Biochimica et Biophysica Acta 1817 (2012) 2005–2015

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

The ScoI homologue SenC is a copper binding protein that interacts directly with the cbb_3-type cytochrome oxidase in *Rhodobacter capsulatus*

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ARTICLE INFO

Article history:
Received 23 April 2012
Received in revised form 27 June 2012
Accepted 28 June 2012
Available online 4 July 2012

Keywords:
Sco proteins
Copper insertion
Cytochrome *c* oxidase
Copper transport
Respiratory growth
Rhodobacter capsulatus

ABSTRACT

Sco proteins are widespread assembly factors for the Cu_A centre of aa_3-type cytochrome oxidases in eukaryotic and prokaryotic organisms. However, Sco homologues are also found in bacteria like *Rhodobacter capsulatus* which lack aa_3-type cytochrome oxidases and instead use a cbb_3-type cytochrome oxidase (cbb_3 Cox) without a Cu_A centre as a terminal oxidase. In the current study, we have analyzed the role of Sco (SenC) during cbb_3 Cox assembly in *R. capsulatus*. In agreement with earlier works, we found a strong cbb_3 Cox defect in the absence of SenC that impairs the steady-state stability of the CcoN, CcoO and CcoP core subunits, without the accumulation of detectable assembly intermediates. *In vivo* cross-linking results demonstrate that SenC is in close proximity to the CcoP and CcoH subunits of cbb_3 Cox, suggesting that SenC interacts directly with cbb_3 Cox during its assembly. SenC binds copper and the cbb_3 Cox assembly defect in the absence of SenC can be rescued by the addition of least 0.5 μM Cu. Neither copper nor SenC influenced the transcription of the *ccoNOQP* operon encoding for cbb_3 Cox. Transcription of *senC* itself was also not influenced by Cu unless the putative Cu-export ATPase CcoI was absent. As CcoI is specifically required for the cbb_3 Cox assembly, these data provide a direct link between Cu delivery to cbb_3 Cox and SenC function.

1. Introduction

Sco (synthesis of cytochrome *c* oxidase) proteins are single-spanning membrane proteins with a large soluble domain that reaches into the inter membrane space of mitochondria, or the periplasmic space of bacteria. The soluble domain has a typical thioredoxin-like fold, and binds a single copper ion via a diagnostic CxxxC motif and a conserved histidine ligand. Sco proteins were first identified as crucial assembly factors for aa_3-type cytochrome oxidase (aa_3 Cox) in yeast [1], but they are widespread in eukaryotic and bacterial organisms [2]. aa_3 Cox contain two different copper sites: the Cu_A centre is a dinuclear copper site, which is located in the periplasmic domain of subunit II (CoxII) and solvent exposed. In contrast, the Cu_B centre of subunit I (CoxI) is deeply buried within the hydrophobic environment of the membrane [3].

Despite the pronounced conservation, a unifying concept on Sco function in different species is still lacking, partly due to the fact that in some organisms like yeast or humans more than one Sco homologue is found [4]. Yeast ScoI interacts with the Cu_A-containing CoxII subunit of aa_3-Cox in yeast [5,6], and its over-expression rescues the aa_3 Cox deficiency of yeast mutants lacking Cox17, a soluble copper chaperone of the inter membrane space [7,8]. These findings led to a model in which ScoI would transfer Cu from Cox17 to the Cu_A site of the CoxII subunit of aa_3 Cox [9]. Yeast Sco2 does not seem to be required for Cox assembly, but nevertheless, is able to partially suppress the phenotype of a *sco1* deletion when over expressed [10]. However, in humans, mutations in either Sco1 (corresponding to yeast Sco2) or Sco2 cause Cox deficiencies that are not rescued by their respective homologues, suggesting that both human homologues function at different steps of Cox assembly [4]. In addition, both human Sco homologues are involved in maintaining cellular copper homeostasis [11], and in redox signaling [12].

Spectroscopic analyses indicated that bacterial Sco homologues from different species also bind Cu [13–15], but a direct transfer of Cu from Sco to the Cu_A centre of *Thermus thermophilus* ba_3-Cox was not observed [16]. Instead, it was suggested that Sco is required for reducing a disulfide bridge within the Cu_A-binding site of CoxII, allowing subsequent

Abbreviations: aa_3-Cox, aa_3-type cytochrome *c* oxidase; BN-PAGE, blue-native polyacrylamide gel electrophoresis; cbb_3-Cox, cbb_3-type cytochrome *c* oxidase; cyt, cytochrome; DDM, n-dodecyl-maltoside; ICM, intracytoplasmic membrane; NADI, α-naphtol and N′-N′-dimethyl-p-phenylenediamine (DMPD); Qox, hydroquinone oxidase; PBS, phosphate-buffered saline; PFA, para-formaldehyde; TCA, trichloroacetic acid; TMPD, *N,N,N,N*-tetramethyl-*p*-phenylenediamine; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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doi:10.1016/j.bbabio.2012.06.621

Cu insertion by another periplasmic copper chaperone, called PCu_AC [16,17]. A general thiol-disulfide oxidoreductase activity of Sco was initially proposed by Chinenov [18], and was experimentally confirmed for the *Rhodobacter sphaeroides* Sco homologue PrrC [19] and for *Pseudomonas putida* Sco1 [20]. Whether bacterial Sco homologues mainly function as a copper chaperone or as an oxidoreductase was suggested to depend on the conformational flexibility of a conserved histidine acting as a Cu ligand in the active site of this protein [2]. The thiol-disulfide oxidoreductase activity of bacterial Sco1 does not seem to act exclusively on CoxII, but probably also on other periplasmic proteins. For example, deleting Sco resulted in an increased sensitivity towards paraquat-induced oxidative stress in *Neisseria* species, which lack a Cu_A-containing aa_3 Cox [21]. A Cu_A-CoxII independent function of Sco is also in line with the observation that in approximately 6% of the available bacterial genomes *sco* homologues are present, but genes encoding CoxII are missing [2,22].

In some bacteria like *Rhodobacter capsulatus* [23], *R. sphaeroides* [24] and *Pseudomonas aeruginosa* [25] the cbb_3-type Cox enzymes have been identified as potential targets for Sco function, although these Cox enzymes lack a Cu_A-containing subunit. The cbb_3 Cox terminal oxidases are frequently encountered in proteobacteria [26,27], and are encoded by the *ccoNOQP*-operon (also named *fixNOQP* in some species) [28]. Instead of the Cu_A-containing CoxII subunit, cbb_3 Cox uses two membrane-bound *c*-type cytochromes (cyt), CcoO and CcoP, to convey electrons to its catalytic subunit (CcoN) [28]. CcoN is highly similar to the CoxI subunit of aa_3 Cox, and contains a low-spin heme *b* that receives electrons from CcoO, and a high-spin heme b_3-Cu_B binuclear centre where oxygen is reduced to H_2O [29]. The assembly of cbb_3 Cox has been intensively studied in *R. capsulatus*, which lacks aa_3 Cox and instead uses the cbb_3 Cox and a hydroquinone oxidase (Qox) as the two terminal oxidases [30–34]. Deletion of SenC, the Sco homologue of *R. capsulatus*, impairs respiration [35] due to reduced cbb_3 Cox activity, which can be restored by the addition of 20 μM Cu [23]. A similar copper-dependent rescue of cbb_3 Cox activity has also been observed in a *P. aeruginosa* SenC mutant [25]. These observations suggest a possible role for Sco as a copper chaperone for the Cu_B centre, especially at low Cu concentrations [36]. However, a direct interaction between SenC/Sco and subunits of cbb_3 Cox has not been observed so far. The periplasmic copper chaperone PCu_AC might cooperate with Sco during Cu_B assembly, as a PCu_AC deletion mutant shows a similar cbb_3 Cox phenotype as a Sco deletion in *R. sphaeroides* [36].

