondrial Cox, most insight has been gained from assessing this process in *Saccharomyces cerevisiae*, for its highly developed genetic techniques available (e.g. [12–14]). In this organism, the copper chaperone Cox17 mediates copper transfer to the membrane bound Cox11 and the two Sco proteins which are anchored to the inner membrane of mitochondria by a single transmembrane helix, and contain a globular copper binding domain protruding into the intermembrane space [15–17]. Cox11 has been implicated in copper insertion to the Cu_B site of subunit I [18–20], while Sco1 is suggested to be directly involved in copper delivery to the Cu_A site of subunit II [15, 21–24]. Three-dimensional structures of truncated, soluble domains of Sco homologues from human, yeast, *Thermus thermophilus*, and *Bacillus subtilis* have been obtained [25–32] revealing that the globular domain consists of a thioredoxin-like fold to bind both Cu^+ and Cu^{2+} ions via a histidine and the two cysteines of the conserved CXXXC motif. Further

Abbreviations: Cox, cytochrome *c* oxidase; Sco, synthesis of cytochrome oxidase; PCu, periplasmic copper proteins; TXRF, total reflection X-ray fluorescence; EPR, electron paramagnetic resonance; BN-PAGE, blue native polyacrylamide gel electrophoresis; DDM, *n*-dodecyl-β-D-maltoside; S.D., standard deviation

* Corresponding author at: Institute of Biochemistry, Molecular Genetics, Goethe University, Max-von-Laue-Str. 9, D-60438 Frankfurt, Germany.
E-mail address: ludwig@em.uni-frankfurt.de (B. Ludwig).
[1] Present address: Biotest AG, D-63303 Dreieich, Germany.

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in-vivo and in-vitro studies in both yeast and mammalian cells demonstrated the direct interactions between Sco1, Sco2 and subunit II of Cox, and suggested that Sco1 specifically delivers copper to the Cu_A site [19,33–36]. Deletion of the *sco1* gene leads to respiratory deficiency whereas deletion of the *sco2* gene does not impair cellular respiration, indicating an unknown function of this gene during oxidase assembly [15]. Also in humans two *sco* genes, *sco1* and *sco2*, are required for Cox assembly [24]. Mutations or deletions in both genes cause different pathological disorders associated with a severe Cox deficiency, and are not rescued by their respective counterparts, indicating independent functions during different stages of Cox biogenesis [37–41]. Subsequent studies proposed a model in which Sco2 acts as a thiol-disulfide oxidoreductase to reduce the cysteines in Sco1 for copper binding and subsequent delivery to the Cu_A site (36). Moreover, both human Sco homologues were also suggested to participate in cellular copper homeostasis [42], and in redox signaling [27].

Apart from mitochondria, Sco proteins have also been implicated in the metallation of the Cu_A center in bacterial oxidases. Deletion of *ypmQ*, encoding the Sco homologue of *B. subtilis*, strongly diminished oxidase activity of a Cu_A containing caa_3-type Cox, but not the activity of its quinol oxidase [43], suggesting a role in Cu_A site biosynthesis in this Gram-positive bacterium. Based on biochemical characterization, the YpmQ has been reported to exhibit both a redox and a copper binding activity during the Cu_A site maturation process [44–46]. Subsequent studies in *Bradyrhizobium japonicum* have shown that the deletion of Sco (termed ScoI) strongly reduces the activity of the aa_3-type Cox while the loss of PcuC (a PCu_AC homologue) showed no significant effect in metallating the Cu_A site [47,48]. An alternative function, that of a thiol-disulfide reductase, has been suggested for some bacterial Sco proteins as well [26,32,49].

Many bacteria contain genes encoding PCu_AC-like copper proteins with a highly conserved $H(M)X_{10}MX_{21}HXM$ copper binding motif [31,50]. These proteins appear to function upstream of Sco and may act as Cu(I) chaperones, possibly in a way comparable to the mitochondrial Cox17. In-vitro studies have suggested that in *T. thermophilus* Sco is required for reducing a disulfide bond in the Cu_A center, facilitating copper insertion into the Cu_A fragment of subunit II by PCu_AC which selectively and sequentially inserts two Cu^+ ions into the Cu_A site of this ba_3-type oxidase; Sco1 was found unable to deliver copper ions to the Cu_A site [31]. However, in-vivo studies in *Rhodobacter sphaeroides* confirmed that both copper chaperones PCu_AC and Sco (PrrC) contribute to metallating the Cu_A center in its aa_3-type oxidase (51); moreover, an involvement of the PCu_AC in the formation of the Cu_B center of the cbb_3-type oxidase was noted. Furthermore, recent studies in *Streptomyces lividans* have pointed at a significant role of Sco as a copper chaperone in metallating the Cu_A site while the predominant role of ECuC (potential homologue of PCu_AC) is to capture and deliver copper to Sco in populating the Cu_A site in Cox [52].

The soil bacterium *Paracoccus denitrificans* has proven a suitable model organism for the mitochondrial respiratory chain [53]. Its aa_3-type Cox closely corresponds to the mitochondrial enzyme and the high sequence identity of subunits I, II and III with their mitochondrial counterparts [54,55] may also indicate similar assembly pathways. A comprehensive bioinformatic survey [56] established that *P. denitrificans* shares a basic set of Cox-specific assembly proteins for metal cofactor insertion into both relevant oxidase subunits: (i) CtaB and CtaA (functional equivalents of mitochondrial Cox10 and Cox15) required for the biosynthesis of heme *a* [57], (ii) a Surf1 homologue involved in an early step of cofactor heme *a* insertion into subunit I [58], (iii) CtaG (homologous to Cox11 of mitochondria), and (iv) two copies of Sco [59].

Using deletion and complementation techniques, we have analyzed the two *P. denitrificans* genes encoding Sco homologues along with two PCu_AC-like genes, focussing on their specific in-vivo roles for generating a functional aa_3-type oxidase. While only one of the two Sco proteins clearly affects the biosynthesis of the Cu_A center, neither of the two PCu_AC chaperones shows an immediate involvement in Cox metallation in *P. denitrificans*.

2. Materials and methods

2.1. Construction of sco and pcu deletion strains

All single as well as double deletion strains of *sco* and *pcu* genes were obtained by double-homologous recombination with derivatives of the suicide plasmid pRvS1 [60]. Homologous flanking regions were amplified by PCR from genomic DNA of *P. denitrificans* strain Pd1222, also introducing restriction sites for subsequent cloning (see Table 1).

The *scoA* 5′-flanking region was amplified with the primer pair scoA-up-f/ScoA-up-r and cloned with SphI, SacI into pUC18, while the 3′-flank, generated with scoA-dw-f/scoA-dw-r, was cloned with SacI, SphI into pUC19. Both pUC-derivatives were cut with SacI/ScaI, and the flank-carrying vector fragments were combined in a three-component ligation with a SacI-flanked kanamycin gene (from transposon Tn5) generating a kanamycin-selectable plasmid. The flanks enclosing kanamycin were cut with SphI and cloned into appropriately digested pRvS, giving pRB01.

