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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*

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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

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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