In both *R. capsulatus* and *R. sphaeroides*, SenC/PrrC are part of a regulatory gene cluster consisting of the histidine sensor kinase RegB and the response regulator RegA [35,37]. The RegB–RegA couple being a global regulator of energy metabolism in *Rhodobacter* species [38], a possible role for SenC/PrrC in regulating gene expression has also been proposed [37]. However, a general requirement for the Sco homologues in cbb_3 Cox expression or assembly has not been observed as the absence of Sco in *Neisseria* does not influence cbb_3 Cox assembly [21], and only reduces aa_3 Cox, but not cbb_3 Cox, activity in *Bradyrhizobium japonicum* [15]. Thus, the exact role(s) of Sco proteins during cbb_3 Cox assembly remains to be determined.

2. Material and methods

2.1. Bacterial strains and growth conditions

The following *R. capsulatus* strains were used in this study: MT1131 (wild type; [39]); LS01 (Δ*senC*; [23]); GK32 (Δ*ccoNO*, [40]), CW6 (Δ*ccoI*; [30]) and Y262 (GTA-overproducer, [41]). *R. capsulatus* strains were grown in Sistrom's minimal medium A or in enriched medium MPYE [42,43] at 35 °C in liquid cultures in the dark with appropriate antibiotics (10 and 2.5 μg/ml for kanamycin and tetracycline, respectively). For semi-aerobic growth, 500 ml cultures were grown in the dark in 1000-ml flasks and were shaken at 110 rpm. Cu-free MPYE medium was generated by mixing 100 ml MPYE medium with 5 g Chelex-100 resin and stirring for 1 h at room temperature before the Chelex-100 resin was removed by filtration. The *E. coli* strains DH5α and BL21(DE3) have been described previously [44]. *E. coli* cells harboring plasmids were grown in LB medium supplemented with appropriate antibiotics (100, 50, and 12.5 μg/ml for ampicillin, kanamycin, and tetracycline, respectively).

2.2. Molecular genetic techniques

For the construction of plasmid pRK415-SenC, the *senC* gene was amplified from the genomic DNA of *R. capsulatus* strain MT1131 using the primer: 5′-ccattcctgagagctcaaactt-3′ and 5′-attttgtctagatcaatttccgttg-3′ (or 5′cctaatctagatcagtggtggtggtggtggtgatttccgttgcccgccgcgg-3′ to insert a hexa-histidine tag). The obtained PCR product was digested with SacI/XbaI restriction enzymes and cloned into a SacI/XbaI digested pRK415 vector. For the construction of pRK415-PCu_AC the primers 5′-cgggcgccatggggctttgct-3′ and 5′-gccttttaggtaccgatcaggggtca-3′ were used and the PCR product was cloned into pRK415 after NcoI and KpnI digestion. For a hexa-histidine version of PCu_AC the primer 5′-ggcaaatctagatcacttctcgaactgcggatgcgaccaggggtcatcttgtg-3′ was used as a second primer. For the construction of pET19b-SenC and pET22b-SenC, the *senC* gene was amplified from the genomic DNA of *R. capsulatus* MT1131 using the following primer pairs: SenC-1: 5′-aaggagagcatatgaacgtttcgagcaag-3′ and SenC-2: 5′-aatctatatctcgagctggtcaatttccgtt-3′ or pet22b_SenCHis_for 5′-atttccgttgcccgccgcgggggtatc-3′ and pet22b_SenCHis_rev 5′-ctcgagcaccaccaccaccaccac-3′. After NdeI/XhoI digestion, the PCR product was cloned into either pET19b or pET22b (Novagen, Bad Soden, Germany). For the construction of a SenC derivative lacking the transmembrane domain, an inverse PCR reaction using pET22b-SenC as template and the following primer pair pet22b-SenCHis-TM_for 5′-ggtcttgctcgaaacgttcat atg-3′ and pet22b_SenCHis-TM_rev 5′-ccccatgagacggatcgctttgcc-3′ was used. The resulting plasmid was called pET22b-SenCΔTM. This plasmid was also used as template for constructing a SenC derivative (pET22b-SenCΔTMC) in which the canonical cysteine residues were replaced by serine residues together with the following primer pair SenC_cys_For 5′-cgtcagcccgatcgacagcac-3′ and SenC_cys_Rev 5′-tcgggcgaatagctgtaaccgaa-3′.

2.3. RNA isolation and RT-PCR

Semi-aerobically grown *R. capsulatus* cultures in MPYE medium were harvested at the mid log phase (OD_{685} 0.8–1.0). Approximately 1×10^9 cells were pelleted, resuspended in TE buffer (10 mM Tris, 0.5 mM EDTA pH 7) containing 10 mg/ml lysozyme, incubated for 15 min at 37 °C, and lysed by 5 passages through a needle with 0.8 μm diameter into an RNase free tube. Total RNA was isolated using GE Healthcare Mini Spin kit following the manufacturer's protocol, and 2 μg of total RNA were digested with DNaseI for 30 min at 25 °C in the presence of RNase inhibitor RNasin. 50 ng of DNaseI treated RNA was used for RT-PCR reactions with the One Step RT-PCR kit (Qiagen, Hilden, Germany). The following primer pairs were used: *ccoN* 5′-ggcgaactggttctacct-3′ and 5′-gtaccaccattgcgtcat-3′; 16sRNA 5′-gtaatacggaggggcta-3′ and 5′-tcacctctctcgacctca-3′, *senC*: 5′-aaggagagcatatgaacgtttcgagc-aag-3′; 5′-aatctatatctcgagctggtcaatttccgtt-3′. As a control for detecting genomic DNA contaminations, PCR reactions were also performed without the reverse transcriptase step, using the PHUSION DNA-Polymerase (Finnzymes, NEB, USA). Samples were separated on a 1.2% agarose gel.

2.4. Preparation of intracytoplasmic membranes (ICMs) and BN-PAGE analyses

Intracytoplasmic membranes (ICMs) from wild type *R. capsulatus* strain MT1131 and mutant strains were prepared essentially as previously described [45].

For blue native-polyacrylamide gel electrophoresis (BN-PAGE) analyses, ICM (50 μg of total proteins) were resuspended in 10 μl of 2× lysis buffer (50 mM NaCl, 5 mM 6-aminohexanoic acid, 50 mM imidazole/HCl, pH 7.0), adjusted to 20 μl with water and solubilized with *n*-dodecylmaltoside (Roche Diagnostics, Mannheim, Germany) at a 1:1 (w/w) ICM proteins/detergent ratio from a 10% dodecylmaltoside stock solution in lysis buffer (final DDM concentration 1%). After 10 min incubation at 25 °C, the samples were centrifuged for 15 min at 70,000 rpm in a TLA100.3 rotor, and 15 μl of the supernatant thus obtained was supplemented with 2 μl of loading buffer (5% Coomassie blue in 500 mM 6-aminohexanoic acid) and 5 μl of 50% glycerol (in H_2O) and loaded onto a 5 to 20% BN-polyacrylamide gel.

2.5. Activity assays

Oxygen uptake: Ascorbate–TMPD (*N*,*N*,*N*,*N*-tetramethyl-*p*-phenylenediamine) oxidase activity was measured at 28 °C in a closed reaction chamber (1-ml volume) with a fiber optic oxygen meter (Fibox 3; PreSens GmbH, Regensburg, Germany). *R. capsulatus* membranes were dissolved in ICM buffer (50 mM triethanolamine, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF) to a final concentration of approximately 0.1 mg/ml. Oxygen consumption was initiated by the addition of 10 μl of 1 M sodium ascorbate (final concentration of 10 mM) and 5 μl of 24 mM TMPD (final concentration of 0.12 mM). Oxygen consumption was recorded at 28 °C using the OxyView 3.5.1 software (PreSens GmbH; Germany), and terminated after several minutes of recording by the addition of 0.1 mM NaCN (final concentration). Net ascorbate-TMPD oxidase activity was determined by subtracting the endogenous respiration rate from that induced by ascorbate-TMPD mixture.

In-gel heme staining, and on-plates/in-gel α-naphthol and N,N-dimethyl-p-phenylenediamine (NADI) staining: SDS–Tris–Tricine polyacrylamide gels were treated with 3,3,5,5-tetramethylbenzidine (TMBZ) to reveal the *c*-type cytochromes according to the method of Thomas et al. [46]. For activity staining of cbb_3 Cox in intact cells via the NADI reaction, colonies obtained on MPYE plates containing appropriate antibiotics at 35 °C, were stained using a 1:1 (v/v) mixture of 35 mM α-naphthol dissolved in ethanol and 30 mM *N*,*N*-dimethyl-*p*-phenylenediamine in water, and those with an active enzyme produced a blue color. NADI staining of BN-PAGE gels was performed as described earlier [31].