The suicide construct for *sco*B was obtained similarly, with the following differences: the PCR fragments (generated with scoB-up-f/scoB-up-r or scoB-dw-f/scoB-dw-r) were cloned into pUC18 or pUC19 digested with SphI/HindIII. The flank-carrying vector fragments (resulting from HindIII/AhdI digestions) were ligated to a HindIII enclosed gentamycin resistance cassette (from pBBR1MCS-5; [61]). The segment with the flanks and the gentamycin gene was cut with SphI and cloned into SphI-digested pRvS, yielding pAL2.

The deletion construct for *pcu*1 was obtained sequentially. At first, the PCR product for the upstream flank (primers pcu1 up-f/pcu1 up-r) was PstI/XbaI cloned into pUC18. This pUC18 derivative was KpnI/EcoRI digested to accommodate the downstream flank as a correspondingly cut PCR fragment (primers pcu1 dw-f/pcu1 dw-r), followed by insertion of a kanamycin gene (see above) via KpnI/XbaI. Finally, the fragment containing both *pcu1* flanks and the Km^r cassette was SmaI cloned into SmaI opened pRvS, yielding pBD3.

The suicide plasmid for *pcu2* was generated exactly as described for *pcu*1 with the exception of the primers (upstream-flank: pcu2 up-f/pcu2 up-r; downstream-flank: pcu2 dw-f/pcu2 dw-r) and the resistance gene (gentamycin, see above). The final plasmid was called pBD4.

Table 1
Oligonucleotides and restriction sites used for cloning.

Name	Sequence (5′ → 3′)
scoA-up-f	ATTATTAATGCATGCCGGCGACCACGCCGCCCAGATGTTG; SphI
scoA-up-r	ATATGAGCTCCTCCAGTCCGTGGGCGCATTGAT; SacI
scoA-dw-f	TATTGAGCTCCCGAATGGGCAAAACACTCTAATCAACAGTGAA; SacI
scoA-dw-r	ATATTATAGCATGCGTCCAGTTCGATGCTCATATCGTT; SphI
scoB-up-f	ATTATTAATGCATGCCGGTAAAGGGCCCCTATGCCCAG; SphI
scoB-up-r	ATAATAAGCTTGGCCATCTCCTTTTTGCGCGCCCGCGTT; HindIII
scoB-dw-f	ATTATAAGCTTCCCTTTGTGCGGCGCCGGGTTT; HindIII
scoB-dw-r	ATATTATAGCATGCGTCCCCGCCGCCAGCCACAG; SphI
pcu1 up-f	TGCACTGCAGCCCGGGCTGCTCAGCAGGCGGCG; PstI, SmaI
pcu1 up-r	TGACTCTAGAACGCAAATCCGGGCTGA; XbaI
pcu1 dw-f	AGTCGGTACCCTGATGGGAATAGCGCC; KpnI
pcu1 dw-r	ACTAGAATTCCCCGGGCTATGAGGACGCGGCCA; EcoRI, SmaI
pcu2 up-f	TGCACTGCAGCCCGGGGATAGGGCAGGAACCCG; PstI, SmaI
pcu2 up-r	GTCATCTAGAGGAAAAGGCCCGCCGGT; XbaI
pcu2 dw-f	AGTCGGTACCCGGCGGGCTTCGGCCTT; KpnI
pcu2 dw-rev	TAGTGAATTCCCCGGGATGGGGGCGCTGGTGCC; EcoRI, SmaI
scoA-His8-TEV-f	CCGTCTAGACATATGCATCACCATCACCATCACCATCACGGCCCGGGCG AGAACCTGTATTTCCAGG GCATGCGCCGGATGGGCAAAGAC; NdeI
scoA-rev	ATTGACGCTGCGTCAAGCACCGGGGTTTGAGAGCTCAAGCTTCTG; SacI
scoB-His8-TEV-f	CCGTCTAGACATATGCATCACCATCACCATCACCATCACGGCCCGGGCG AGAACCTGTATTTCCAGGG CATGGCGGGCACTGAACGCAAA, NdeI
scoB-rev	AGCCTGCGCCGCCTGCTGAGCAGCTGAGAGCTCAAGCTTCTG; SacI

All suicide derivatives of pRvS1 were conjugated into *P. denitrificans* wild type strain Pd1222 by triparental mating [62] and selected for double-homologous recombination [60]. To test for proper incorporation of the antibiotic resistance marker, corresponding genomic DNA was isolated, and the deletion was verified via PCR (with gene specific primers or in combination with antibiotic gene primers) and Southern hybridization, and finally by sequencing (SRD, Oberursel, Germany).

For the generation of the *scoA* and *scoB* deletion strains (AL1 or AL2) the suicide plasmids pRB01 or pAL2 were conjugated into *P. denitrificans* 1222. pAL2 was additionally recombined into AL1 to generate the double-deletion strain AL3. The *pcu1* and *pcu2* deletion strains were named BD1 and BD2. The double deletion strain BD3 was obtained via conjugation of pBD2 into BD1, and verified as above.

Generation of the *scoA* and *scoB* complementation plasmids and strains:

For complementation studies, the *scoA* and *scoB* genes were amplified with N-terminal introduction of a TEV-cleavage site and a His_8-tag (primers *scoA*-His_8-TEV-f/*scoA*-rev or *scoB*-His_8-TEV-f/*scoB*-rev). The *sco*A PCR-product was NdeI/SacI cloned into pAL7 (see below), resulting in the *Paracoccus* expression plasmid pBD5. The amplified *scoB* product was NdeI/SacI digested and cloned behind the *cta*2-promoter in pFA39 [63] resulting in pAL6. Finally, the *cta*2/*scoB*-fragment was cloned with SalI and SacI into pRI [64] resulting in pAL7. The complementation plasmids pBD5 and pAL7 were verified by sequencing and recombined into the deletion strains by conjugation.

2.2. Bacterial growth and membrane preparation

All *Paracoccus* strains were grown in the presence of the appropriate antibiotic at 32 °C in 2 l succinate medium [65], using 0.2 ml of the trace element solution per liter of growth medium, to yield a final copper concentration of 1 μM. For growth under copper depletion conditions, copper sulfate was omitted from the trace element stock, and the residual copper level (introduced from impurities of the media components) as determined by TXRF dropped to 0.07 μM. Cells were harvested after 20 h of growth and membranes prepared by established methods. The protein concentration was determined using a modified Lowry protocol [66].

2.3. Purification of cytochrome c oxidase and Cox activity measurement

Cox was purified from *Paracoccus* membranes as described previously [67]. Cox activity measurements were performed at room temperature on a Hitachi U-3000 spectrophotometer in 20 mM KP_i (pH 8), 20 mM KCl, and 0.05% DDM (*n*-dodecyl-β-D-maltoside) with 20 μM reduced horse heart cytochrome *c* as electron donor ($\Delta\varepsilon_{cyt\ c\ (550\ nm)} = 19.4\ mM^{-1}\ cm^{-1}$); due to the use of this electron donor, the assay is specific for the aa_3-type Cox in membranes, excluding any hypothetical cbb_3 Cox activity.