2.6. Formaldehyde cross-linking

For *in vivo* formaldehyde cross-linking, *R. capsulatus* was grown semi-aerobically in MPYE medium with appropriate antibiotics. The formaldehyde solution was freshly prepared by dissolving paraformaldehyde (PFA) in preheated (60 °C) PFA buffer (2.7 mM KCl, 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, pH 6.5) to a final PFA concentration of 4% and then cooled down to 35 °C. When the *R. capsulatus* cultures reached an OD_{685} of 0.9–1.0, the freshly prepared PFA solution was added to the cultures (441 ml of PFA solution to each 2500 ml culture), resulting in a final PFA concentration of 0.6%. Controls were performed by adding the same amount of PFA buffer. After the addition of PFA or buffer, the cultures were incubated for additional 20 min at 35 °C and 110 rpm and chilled on ice before harvesting them in a SLC-6000 rotor for 10 min at 14000 g. The pellets were resuspended in 80 ml ice-cold PBS (50 mM Na-Phosphate, pH 7.0, 300 mM NaCl), then centrifuged again and resuspended in 30 ml ice-cold PBS containing 0.5 mM PMSF and the *Complete* protease inhibitor cocktail (Roche, Mannheim, Germany). Cell suspensions were disrupted by using a French press at 8000 psi (pounds per square inch) followed by centrifugation in the SS-34 rotor for 30 min at 28,720 g to remove cell debris. The supernatant was ultracentrifuged for 2 h at 174,000 g in the Ti50 rotor (Beckmann-Coulter, Krefeld, Germany). The pellet containing the intracytoplasmic membranes was resuspended in 4 ml solubilization buffer (50 mM Na-Phosphate, pH 7.0, 300 mM NaCl, 20% Glycerol, 0.5 mM PMSF and *Complete* protease inhibitor cocktail and homogenized with a potter. Resuspended membranes were either frozen in liquid nitrogen, and stored at −70 °C until further treatment, or solubilized directly by the addition of dodecyl-maltoside (1% final concentration; 25 ml total volume) and incubation for 1 h on a rotating wheel at 4 °C. After ultracentrifugation for 30 min and 38,000 rpm in the Ti50 rotor, the supernatant was mixed with 2 ml of prepared Talon material in washing buffer (50 mM Na-Phosphate, pH 7.0, 300 mM NaCl, 20% glycerol, 0.1% DDM, 0.5 mM PMSF) and incubated on a rotating wheel for 45 min at 4 °C. After centrifugation for 2 min at 4000 *g*, the supernatant was removed, mixed with 1 ml washing buffer and poured into disposable polypropylene columns. After four washing steps with 1 ml washing buffer, elution was started with elution buffer (50 mM Na-Phosphate, pH 7.0, 300 mM NaCl, 12% Glycerol, 1% DDM, 200 mM imidazole, pH 7.0, 0.5 mM PMSF) and fractions were collected.

2.7. Immune detection methods

For immunoblot analyses, proteins were electro-blotted onto Immobilon-P transfer membranes, and polyclonal antibodies against CcoP, CcoN and CcoH were used with either horseradish peroxidase-conjugated goat anti-rabbit antibodies (Caltag Laboratories, Burlingame, CA) or alkaline phosphatase-conjugated goat anti rabbit antibodies (Sigma, St. Louis, USA) as secondary antibodies, and ECL (GE Healthcare, Munich, Germany) or NBT/BCIP (Roche, Germany) as detection substrate. Peptide antibodies against CcoI, PCu_AC, and SenC were generated by GeneScript (New Jersey, USA), using the following peptides: CcoI (RIAGQARRRIKDNFC); PCu_AC (CNAGDSADRLTAVEV), SenC (CLQTPGDTPAAGNGN). Antibodies against the $(His)_6$-tag were obtained from Roche (Mannheim, Germany).

2.8. Measuring copper binding to SenC

For measuring copper binding to SenC, pET22b-SenCΔTM and pET22b-SenCΔTMC were expressed in *E. coli* Bl21 (DE3) and purified via their C-terminal His-tag, basically using the same protocol as described for the Talon purification of the formaldehyde-cross-linking products. Copper binding was analyzed by UV/Vis spectroscopy following the protocol described by Bühler et al. [15]. In brief, soluble SenC was adjusted to a protein concentration of 10 μM in elution buffer lacking DDM (see above; corresponding to approx. 250 μg/ml) and was reduced under aerobic conditions with 2 mM DTT and 5 mM sodium dithionite for 4 h at 4 °C. Subsequently, the sample was diluted 1:1 with 1 ml Chelex-100 treated phosphate buffered saline (Cu-free PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2.0 mM KH_2PO_4, pH 7.4). DTT and sodium dithionite were removed by buffer exchange using a 2 ml Vivaspin micro-concentrator (Sartorius, Germany; cut-off MW 3 kDa). After three washing steps using Cu-free PBS, the protein was concentrated to a final concentration of 10 μM in 1 ml Cu-free PBS. Apo-SenC was then incubated under aerobic conditions with different concentrations of $CuSO_4$ (between 0.5 and 10 μM final concentration) for 1 h at 4 °C. Unbound Cu was removed by buffer exchange against Cu-free PBS using a micro concentrator as above and UV/Vis spectra of Cu(II)-SenC were recorded between 300 and 420 nm under aerobic conditions using a TIDAS-100 spectrophotometer (*j & m* Analytics, Germany).

2.9. Measurement of cellular copper accumulation

R. capsulatus cells were grown in MPYE media under aerobic conditions at 35 ºC, up to an OD_{685} of 1.0. 100 ml of the cell culture was harvested by centrifugation at 4 °C, 6000 rpm for 10 min and the cell pellet was washed with 50 ml of Cu-depleted MPYE and centrifuged again. The pellet was then resuspended in 1 ml of Cu-depleted H_2O and 1 ml of this suspension was transferred to a new 15 ml tube. 20 μl of cell suspension was collected and protein concentration was

measured by using the Lowry assay. Per 1 ml cell suspension 0.75 ml concentrated nitric acid was added and incubated at 80 °C for 1 h and at 60 °C overnight to obtain a clear, transparent liquid. The reaction was terminated by adding 300 μl H_2O_2. Cu-free water was added up to a final volume of 10 ml and the samples were measured by using an atomic absorption spectrophotometer Perkin-Elmer 4110 ZL Zeeman. If any insoluble impurities were observed in the solution, samples were filtered through 0.45 μm filters.

3. Results

3.1. The effect of SenC deletion on active cbb_3-type cytochrome oxidase production

NADI-staining (see Materials and Methods) of *R. capsulatus* colonies is a simple assay for assessing cbb_3 Cox activity directly, because cbb_3 Cox is the only Cox present in this organism and because the alternative Qox does not respond to the NADI reagent [40]. In the presence of active cbb_3 Cox, colonies of a wild type *R. capsulatus* strain like MT1131 turn blue in less than 30 s ($NADI^+$), while cbb_3 Cox deficient strains like the *ccoNO* deletion mutant GK32 retain their original color even after more than 10 min ($NADI^-$). The Δ*senC* strain LS01 did not respond to the NADI reagent unless it contained a plasmid-borne *senC* copy (Fig. 1A). In agreement with earlier data [23], cbb_3 Cox activity in LS01 was also restored upon supplementation of MPYE agar plates with at least 0.5 μM $CuSO_4$ (Fig. 1B). On the other hand, addition of up to 20 μM $MgCl_2$ or $FeSO_4$ did not influence the NADI reaction of LS01 (data not shown). The need for Cu supplementation to detect cbb_3 Cox activity in LS01 was not changed when the cellular amount of the periplasmic copper chaperone PCu_AC was increased by expressing it from a plasmid-borne copy (Fig. 1B and C). Similar data were recently also observed in *R. sphaeroides* and it was concluded that the presence of PrrC, the SenC/Sco1 homologue in *R. sphaeroides*, is required for PCu_AC to enhance cbb_3 Cox assembly [36]. These data would also be in line with the suggestion that PCu_AC acts downstream of SenC/Sco1 during copper delivery to the aa_3 Cox [16]. A *R. sphaeroides* ΔPCu_AC mutant is viable and displays a cbb_3 Cox phenotype similar to ΔPrrC mutant [36]. We tried to isolate a *R. capsulatus* ΔPCu_AC mutant, but for unknown reasons several attempts failed so far. A possibility is that PCu_AC might be essential for *R. capsulatus* growth under the conditions tested. Interestingly, LS01 turned $NADI^+$ even in the absence of additional Cu when grown at low temperature (20 °C) or upon longer incubation at room temperature subsequent to growth at 35 °C (Fig. 1D). In contrast, the *ccoNO*-deletion strain GK32 remained $NADI^-$ after extended incubation at low temperature. This finding suggests that at slower growth rates, production of active cbb_3 Cox proceeds even in the absence of SenC.

In order to determine the role of SenC in cbb_3 Cox production, cbb_3 Cox activity was measured in intracytoplasmic membranes (ICMs) isolated from appropriate *R. capsulatus* strains grown under semi-aerobic conditions on MPYE medium. In LS01, cbb_3 Cox activity was reduced to about 20% of the wild type activity, but it was fully restored by providing a plasmid-borne copy of SenC (Fig. 1E). Almost complete restoration of cbb_3 Cox activity was also observed when LS01 was grown in the presence of 1 μM $CuS0_4$ (Fig. 1E), while the addition of Cu to MT1131 did not change the cbb_3 Cox activity. Finally, increasing the SenC amounts in wild type cells by expressing additional SenC from a plasmid-borne copy (Fig. 1F) also increased cbb_3 Cox activity (Fig. 1G), suggesting that SenC might be limiting for cbb_3 Cox production under the conditions tested. Increasing the cellular concentration of PCu_AC did not increase the cbb_3 Cox activity in wild type cells and also had no effect on the cbb_3 Cox activity in LS01 (Fig. 1G).

SDS-PAGE analyses showed that the catalytic subunit CcoN was almost undetectable in LS01 but restored to wild type level when LS01 was grown in the presence of additional Cu (Fig. 2A). Heme staining for revealing the *c*-type cytochrome subunits of cbb_3 Cox demonstrated the lack of the CcoP and CcoO subunits of cbb_3 Cox in LS01 (Fig. 2A), but the steady-state concentrations of cyt c_1 of the bc_1 complex or of the membrane-bound electron carrier cyt c_y were identical to the wild type. In the presence of Cu, CcoP and CcoO levels were comparable to wild type. Cbb_3 Cox forms on BN-PAGE an active 230 kDa complex that contains the core subunits CcoN, CccO, CcoQ, CcoP and the recently identified accessory subunit CcoH [33]. For analyzing whether the same 230 kDa complex was detectable in LS01 complemented with Cu, we performed BN-PAGE analyses combined with NADI activity staining. The active cbb_3 Cox complex was detectable as a blue band at approx. 230 kDa in wild type membranes (Fig. 2B) and in membranes derived from wild type cells expressing *senC* from a plasmid. This band was not detectable in GK32 or LS01 (Fig. 2B), but by adding Cu to LS01, the same active 230 kDa complex was observed, indicating that normal cbb_3 Cox assembly proceeds in the absence of SenC as long as Cu is present in sufficient amounts. This was further verified by immune detection. ICMs were separated on BN-PAGE and after western transfer probed with antibodies against the CcoP subunit. The 230 kDa cbb_3 Cox and the CcoPQH intermediate were only weakly detectable in LS01, but in ICMs from LS01 grown in the presence of Cu, both the 230 kDa and the CcoPQH intermediate were readily detected (Fig. 2C). Wild type ICMs and ICMs from wild type expressing *senC* from a plasmid served as a control. The latter ICMs showed an increased amount of the 230 kDa complex, which is in line with the higher activity (Fig. 1G).

3.2. The absence of senC does not affect ccoNOQP transcription and translation initiation

In the *R. capsulatus* genome the structural genes corresponding to the trans-acting regulatory proteins RegA and HvrA are co-transcribed together with that of SenC [47]. We therefore inquired whether the absence of SenC impaired *ccoNOQP* transcription. Total RNA isolated from appropriate *R. capsulatus* strains grown on MPYE medium was subjected to RT-PCR analyses using a *ccoN* specific primer pair (Fig. 3A). A DNA fragment of the expected size was detected using the RNA from a wild type strain, but not from a Δ*ccoNO* (GK32). In the case of LS01, or LS01 carrying a plasmid-encoded *sen*C copy, the same DNA fragment was also detected in amounts comparable to those seen with a wild type strain. We therefore concluded that the absence of SenC had no significant effect on *ccoNOQP* transcription. This is in agreement with previous experiments using a *ccoN::lacZ* transcriptional–translational fusion, which also suggested that the deletion of *senC* has no significant effect on ccoNOQP transcription and translation initiation [23]. Thus, SenC appears to function at a post-transcriptional and post-translation initiation step during cbb_3 Cox maturation.

3.3. senC expression is affected by copper in the absence of the Cu export ATPase CcoI

The defect inflicted by the absence of SenC can be alleviated by Cu supplementation, suggesting that its role might be more important at low Cu concentrations. We therefore tested whether *senC* expression itself was copper dependent. Total RNA was isolated from wild type cells grown either on MPYE media, which contains approx. 150 nM Cu [30] or on MPYE media supplemented with 1 μM $CuSO_4$ for 1 h. RT-PCR analyses using *senC* specific primers showed that in wild type cells, the amount of *senC* mRNA was only slightly increased upon copper addition, suggesting that *senC* expression is not significantly influenced by copper concentrations above 150 nM (Fig. 3B). As a control, Cu supplementation did also not affect significantly the *ccoN* or the 16S rRNA expression in a wild type strain. Remarkably though, different results were seen with a mutant lacking the Cu export ATPase CcoI, which has been implicated in Cu delivery to cbb_3 Cox [30]. CcoI belongs to the P_{1B}-class ATPases and is highly homologous to CopA2 of *P. aeruginosa*, which catalyzes cytoplasmic Cu^+ efflux into the periplasm [48]. When the Δ*ccoI* strain CW2 was tested