2.4. Electrophoretic methods

For Western blot analysis, membrane samples were denatured in SDS-containing buffer for 20 min at 37 °C. Samples were separated on 12% polyacrylamide gels according to Laemmli [68] and proteins blotted to nitrocellulose membranes. Blue native gel electrophoresis (BN-PAGE) and two dimensional PAGE were run as described [69] and analyzed by immunoblotting or silver staining. Cytochrome *c* oxidase was identified using a polyclonal antibody directed against subunits I and II. Densitometric analyses were performed with the NIH ImageJ 1.47 Java-based image processing software (http://rsb.info.nih.gov/ij/).

2.5. UV–Vis spectral analysis

Redox difference spectra for solubilized membrane proteins were recorded from 500 to 650 nm on a Hitachi U-3000 spectrophotometer using potassium ferricyanide for oxidation and sodium dithionite for reduction, and the concentration of cytochrome *c* oxidase (heme aa_3) determined ($\Delta\varepsilon_{(red\text{-}ox,605\ nm)} = 23.4\ mM^{-1}\ cm^{-1}$ [70]).

2.6. Metal content analysis

The copper content of the growth medium and of purified Cox protein was determined by total reflection X-ray fluorescence spectrometry (TXRF; 50 mM Tris/acetate pH 8, containing 0.05% DDM) essentially as described earlier [71], using a Bruker nano Picofox S2 operating at Mo Kα (50 kV, 600 μA).

2.7. EPR measurements

X-band (9.4 GHz) continuous wave EPR spectra were recorded with a standard Bruker ESP300 setup equipped with an Oxford Instruments ESR A 900 helium flow cryostat and a rectangular TE_{102} microwave cavity Bruker ER 4102 ST including a PC-based data acquisition unit; for further details, see Fig. 7.

3. Results

3.1. P. denitrificans encodes two different Sco and two PCu proteins

BLAST searches of the *P. denitrificans* genome reveal two genes, Pden_2780 (ScoA, 216 aa) and Pden_4443 (ScoB, 210 aa), encoding proteins of significant sequence similarity with corresponding Sco proteins of other species. As sequence comparisons did not allow an unambiguous assignment of the two *P. denitrificans* Sco proteins to their mitochondrial counterparts, we have arbitrarily named their gene products ScoA and ScoB. Topology predictions using bioinformatic algorithms show that both *P. denitrificans* Sco proteins contain an N-terminal transmembrane domain and a C-terminal, periplasm-exposed thioredoxin-like domain with a conserved CPXXCP motif and a conserved histidine for copper binding. ScoA shows 33% sequence identity and 54% sequence similarity with ScoB. A comparison of ScoA with both human Sco proteins [27] reveals about 35% identical residues, whereas blasting against the yeast homologues [28] gives around 36% sequence identity. Comparison of ScoB with the mitochondrial proteins yields sequence identities of around 35% (human), and 32 or 37% (yeast) respectively. On the other hand, the ScoA and ScoB amino acid sequences share roughly 50% and 30% identity to the bacterial Sco proteins SenC/PrrC from *Rhodobacter capsulatus* [72] and *R. sphaeroides* [73] respectively (Fig. 1 supplement). Similar to the situation in *Rhodobacter*, where the SenC (PrrC) gene is part of a *regBCA* cluster, the *scoA* gene of *P. denitrificans* is also associated with a similar two-component regulatory RegB/RegA gene cluster while the *scoB* gene is located adjacent to Pden_4444, a gene which is a potential periplasmic copper chaperone.

In addition to both Sco proteins, sequence similarity searches identified two putative genes, denoted as *pcu1* (Pden_4444) and *pcu2* (Pden_0519), encoding products related to the PCu_AC family of proteins [31,50]. The mature form of PCu1 comprises 300 amino acids and has a molecular mass of 31.1 kDa, whereas the calculated molecular weight of the mature PCu2 (127 amino acids) is 13.4 kDa. PCu1 and PCu2 show pronounced sequence conservation to prokaryotic PCu_AC copper binding proteins including an evolutionary well conserved motif H(M)$X_{10}MX_{21}$HXM, which forms a copper binding domain (Fig. 2, supplement; [31]). The predicted PCu1 is made up of an N-terminal YcnI-like [74] domain (residues 28–170), whereas the C-terminal part (residues 201–309) is clearly homologous to the known copper chaperone PCu_AC (sharing 43% amino acid sequence identity and 54% similarity with the *T. thermophilus* PCu_AC protein (Fig. 2 supplement)). The predicted PCu2 protein shares 41% amino acid sequence identity with the C-terminal half of the PCu1 protein, and shows 34% sequence identity with the PCu_AC protein from *T. thermophilus* (Fig. 2, supplement).

3.2. Gene deletions do not affect the biogenesis of the aa_3-type Cox in copper-rich medium

To address the role of Sco and PCu proteins on the assembly of the aa_3-type Cox in *P. denitrificans*, we constructed several chromosomal deletion strains by double homologous recombination. At first, we tested the role of the Sco and the PCu proteins in the formation of functional oxidase in a normal copper (1 μM $CuSO_4$) medium. Cytochrome *c* oxidase activities of both *sco* deletion strains (single and double) in isolated membranes as well as of the purified oxidase show no significant effect, indicating that there is essentially no contribution of the Sco proteins in Cox biogenesis under normal copper concentrations in the medium (Fig. 1). Furthermore, redox spectra of membrane preparations from *sco* single and double deletion strains demonstrate no major loss of the heme *a* peak with respect to wild type (data not shown). To assess the effect of copper depletion on Cox activity in the deletions of *P. denitrificans sco* genes, copper was omitted from the medium, which resulted in a residual copper concentration of 0.07 μM as determined by TXRF (see Materials and methods section). To investigate the effect of *sco* deletions on overall bacterial metabolism, we measured growth rates under copper limiting conditions. Wild-type and *sco* deletion strains reveal no growth differences (data not shown) in medium containing 0.07 μM copper.

3.3. Strains with a deletion in scoB show a drastic reduction of Cox activity and of the heme a signal in membranes of cells grown in low copper medium

To investigate the role of ScoB in the maturation of aa_3-type oxidase in *P. denitrificans*, we measured oxidase activity (see Materials and methods section) in isolated membranes prepared from wild-type, the *sco* and *pcu* deletion as well as from complementation strains grown aerobically in succinate medium without copper supplementation. In the low copper situation, Cox activities of the deletion strains *scoB* and *scoA/B* are found to be drastically reduced, both showing only 3–6% residual activity as compared to wild-type (Fig. 1), clearly suggesting that Sco proteins act specifically on Cox. The addition of copper to the medium fully restores the oxidase activity to wild-type level in the *sco* deletion strains and functionally complemented the *scoB* deletion phenotype of strains *scoB* and *scoA/B* (Fig. 1). As a control, wild-type cells grown in the absence of copper show an oxidase activity of 74% compared with the wild-type cells grown in the normal copper medium. As deletions of *scoA* alone and any *pcu* gene show little effect on the Cox activity, it is obvious that ScoB has a definite function in the formation of the oxidase under low copper concentrations, while at normal copper levels ScoB is not critically required during Cox assembly.

To further check the effect of *sco*/*pcu* deletions on the synthesis of a functional Cox, we also analyzed the heme content of membranes of the different strains and compared it to that of wild-type using cells grown aerobically with and without copper addition. In the wild-type redox spectrum, peaks are detected for hemes *c*, *b* and *a* at around 550 nm, 560 nm and at 605 nm. By contrast, spectra of membranes from cells deleted in *scoB* and *scoA/B* show drastically diminished levels of heme *a*, while *b*- and *c*- type cytochromes are still present in comparable amounts (Fig. 2). In the *scoB* and in the *scoA/B* deletion mutants, the diagnostic heme *a* peak at 605 nm reappears when cells are supplemented with 1 μM $CuSO_4$ in the medium or when cells are complemented with *scoB in trans* (not shown). These results indicate that the deletion of *scoB* alone causes an almost complete loss of a functional oxidase in membranes.

3.4. In the ΔscoB strains, Cox gets fully assembled, but steady-state levels of the protein complex in membranes are severely diminished

The levels of Cox subunits in membranes of wild-type and *sco* deletion strains were assessed by immunoblotting using antibodies specific for subunit I and subunit II of cytochrome *c* oxidase. We find that the amounts of both subunits are significantly reduced in membranes of the Δ*scoB* and ΔΔ*scoA/B* mutants compared to wild-type (Fig. 3). Densitometric analysis indicates a parallel decrease in subunits I and II to about 25% in the double deletion mutant compared to wild-type.

To address the assembly state of Cox subunits in the *sco* and *pcu* deletion mutants under low-copper conditions, solubilized membranes were also analyzed by two dimensional gel electrophoresis with BN-PAGE in the first dimension, then in the second dimension SDS-PAGE, followed by silver staining or by Western blotting (see Materials and methods section; the ΔΔ*scoA/B* mutant is given in Fig. 4). In silver stained gels, Cox subunits are perfectly aligned on a vertical line (Fig. 4a), as confirmed by Western blotting; individual subunits of Cox are detected by a polyclonal antibody directed against subunits I and II (Fig. 4b and 4c). This blot shows no evidence for the presence of unassembled Cox subunits, which means that the oxidase migrated as an intact complex in the first dimension BN-PAGE and dissociated into individual subunits in the second dimension SDS-PAGE. In line with this BN-PAGE analysis, Western blot analysis shows only fully assembled Cox complexes during chromatographic purification of Cox from

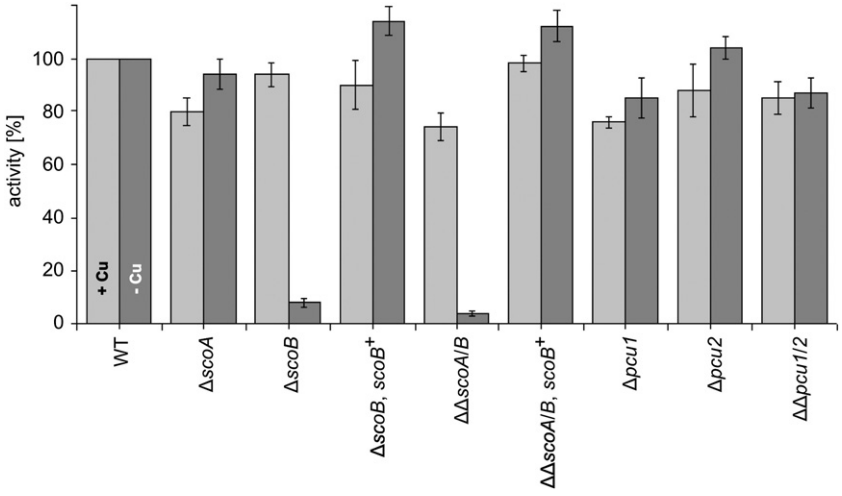

Fig. 1. Cytochrome *c* oxidase activity in *P. denitrificans* membranes from different strains. Activities of the *scoA*, *scoB* single deletions, the *sco* double deletion, *pcu1* and *pcu2* single and double deletions as well as the *scoB* complementation were compared to the wild-type activity. 100% corresponds to an activity of 2.5 units per mg membrane protein (+ Cu) and to an activity of 1.85 units per mg membrane protein (− Cu) respectively. Light gray (+) bars refer to cells grown in the presence of 1 μM copper in the succinate medium, dark gray (−) bars to cells grown without external copper addition, see Section 2.2. Values are the mean ± S.D. from 4 independent preparations.

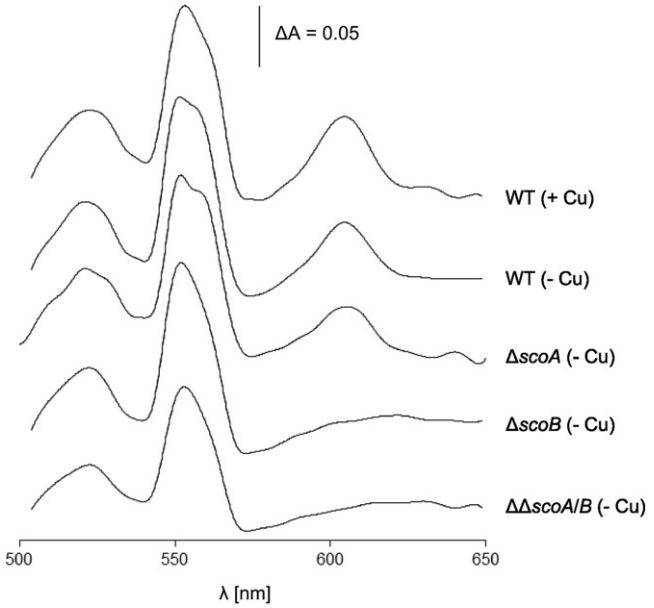

Fig. 2. Absorbance difference spectra of membranes (dithionite-reduced minus ferricyanide-oxidized; the protein concentration was 5 mg/ml) from aerobically grown cells of *P. denitrificans* WT and *sco* deletion mutants grown in medium with (+) or without (−) copper.

membranes of a *scoA/B* double deletion mutant, when all resulting column elution fractions were assayed for the presence of Cox subunits (not shown). Most importantly, dramatically lower yields of Cox are obtained for the *scoB* (Δ*scoB*) and *sco*-double deletion mutants (ΔΔ*scoA/B*) relative to wild-type, confirming the densitometry measurements of Cox subunits described above (Fig. 3).