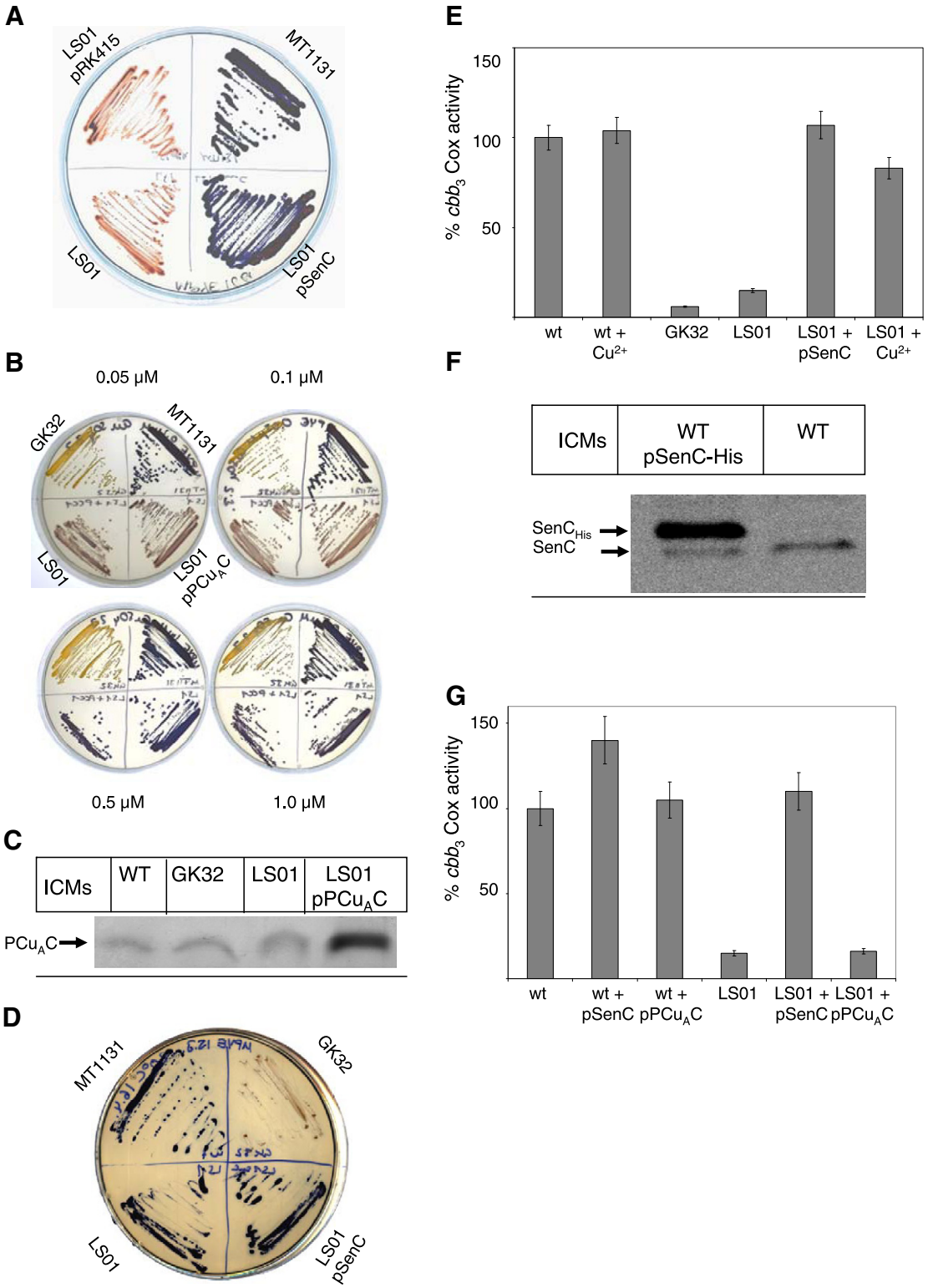

Fig. 1. Role of SenC in cbb_3 Cox assembly. A: The indicated strains were streaked onto MPYE agar plates and incubated overnight at 35 °C before they were overlaid with the NADI reagent. Cells containing cbb_3 Cox activity turn blue in less than 30 s, while cells lacking cbb_3 Cox activity do not change color even after more than 10 min. MT1131 corresponds to wild type *R. capsulatus* and LS01 to a Δ*senC* strain. pRK415 is a medium copy plasmid into which *senC* was cloned under its own promoter to yield pSenC. B: As in A, but cells were grown on MPYE media supplemented with the indicated $CuSO_4$ concentrations. GK32 corresponds to a Δ*ccoNO* strain lacking cbb_3 Cox and plasmid pPCu$_A$C encodes the putative *R. capsulatus* copper chaperone PCu$_A$C under its own promoter. C: The presence of PCu$_A$C in intracytoplasmic membranes (ICMs) isolated from different *R. capsulatus* strains was determined by Western blotting using peptide-directed antibodies. D: NADI staining of *R. capsulatus* colonies grown for 7 days at 20 °C. E: The activity of cbb_3 Cox in ICMs from different strains was analyzed by measuring oxygen uptake activities in at least three different experiments. The activity of cbb_3 Cox in wild type membranes (wt) corresponded to 17 µmol O_2/min mg protein and was set to 100 %. When indicated, LS01 or MT1131 (wt) were grown in the presence of 20 µM $CuSO_4$ for ICM preparation. G: The expression level of His-tagged and plasmid encoded SenC was analyzed by Western blotting using antibodies directed against *R. capsulatus* SenC peptides. F: The activity of cbb_3 Cox in ICMs from different strains was analyzed as in E. When indicated, wt and LS01 carried a plasmid encoded copy of either *senC* or *pCu$_A$C*.

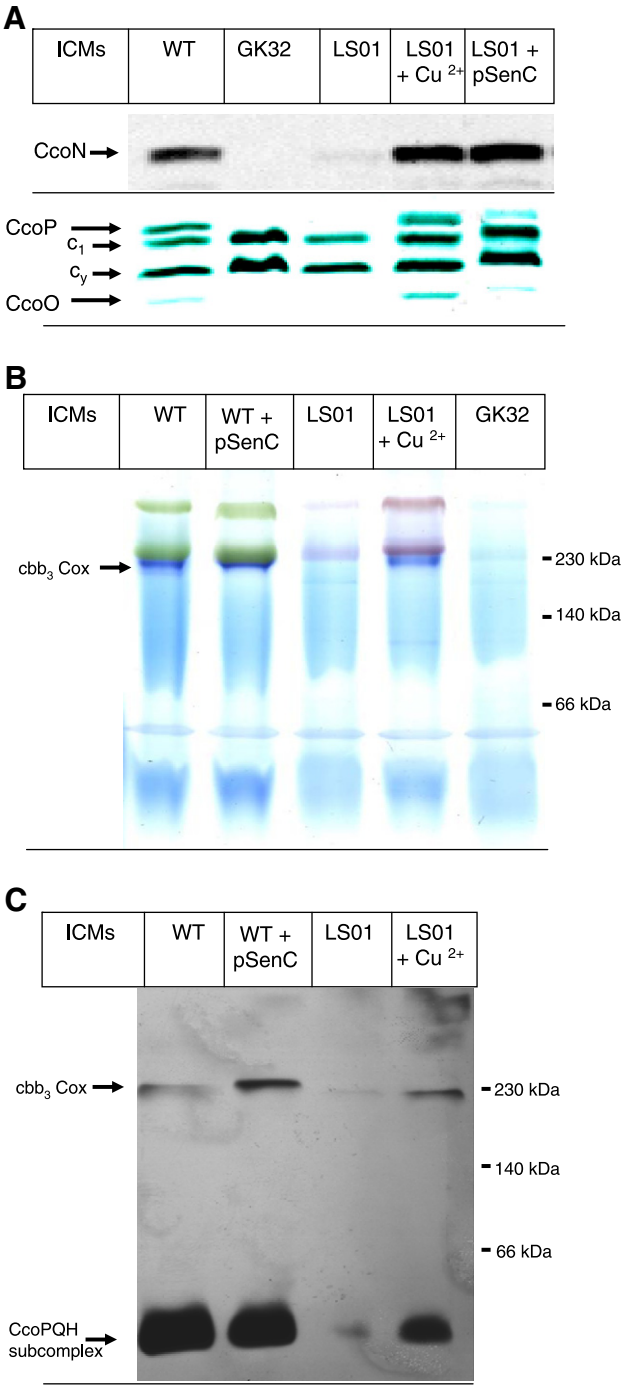

Fig. 2. The steady-state stability of cbb_3 Cox is drastically reduced in the absence of SenC. A: The presence of cbb_3 Cox subunits was determined by either Western blotting using antibodies against the catalytic subunit CcoN (upper panel) or by heme staining, which visualizes the membrane-bound *c*-type cytochrome profile of *R. capsulatus* ICMs (lower panel). CcoP and CcoO correspond to the membrane-bound *c*-type cytochrome subunit of cbb_3 Cox, while c_1 is part of the cyt. bc_1 complex and c_y a membrane bound electron carrier. Approximately 50 μg of ICMs were loaded per lane and separated on a 16.5% Tris–Tricine SDS-PAGE. When indicated, LS01 was grown in the presence of 20 μM $CuSO_4$ for ICM preparation. B: Approximately 100 μg of ICMs of the indicated strains were solubilized with dodecyl-maltoside, separated on a Blue-Native PAGE (BN-PAGE) and cbb_3 Cox activity was subsequently visualized by NADI-staining of the BN-PAGE. Active cbb_3 Cox is visible as a blue band at approx. 230 kDa. C: BN-PAGE was performed as in C, but proteins were transferred to a polyvinylidene difluoride (PVDF) membrane and decorated with antibodies against the CcoP subunit. CcoP recognizes the fully assembled cbb_3 Cox at approx. 230 kDa and a sub-complex consisting of CcoP, CcoQ and CcoH below the 66 kDa marker band.

for *senC* transcription in response to Cu addition, a pronounced increase in *senC* mRNA in CW2 was seen upon Cu addition (Fig. 3B, upper panel). In CW2, cbb_3 Cox is not stably assembled and therefore the increase in *senC* expression could primarily reflect the lack of cbb_3 Cox rather than the lack of CcoI. However, when we repeated the RT-PCR using mRNA isolated from GK32, we did not observe a significant difference in *senC* expression (Fig. 3B, lower panel). Considering that CcoI is likely to export Cu from the cytoplasm into the periplasm, we determined whether the *senC* up-regulation was the result of an altered intracellular copper concentration. However, when the cellular Cu content in wild type and CW2 cells was determined by atomic absorption spectroscopy, no significant difference between these two strains was observed, independently of whether additional Cu was added to the growth medium (Fig. 3D). These data indicated that CcoI apparently is not involved in maintaining copper homeostasis in *R. capsulatus*, a finding which is also supported by the fact that CW2 is neither more sensitive nor more resistant towards Cu addition (data not shown, [30]). The increased expression of *senC* in the absence of CcoI therefore does not seem to indicate any change in the overall cellular copper concentration, but it possibly reflects the lack of Cu specifically required for insertion into the Cu_B centre of cbb_3 Cox.