3.5. Cox complex purified from the ΔscoB strains lacks full WT activity

To further investigate the effect of the *sco* deletions on Cox, the enzyme was purified by established methods from wild type, single deletion strains of individual *sco* genes (Δ*scoA* and Δ*scoB*), the double deletion strains (ΔΔ*scoA/B* and ΔΔ*pcu1/2*) and *scoB* complementation strains. Activity measurements of purified Cox show a decrease of about 70% for the Δ*scoB* single as well as the ΔΔ*scoA/B* double deletion strain, whereas complementation with ScoB completely restores Cox

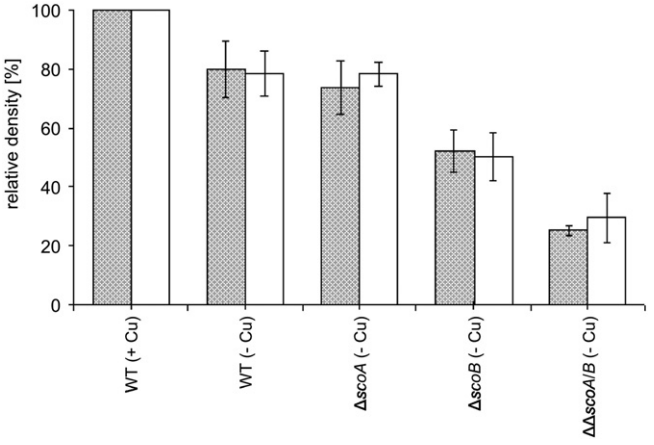

Fig. 3. Quantification of Cox I and II subunits on Western blots after SDS-PAGE of isolated membranes. Protein bands of Cox subunits I and II were quantified by densitometry using NIH image software (http://rsb.info.nih.gov/ij/) for three separate experiments (mean ± S.D.), and band intensities are shown in gray (subunit I) and white (subunit II) on the graph. "+" and "−" indicate the presence or absence of copper in the medium. The relative density is individually normalized for CoxI and for CoxII bands in the wild-type grown in normal copper medium, and is set to 100% for each subunit.

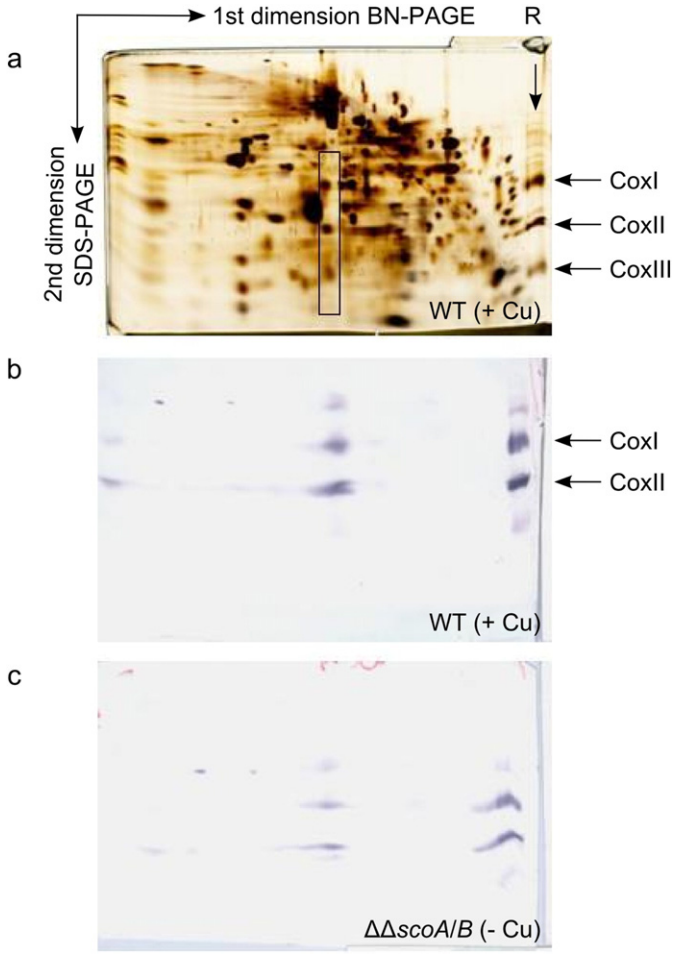

Fig. 4. Migration behavior of Cox subunits after 2-D electrophoresis of membranes. Membranes isolated from WT and ΔΔ*scoA/B* cells grown in normal copper or copper-depleted medium were DDM solubilized (protein/detergent ratio 1:2) and subjected to BN-PAGE in the first dimension followed by a second dimension SDS-PAGE gel. Gels were either silver stained (a, 50 μg protein) or probed with antibodies specific for Cox subunits I and II by Western blotting (b, c, 5 μg protein). The location of the three Cox subunits is indicated by a box; lane R is a reference of 0.5 μg purified Cox.

activity. On the contrary analysis of *scoA* and *pcu* deletion mutants reveals no significant loss of Cox activity compared to wild-type enzyme (Fig. 5).

In summary, analysis of the purified aa_3-type oxidase indicates that the loss of ScoB alone significantly lowers the oxidase activity compared to the absence of ScoA or PCu in cells grown in copper-depleted medium. The results also suggest that PCu and ScoA cannot compensate for the absence of ScoB in the biosynthesis of the aa_3-type oxidase, i.e., the ScoB protein acts specifically on oxidase, and is not interchangeable with ScoA in functional terms.

3.6. Impaired activity of Cox from the ΔscoB strains is based on diminished copper contents, and is traced back to partial loss of the Cu_A signal

In order to determine the extent to which copper was lost from purified oxidase, samples of *sco* and *pcu* deletion mutants grown in medium with or without 1 μM Cu^{2+} were analyzed by TXRF (see Material and methods section). The copper content for the Cox complex isolated from wild-type cells grown in copper-rich medium is 3.2 (Fig. 6), close to the value expected for an intact aa_3-type oxidase with three copper ions (Cu_B and the di-copper Cu_A center). The total copper content of the oxidase purified from wild-type cells grown in copper-depleted medium is 2.75, still within 10% of the expected wild-type value. In the absence of any added copper, the total copper content of the oxidase

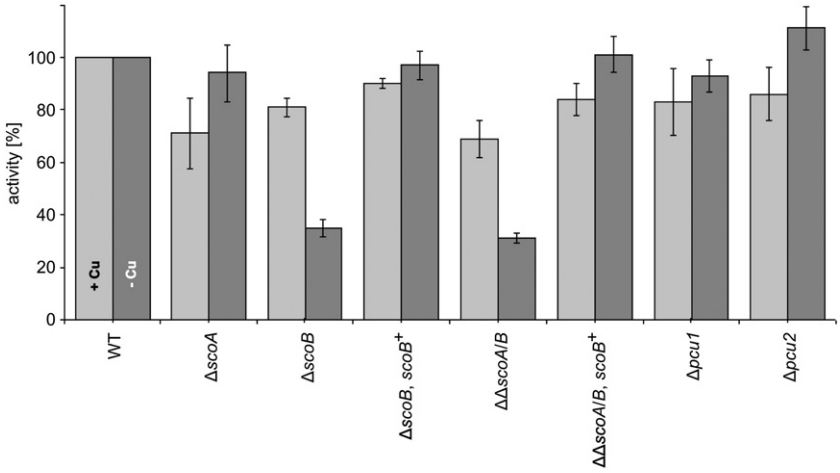

Fig. 5. Effect of the *sco* and *pcu* deletions on the activity of purified aa_3-type oxidase. Turnover numbers of the purified enzyme from WT cells, deletion strains of *sco* and *pcu*, and a *scoB* deletion mutant complemented with a plasmid containing the *scoB* gene are given. Copper addition to the medium is indicated by light gray (+) or dark gray (−) colors in the graph. The 100% value corresponds to a turnover number of 413 s^{-1} (+ Cu) or 363 s^{-1} (− Cu) of the purified enzymes (mean ± S.D. from 4 measurements).

purified from the *scoB* (Δ*scoB*) single deletion and from the double deletion of *sco* (ΔΔ*scoA/B*) ranges between 2.14 and 2.08, suggesting loss of copper is primarily due to the absence of ScoB. The copper content of oxidase purified from both the Δ*scoA* single deletion and the Δ*pcu* deletion (single and double) strains grown in medium with low copper concentration is 2.60, thus only marginally reduced.