Earlier, SenC homologues from *R. sphaeroides* (PrrC) and *B. japonicum* (ScoI) were shown to bind Cu [13,15]. Indirect evidence for Cu-binding of *R. capsulatus* SenC was provided by data showing that a SenC derivative lacking one of the putative Cu-chelating cysteine residues exhibited a NADI-slow phenotype [23]. However, direct evidence for Cu binding by *R. capsulatus* SenC was lacking and we therefore determined Cu binding by a spectrophotometric assay [49]. As a control, we used a SenC derivative lacking the canonical cysteine residues Cys83 and Cys87, which have been shown to be essential for Cu binding in other SenC homologues [15]. Wild type SenC protein and its cysteine-less variant were expressed and purified as soluble proteins from *E. coli*. The purified proteins were reduced under aerobic conditions by DTT and sodium dithionate treatment, incubated in a buffer with or without Cu^{2+}, and their absorption spectra were recorded between 300 and 420 nm (Fig. 3E). With the native protein in the presence of Cu, a peak around 360 nm, which is diagnostic for Cu binding [15,49,50] and is likely due to thiolate-Cu(II) charge transfer [14] was detected. The highest signal was observed at approx. 5 μM Cu (Fig. 3E). This peak was not observed in the absence of Cu, or with the cysteine-less variant of SenC in the presence of Cu, indicating that like its homologues, the *R. capsulatus* SenC also binds Cu via its conserved cysteine residues. It is important to emphasize that although both wild-type SenC and the cysteine-free SenC contained a C-terminal His-tag, copper binding was only observed for wild type SenC, demonstrating that the His-tag did not promote copper binding in this assay. As SenC can be bypassed in the presence of high copper concentrations it is likely that copper binding by SenC is particularly important at low Cu concentrations.

3.4. SenC interacts with the CcoP and CcoH subunits of cbb_3 Cox

The role of SenC for the production of active cbb_3 Cox was further analyzed by *in vivo* cross-linking experiments using formaldehyde, and a His-tagged, functional SenC derivative expressed from its own promoter from the low-copy plasmid pRK415. Formaldehyde was chosen as a cross-linker because of its high permeability with whole cells and its very short spacer-length (2.3–2.7 A), which allows cross-links only between proteins that are in immediate contact with each other (Fig. 4A). Exponentially growing cell cultures were directly treated with para-formaldehyde (PFA); cells were collected and broken by a French pressure cell. His-tagged SenC was then purified from dodecyl-maltoside solubilized ICMs by Talon metal-affinity chromatography and elution fractions containing purified SenC were subjected to SDS-PAGE/immunoblot analyses using SenC specific antibodies. The immuno-blot data revealed a strong $SenC_{His}$ band in both the formaldehyde treated

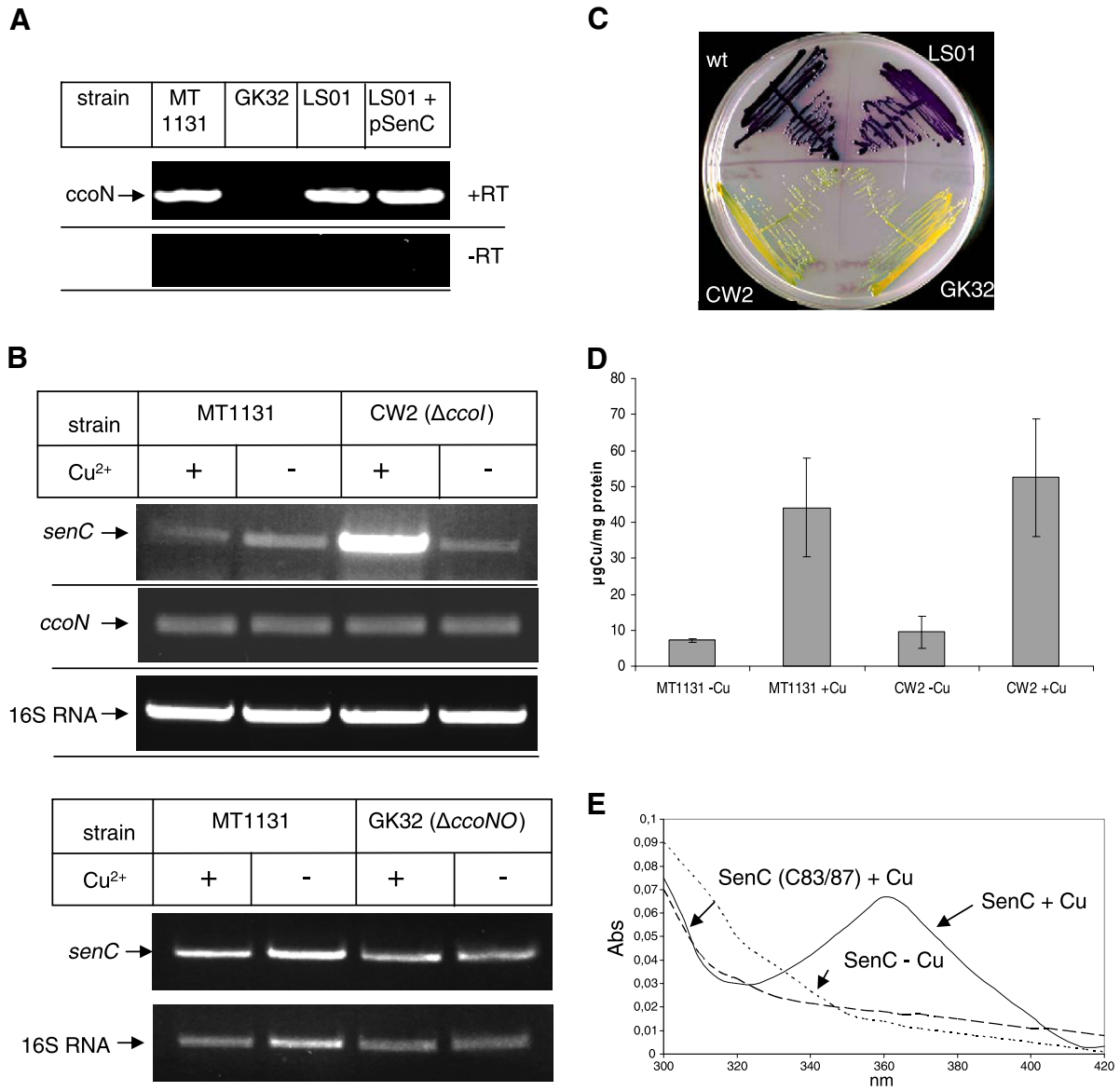

Fig. 3. SenC expression is enhanced in the presence of copper when the putative copper transport ATPase CcoI is missing. A: The expression of *ccoN* was analyzed by reverse transcriptase PCR. Total RNA was isolated from the indicated strains and RT-PCR was performed with a primer pair specific for *ccoN*. A control PCR without reverse transcriptase treatment (−RT) was performed for detecting possible DNA contaminations in the RNA sample. 50 ng of RNA was used for each RT-PCR. B: RT-PCR for detecting *senC* and *ccoN* expression in wild type, the Δ*ccoI* strain CW2 and the Δ*ccoNO* strain GK32. When indicated cells were treated for 60 min with 1 µM $CuSO_4$ prior to RNA isolation. The expression of the 16 S RNA served as control. C: NADI staining of the indicated strains grown on MPYE medium supplemented with 0.5 µM $CuSO_4$. D: Copper concentration in wild type and CW2 cells treated with 1 mM $CuSO_4$ for 60 min. Subsequently, cells were digested with concentrated copper-free nitric acid and the cellular copper concentration was determined by atomic absorption spectroscopy using a Perkin-Elmer 4110 ZL Zeeman spectrophotometer. E: SenC and a SenC-derivative lacking the canonical cysteine residues (SenC(C83/87)) were expressed as soluble proteins in *E. coli* and subsequently purified via metal-affinity purification. The purified protein (10 µM) was reduced under aerobic conditions by the addition of DTT and sodium dithionite and subsequently buffer exchanged against Cu-free phosphate-buffered saline. The purified protein was then incubated with increasing concentrations of $CuSO_4$ and incubated for 1 h at 4° before non-bound copper was removed by filtration. Spectra were taken with a TIDAS 100 spectrophotometer at 25 °C. Shown is the spectra of apo-SenC incubated with 5 µM Cu, which gave the highest optical signal.

sample and in the control sample. We also noticed in both samples a second band running below $SenC_{His}$. This band was recognized by α-SenC antibodies but not by α-His antibodies (data not shown), indicating that some cleavage of the His-tag occurred during protein purification. In the formaldehyde treated sample the α-SenC antibodies recognized several additional bands running between approx. 50 kDa and 80 kDa, which were not present in the control sample without formaldehyde. Thus, the *in vivo* formaldehyde treatment resulted in several SenC cross-linking products, which were purified via the His-tagged SenC. The Talon purification procedure was further controlled by formaldehyde treatment of cells expressing a SenC derivative without His-tag. Neither SenC nor any cross-linked bands were detected by α-SenC antibodies in these samples (Fig. 4B), demonstrating that purification procedure selectively purified $SenC_{His}$ and its cross-linking products.

For detecting possible cross-links between SenC and the cbb_3 Cox subunits, the Talon-purified cross-linked materials were separated on large SDS-polyacrylamide gels. The better resolution allowed the detection of two prominent bands of approx. 55 kDa and 72 kDa which were recognized by α-SenC antibodies (Fig. 5, left panels). The cross-linked material was then probed with antibodies against the CcoN and CcoP subunits of cbb_3 Cox. The α-CcoN-antibodies did not detect any cross-linked material (data not shown), but the α-CcoP antibodies recognized specifically the 72 kDa band (Fig. 5, left panels), indicating that SenC was cross-linked to the CcoP subunit of cbb_3 Cox. A SenC–CcoP

cross-linking product has a predicted mass of approx. 57 kDa (25 kDa + 32 kDa) and thus is expected to migrate below the recognized 72 kDa band. However, the migration behavior of two covalently linked proteins is often difficult to predict using the molecular masses of individual partners, and many membrane proteins display aberrant migration pattern after cross-linking [51]. Nevertheless, it was also possible that the 72 kDa cross-linking product represented a complex in which SenC was cross-linked to more than one protein. A possible candidate was the 17 kDa membrane protein CcoH because we had recently shown that CcoH is in close contact with CcoP [33]. CcoH was originally considered to function as an assembly factor for cbb_3 Cox [31], but unexpectedly, CcoH forms a stable complex with the fully assembled cbb_3 Cox, and thus behaves more like a subunit [33]. When the cross-linked material was probed with antibodies against CcoH, the 72 kDa band was strongly recognized (Fig. 5, left panels). Thus, the 72 kDa band most likely reflects a trimeric SenC–CcoP–CcoH complex. However, we cannot entirely exclude the possibility that the 72 kDa band reflects two cross-linking products of SenC, containing either CcoH or CcoP. The composition of the band at approx. 55 kDa is currently unknown.

The occurrence of a SenC–CcoP–CcoH cross-linking product was further controlled by repeating the formaldehyde cross-linking with the cbb_3 Cox deletion strain GK32, which lacks all subunits of cbb_3 Cox including CcoH [33]. When the formaldehyde cross linking and affinity purification experiments were repeated and compared with the data obtained using wild type cells (Fig. 5, right panels), the approx. 72 kDa band previously detected using the α-CcoP or the α-CcoH antibodies was not detectable (Fig. 5, right panels). This further substantiates that the approx. 72 kDa band represents a SenC–CcoP–CcoH cross-linking product. Interestingly, the 55 kDa cross-linking product was also not detectable in GK32 complex. As SenC is stably expressed in GK32 as deduced from the amount of non-cross-linked SenC that is purified from GK32 (Fig. 5, right panel), the 55 kDa band could reflect a SenC cross-link to the CcoO subunit of cbb_3 Cox, which has a molecular mass of 28 kDa. However, due to the lack of antibodies against CcoO this requires further analyses. Alternatively, it is also possible that the 55 kDa band reflects a SenC-dimer, as dimerization of Sco proteins has been observed before [3,52] and that SenC dimerization requires its interaction with cbb_3 Cox.

We also tried to detect interactions between SenC and other proteins involved in cbb_3 Cox assembly, like PCu_AC, CcoI and CcoS. However, the quality of the currently available peptide antibodies did not allow firm conclusions on possible cross-links between SenC and these proteins. In any event, our data show for the first time a direct physical interaction between SenC and the cbb_3 Cox subunits.

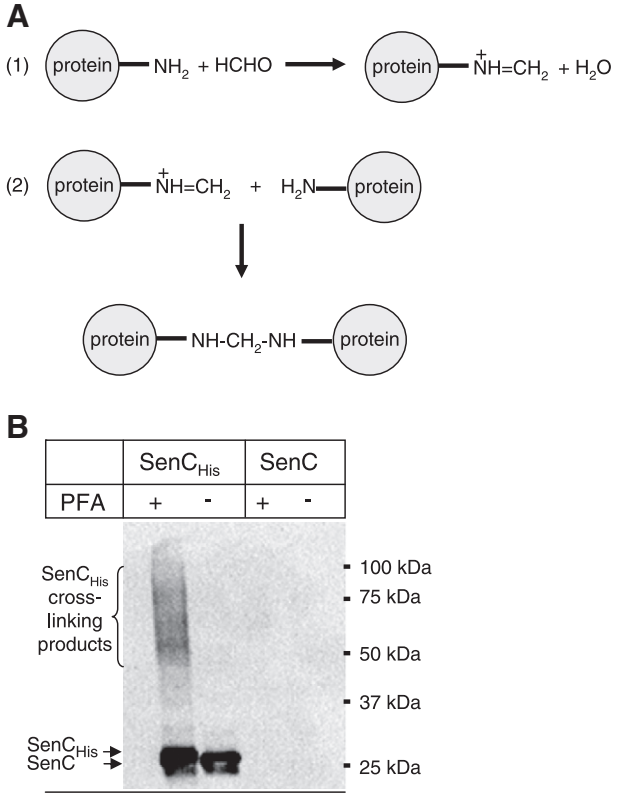

Fig. 4. *In vivo* formaldehyde cross-linking of SenC. A: Principle of formaldehyde cross-linking. Formaldehyde's single carbonyl group functions essentially as a homo-bifunctional reagent and is capable of conjugating targets through two different chemical pathways. In Mannich-type reactions formaldehyde condenses with amines or active hydrogens at elevated temperatures (>37 °C for 2–24 h) to from stable cross-links. Formaldehyde also reacts with amines to form highly reactive immonium cations (reaction 1) that are reactive toward protein-containing nucleophiles, including sulfhydryls, amines, phenols, and imidazoles (reaction 2). The latter reaction is rapid and is the typical protein cross-linking reaction at ambient temperatures. B: Plasmid-borne copies of His-tagged or non-tagged SenC were expressed in *R. capsulatus* and cells were either treated with para-formaldehyde (PFA) or with buffer. Subsequently, cells were harvested, french-pressed and ICMs were isolated by ultracentrifugation. ICMs were solubilized by dodecyl-maltoside and SenC was purified by metal-affinity chromatography. The purified material was then separated on a 15% SDS-PAGE (10 cm × 10 cm), blotted onto PVDF membranes and decorated with antibodies against SenC. During protein purification, some proteolysis occurred, resulting in the loss of the His-tag. When non-tagged SenC was expressed, no protein was detectable in the elution fraction.