EPR spectroscopy is a means to attribute the loss of copper to the Cu_A center on the basis of a signal in the g = 2.0 region, as Cu_B is EPR silent due to magnetic coupling to heme a_3 [75]. Therefore, EPR spectra of oxidized oxidase preparations from wild-type, and from *sco* and *pcu* deletion strains were recorded and normalized for the heme *a* peak (g = 2.8). The oxidase purified from the *scoA* and *pcu* deletion strains grown in low copper medium reveals wild-type amounts of the Cu_A signal. However, the EPR spectrum of oxidase from the Δ*scoB* deletion strain shows significant reduction of the above-mentioned Cu_A peak relative to the wild-type reference (Fig. 7a). The Cu_A signal of purified oxidase from the Δ*scoB* and ΔΔ*scoA/B* deletion strains is reduced by 22 and 37% of that present in the wild-type protein. Therefore the decreased oxidase activity of purified enzymes from the *scoB* deletion and the *sco* double deletion strains grown in the absence of additional copper is mostly due to the decreased insertion of copper into the Cu_A center (Fig. 7b). The effect is most pronounced when both *sco* genes are deleted, although the role of ScoA remains unclear (see Discussion section), as deletion of *scoA* alone shows only marginal consequences, leaving the absence of ScoB as the main reason.

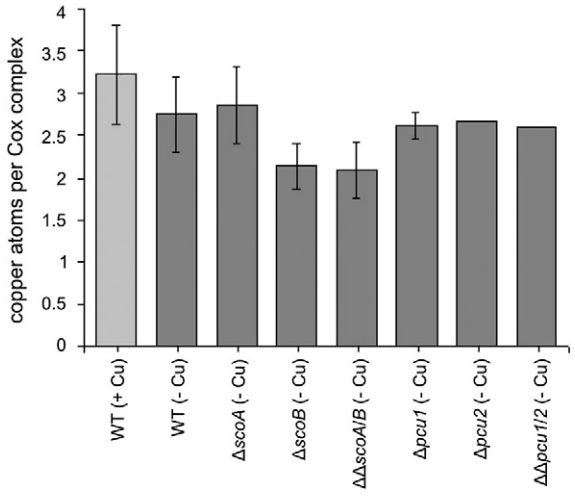

Fig. 6. Analysis of total copper content of the aa_3-type oxidase isolated from WT, *sco* and *pcu* deletion strains. The number of copper per oxidase was determined by TXRF as described in the Materials and methods section. Five independent measurements are shown for each sample (Δ*pcu2* and ΔΔ*pcu1/2* are single measurements). Error bars show S.D.

4. Discussion

Copper is an essential trace element required as a cofactor for a small number of copper-containing enzymes that carry out fundamental biological functions such as oxidative phosphorylation and protection against oxidative damage (for reviews, see [76,77]). The aa_3-type cytochrome *c* oxidase is the terminal electron acceptor of the respiratory chain of mitochondria and of many bacteria (see Introduction section); its three key subunits contain the two copper centers, Cu_A and Cu_B (see Fig. 8 for a first structural orientation). In mitochondria and in some bacteria, a number of copper chaperones have been identified in copper delivery, among them two Sco proteins, Sco1 and Sco2, implicated in the delivery of copper to the Cu_A center of Cox (see Introduction section).

Paracoccus encodes two different *sco* genes termed A and B here, as their derived amino acid sequences do not allow a clear-cut assignment to the canonical Sco1/Sco2 proteins of mitochondria. In addition, two genes encoding PCu_AC-like proteins, termed PCu1 and PCu2, are identified in *Paracoccus*. Using various deletion strains, we analyze the in-vivo role of these four copper chaperones for the biosynthesis of the aa_3-type oxidase, by assessing the biochemical properties of Cox both at the level of membranes as well as in purified form, to derive a working model for delivering copper to the Cu_A center of Cox.

4.1. ScoB is the copper chaperone primarily responsible for metallating Cu_A at low copper availability

Taking membrane activity of Cox in various *Paracoccus* deletion strains as the most comprehensive criterion for an undisturbed biosynthesis of the complex, it gets obvious that in normal growth medium, i.e., at a 1 μM $CuSO_4$ concentration, the absence of ScoB has no major effect on Cox activity in membranes (Fig. 1). However, significant effects are observed when copper is omitted from the medium, which results in a residual copper level of 0.07 μM, originating from impurities introduced with the various growth medium components. Under such copper depletion conditions, Cox activity in membranes from the Δ*scoB* mutant is drastically affected (Fig. 1), dropping to between 6 and 3% for the Δ*scoB* and the ΔΔ*scoA/B* double deletion mutant, resp. Therefore, loss of activity is a clear consequence of the *scoB* deletion only under copper limitation, as also reported in a similar study in *R. sphaeroides* [51]. Yet, there is no general growth defect observed for this *Paracoccus*

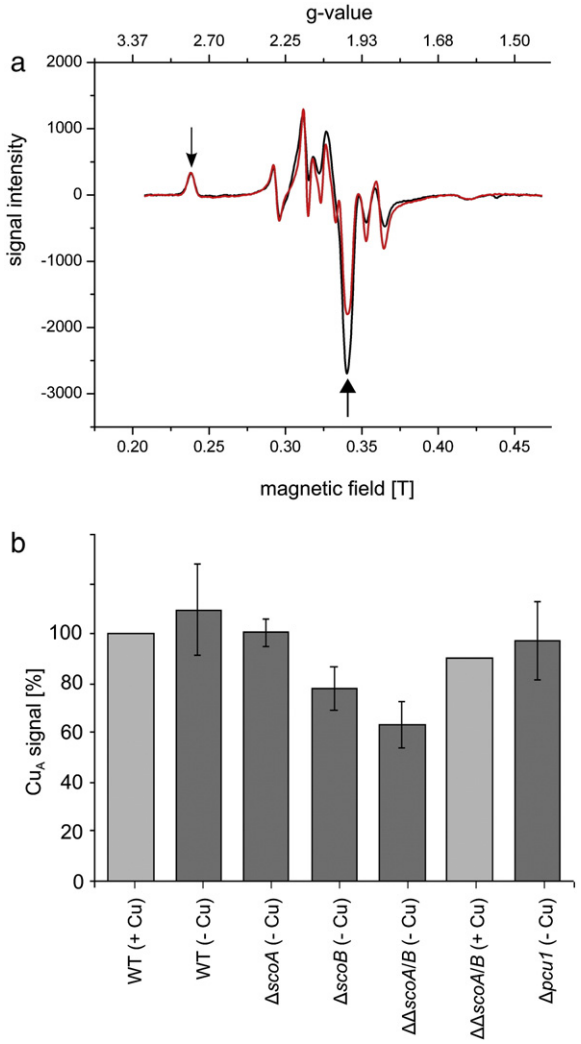

Fig. 7. Quantification of Cu_A in purified Cox by EPR. (a) 40 μM Cox isolated from wild-type cells (black trace) grown in medium containing normal copper and from cells lacking both Sco proteins in copper-depleted medium (red trace) were recorded at X-band continuous-wave (cw) using a Bruker ESP300 spectrometer. The experimental conditions were as follows: the spectra were taken at 20 K using a microwave power of 2 milliwatt at 9.44 GHz; field modulation amplitude, 30 G; effect modulation frequency, 100 kHz; time constant, 81 ms and the sweep time was 21 s. The resulting spectra were normalized for the heme *a* peak (at g = 2.8, downward facing arrow), and the Cu_A signal at g ~ 2.0 highlighted by an upward arrow. (b) The Cu_A to heme *a* ratios derived from EPR spectra for Cox isolated from different backgrounds are plotted; the value for wild-type (WT + Cu) oxidase is set to 100%. Values are given as means ± S.D. with two different preparations of protein used (except for Δ*scoA*/*B* (+Cu)).

strain (nor for any of the other deletion strains), based on measuring generation times, whether copper is supplemented or not (data not shown). This is explained by the fact that this bacterium, with its branched electron transfer chain, resorts to several terminal oxidases, maintaining efficient electron transfer to oxygen for energy transduction.

Somewhat unexpectedly, on analyzing the heme redox spectra obtained for the Δ*scoB* deletion mutant (Fig. 2), we observe an almost complete loss of the diagnostic heme *a* peak, originating from both hemes associated with subunit I. To exclude any concerted biogenesis or assembly defect on Cox, we analyzed the steady-state levels of Cox subunits in membranes both by 1-D and by 2-D SDS-PAGE. Fig. 3 clearly documents that in the single-deletion mutant (and more severely in the double *sco* deletion) the amounts of both subunits I and II are extensively diminished; yet this observation alone does not explain the full extent of the more than tenfold loss of activity (discussed below).

An important finding comes from 2D gel electrophoresis: no free Cox subunits are seen for either of the two Δ*scoB* mutant membranes (Fig. 4), suggesting that all subunits present in the membrane are assembled into a physically intact complex, and neither any evidence for a pile-up of potential assembly intermediates nor, on the other hand, any degradation products of individual subunits are revealed immunologically. Along the same lines we also never observe the presence of unassembled subunits during the high detergent-based conventional chromatographic purification from the ΔΔ*scoA*/*B* membranes (checked by Western blotting of all relevant elution fractions; data not shown), but we noted an unusually low yield for the Cox complex isolated from such mutant membranes.

In terms of its enzymatic activity of the purified Cox, it may be surprising to observe a residual electron transfer activity of around one third compared to wild-type (Fig. 5), which can only be rationalized by a full cofactor equipment, despite the lack of ScoB, for this subfraction of Cox. The drastic loss of Cox activity in membranes can at this point be explained and quantitatively accounted for by the combination of two defects in the deletion strain grown under copper limitation: (i) a diminished steady-state Cox protein level in membranes, and (ii) in purified Cox only one out of three complexes shows enzymatic activity.

To shed some light on the Sco proteins towards recruiting copper ions to Cox, we determined copper stoichiometries for the purified Cox preparations (see Fig. 6), revealing that the *scoB* deletion mutant(s) grown in copper-limiting medium have a lower content of copper compared to the wild-type enzyme from cells grown identically. Assuming a subunit I with its regular metal cofactors (both hemes and the Cu_B site) populated, this would on average point at a copper deficiency in the Cu_A site of subunit II. Evidence from EPR measurements on isolated Cox preparations (Fig. 7) indeed indicates that copper is lost to some extent from the Cu_A center. Our interpretation from the latter two observations, along with the residual enzymatic activity of the purified enzyme now suggests that ScoB is directly responsible for Cu_A metallation under these growth conditions, and its deletion causes multiple defects:

- In the fully assembled "as purified" enzyme, a partial (even though not complete, see below) loss of this redox site, along with a nominal copper stoichiometry of close to 2 per complex, requires about one third of all complexes with a full complement of all redox sites and uncompromised competence in electron transfer. The remaining subspecies are catalytically incompetent for the fact that they are deficient in their Cu_A site with – on average – less than 1 copper ion present in this actual binuclear site.
- During or immediately after translation of subunit II, a delay in the supply of copper ions may impede the proper folding of the Cu_A domain (which in-vitro readily folds in the presence of the metal ions; [78]). As a consequence, we envisage a fast proteolytic degradation of subunit II, making also subunit I (and the remaining two subunits) of Cox prone for degradation, for the lack of interaction partners. Actually, loss of subunit II has been reported earlier to lead to a loss of the subunit I protein in *P. denitrificans* cells [79]. An important aspect derived from our deletion studies is the fact that ScoB is the major facilitator, but not an exclusive nor fully indispensable player to mediate copper recruitment to the Cu_A site. In quantitative terms, this behavior is reminiscent of the role of the Surf1 proteins in transferring the heme *a* moiety to subunit I of Cox, which is not completely abolished either in the respective deletion mutant [80].

The effect of deleting ScoB is similar to results obtained for the mitochondrial (human and yeast; [24,81,82]) and bacterial Sco homologues (*B. subtilis*, *B. japonicum*, *R. sphaeroides* and *S. lividans*) [43,47,51,52]. In more detail, under low copper conditions the absence of the PrrC, the (single) Sco homologue in *R. sphaeroides*, drastically lowers the Cox (aa_3-type) activity to only a few percent in membranes, paralleled by a sharp drop in the content of total copper and of the population of the Cu_A site of the purified enzyme [51]. It was further shown that

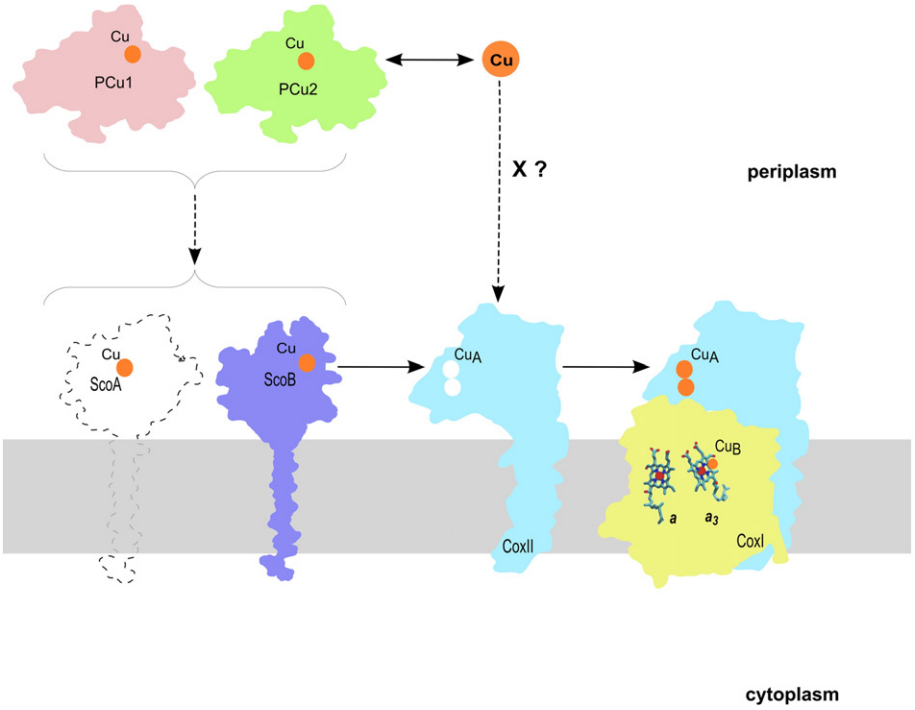

Fig. 8. Working model for Sco and PCu chaperones contributing to copper insertion into the Cu_A site of Cox in *P. denitrificans*. Either of the two PCu proteins may deliver copper to ScoB, which in turn donates it to CoxII. ScoA (stippled outline) participates in an as yet unknown fashion (see text). Copper insertion into the oxidase occuring spontaneously or by an unidentified component is marked by X?. Copper is shown by orange circles, heme cofactors by ball and stick representation. The two oxidase subunits carrying the redox centers are indicated by Roman numbering.

deletions in PrrC or PCu_AC in *R. sphaeroides* also affect the metallation of the Cu_B center in subunit I, but this observation was discussed as an indirect effect due to a perturbed copper delivery to the Cox11 chaperone.

In the current work, we also show that the decreased Cox activity in the Δ*scoB* single mutant (and likewise in the *sco* double deletion mutant as well) grown with limiting copper could be restored to almost wild-type levels by increasing the copper level in the medium from 0.07 μM to 1 μM, or upon complementation by ScoB *in trans* (Fig. 5), indicating that the incorporation of copper into the Cu_A site of Cox is largely independent of ScoB under normal copper conditions. Similar results have been demonstrated in other bacteria (including *B. subtilis*, *B. japonicum*, *R. sphaeroides*, *S. lividans*) on deletion of their Sco protein(s), as well as in humans when a functional Sco2 is absent [43,47,51,52]. Taking together biochemical and biophysical data, our findings suggest a decisive role of ScoB in Cox biosynthesis at the stage of copper insertion and formation of the Cu_A site in subunit II of the *P. denitrificans* aa_3-type Cox.

4.2. ScoA has no immediate effect of its own in metallating the Cu_A site, but may functionally assist ScoB

In cells grown in medium containing normal or low copper, the absence of ScoA alone leads to no substantial loss in the Cox activity in membranes (Fig. 1). On analyzing the heme spectra obtained from the membranes of the Δ*scoA* deletion mutant grown in low copper medium, we observe that the heme *a* peak is only slightly diminished compared to wild-type level (Fig. 2), suggesting that ScoA has no effect on Cox assembly. We also notice that steady-state levels of Cox subunits I and II remain unchanged in membranes of the Δ*scoA* mutant. No significant changes for the purified Cox in terms of its enzymatic activity or its copper content are observed either.

From our single-deletion data we can exclude a redundant function of either Sco protein, as well as a scenario in which each of the two Sco proteins would specifically deliver a single copper ion to the binuclear Cu_A center. Yet, can we observe any additional effects when both genes are deleted at the same time? Compared to the Δ*scoA* single deletion, in a ΔΔ*scoA/B* mutant, Cox activity in membranes drops further by a small percentage compared to the Δ*scoB* (Fig. 1). In this double mutant, we also observe additional defects (i) in the steady-state levels of Cox subunits I and II in membranes, (ii) in the activity of purified Cox, and (iii) a further impairment of the Cu_A metallation (Fig. 7b). Thus, our data suggest a copper delivery pathway in which ScoB delivers copper to the Cu_A site in a mechanism that may be assisted by ScoA. In this respect, our data resemble the copper delivery pathway in yeast, where Sco2 is not required to metallate the Cu_A site since its deletion does not impair respiration [15]. The two *Paracoccus* Sco proteins share even less sequence identity among each other than their yeast counterparts (33 versus 59% identity), and there is no functional overlap in metallating the Cu_A site either. Unlike yeast, both the human Sco proteins have been assigned distinct roles in the assembly of Cu_A, and their loss is associated with Cox deficiency: Leary et al. have provided a model in which Sco1 and Sco2 have cooperative, non-overlapping functions in Cu_A site formation [36,83].

In *Rhodobacter* spp., *prrC/senC* is part of a regulatory gene cluster acting in a two-component signal transduction cascade (PrrAB/RegAB; [72,73]), affecting expression of the cbb_3-type cytochrome *c* oxidase. Therefore it is tempting to speculate that ScoA, with its gene located in the *regAB* operon, may perform similar regulatory functions in *Paracoccus* as well.

4.3. Both PCu1 and PCu2 proteins may be responsible for general Cu metabolism in the cell, but do not directly participate in Cu_A biogenesis

In this study, the results obtained from the activity measurements using membranes and purified oxidase indicate that the loss of PCu1 and PCu2 has no direct effect on the biosynthesis of Cox (Figs. 1 and 5), nor do the copper determinations deviate from wild-type (Figs. 6 and 7). In-vitro experiments with a recombinant form of the *T. thermophilus* PCu_AC have demonstrated direct delivery of two copper ions to the apo-Cu_A fragment of subunit II of the ba_3-type oxidase, while Sco would only function to reduce the Cu_A cysteine ligands [31].

In clear contrast, our in-vivo results for PCu in *Paracoccus* specifically preclude an immediate role in the assembly of the Cu_A site, and we assume that in *Paracoccus* both PCu proteins are most likely engaged in general copper recruitment and homeostasis.

In conclusion, we can now derive a tentative working model on the roles of the four copper chaperones of *P. denitrificans* under copper limitation (Fig. 8). ScoB is the major player in the metallation of the Cu_A site of subunit II, while ScoA clearly differs in this respect: it can by no means functionally substitute ScoB. However, we observe a slight phenotypic reinforcement of oxidase defects, as judged from several experimental criteria for strains with both *sco* genes deleted. From this we hypothesize that ScoA may simply be an intermediate copper recruitment partner for ScoB, at least under the two copper concentrations studied here. Our data cannot exclude the possibility that ScoA, either of the two PCu proteins, or an as yet unidentified factor may be of critical importance under still lower copper concentrations (which, however, are not technically feasible, see Section 2.2). Alternatively, ScoA may act as a thiol oxidoreductase to re-reduce a disulfide bond in ScoB, after having delivered its copper ion to Cu_A. In *B. japonicum* the TlpA protein functions as a disulfide reductase for Sco as well as for the Cu_A domain [84,85], and *P. denitrificans* also encodes such a TlpA-like potential reductase (Pden_2023). Taken together, we therefore suggest that ScoB rather resembles, in many functional aspects, that of its mitochondrial Sco1 counterpart, particularly that of yeast.

While a functional ScoB chaperone may have specifically evolved to overcome low copper availability growth conditions, a somewhat enigmatic feature is the fact that in the *sco*B mutant strain, the Cu_A center of oxidase still recruits sufficient copper ions to display around 30% of wild-type activity. Presently we have no evidence to speculate on the nature of "X" which functionally bypasses ScoB (see Fig. 8), but we do not regard the spontaneous insertion of copper ions a very likely scenario.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2014.11.001.

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