4. Discussion

Our data show that the *R. capsulatus* Sco1 homologue SenC is specifically required for the steady-state stability of cbb_3 Cox at low copper concentrations and at higher (35 °C) growth temperatures. The absence of SenC does not affect the cbb_3 Cox assembly in the presence of at least 0.5 μM of exogenous Cu supplement, or at lower (20 °C) growth temperatures. In addition, this study has revealed three important aspects of SenC/Sco1 function during cbb_3 Cox assembly: (1) SenC can be chemically cross-linked *in vivo* to the CcoP and CcoH subunits of cbb_3 Cox, providing the first evidence for a direct physical interactions between SenC and cbb_3 Cox; (2) The transcriptional regulation of SenC is linked to the Cu delivery pathway to cbb_3 Cox, suggesting a role of SenC during Cu_B insertion into cbb_3 Cox; and (3) SenC binds Cu via a conserved cysteine motif, like its homologues in other species.

Multiple studies in bacteria and eukaryotes have shown that Sco homologues are crucial for aa_3 Cox assembly [5,7,16,36]. Sco proteins can transfer copper from a copper chaperone like Cox17 to the surface exposed Cu binding site Cu_A of the aa_3 CoxII subunit [5,7]. Alternatively, Sco proteins have also been suggested to function as thiol-oxidoreductases, reducing the thiol groups of the Cu binding motif in CoxII, allowing the subsequent Cu insertion by other Cu chaperones like PCu_AC [16]. However, whether PCuAC is able to insert Cu into the Cu_A-centre of Cox is still controversially discussed [16,36]. Although the *sco*/*senC* deletions have been shown to affect also cbb_3 Cox assembly in some bacteria [23,24,35], the function of Sco during this process is largely unknown, in particular because cbb_3 Cox lacks a Cu_A-containing subunit. Using *R. capsulatus* for studying Sco function has the intrinsic advantage that this model organism lacks an aa_3 Cox; hence the role of Sco on cbb_3 Cox assembly is not influenced by secondary effects that might result from impaired aa_3 Cox activity.

The observation that the a SenC knock-out is rescued by the addition of Cu^{2+} ($E_0 = 0.35$ V), and not Fe^{2+} ($E_0 = -0.41$ V), could be the result of a metal-catalyzed oxidation bypassing the need for the thiol:disulfide oxidoreductase activity of SenC. During the maturation of the *c*-type cytochrome subunits of cbb_3 Cox, the thiol-groups present at the heme attachment motifs of the apo-cytochromes are oxidized by the periplasmic DsbA–DsbB system and then subsequently reduced by a specific reduction pathway [53]. This redox-loop is thought to prevent the proteolytic degradation of the apo-cytochromes by DegP before heme insertion [54,55]. A *R. capsulatus* DsbA mutant shows a temperature-sensitive

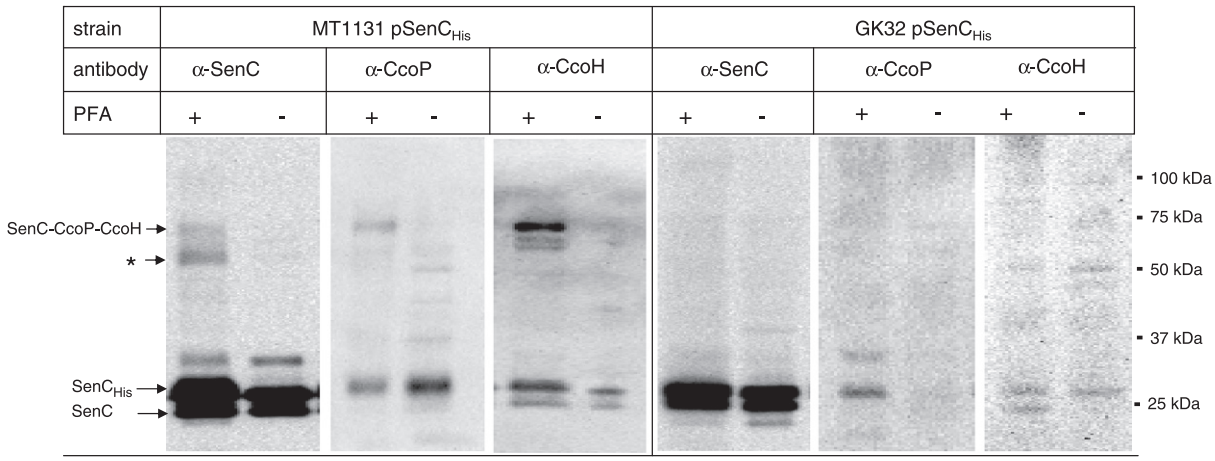

Fig. 5. SenC is in close proximity to the CcoP and CcoH subunits of cbb_3 Cox. The material shown in Fig. 4 was separated on large 15% SDS-PAGE (25 cm × 35 cm), blotted and decorated with antibodies against SenC, CcoP and CcoH. The cross-linking products are indicated. As control, pSenC$_{His}$ was also expressed in the Δ*ccoNO* strain GK32, which lacks CcoP and CcoH. Approximately 20 μg protein was loaded in each lane. The band labeled by a (*) reflects a so far unidentified SenC cross-linking product.

phenotype and exhibits a very low cbb_3 Cox activity at its permissive growth temperature (25 °C) (data not shown) but importantly, this cbb_3 Cox deficiency is rescued by the addition of Cu^{2+} or by other redox-active chemicals (data not shown; [28]). Thus, a mutant lacking DsbA has a phenotype that is reminiscent of that of a SenC mutant. A role of SenC in the oxidation/maturation of apo-CcoP and apo-CcoO would in principle explain the cbb_3 Cox deficiency of the Δ*senC* mutant, and is supported by a recent study showing that the *P. putida* Sco homologue is involved in electron transfer reactions [20]. However, no general cyt *c* maturation defect is observed in the absence of SenC because the synthesis of the membrane bound cytochromes c_1 and c_y is un-impaired. Nevertheless, the cytochromes c_1 and c_y are, like CcoO, mono-heme *c*-type cytochromes, whereas CcoP is a di-heme *c*-type cytochrome [45]. The cbb_3 Cox assembly defect in Δ*senC* might be the result of a particular defect in the maturation of the di-heme CcoP subunit or its assembly into cbb_3 Cox. This would explain the cbb_3 Cox deficiency because in the absence of the CcoP subunit, cbb_3 Cox in *R. capsulatus* is proteolytically degraded [40]. A possible contribution of SenC to CcoP maturation and assembly into the cbb_3 Cox would be in line with the observed CcoP-SenC cross-linking product. The molecular mass of the CcoP-SenC cross-linking product is 72 kDa and the immune detection data suggest that it corresponds to a trimeric SenC–CcoP–CcoH complex. CcoH is a recently identified novel subunit of cbb_3 Cox that is required for its stable assembly [30,32]. *In vitro* data suggested that CcoH is positioned very closely to the CcoP subunit [33], which we now confirm by *in vivo* data.

Although thiol-oxidoreductase activity and Cu chaperone activity are not mutually exclusive [13,19], the observation that SenC cross-links to CcoP but not to CcoN could suggest that it is primarily the thiol-oxidoreductase activity of SenC that is required for cbb_3 Cox assembly. However, both CcoP and SenC are single-spanning membrane proteins with large periplasmic domains, which favor multiple contacts between the two proteins. In contrast, CcoN is mainly membrane embedded and thus a possible SenC–CcoN interaction is most likely limited to the transmembrane domains and more difficult to detect by formaldehyde cross-linking. Thus, the lack of a cross-linking product between CcoN and SenC does not exclude a direct SenC–CcoN interaction or a direct involvement of SenC in Cu-delivery to CcoN. The Cu_B centre within CcoN is the only copper present in cbb_3 Cox [29], but its assembly is largely unknown. Different from the surface exposed Cu_A centre, the Cu_B centre is deeply buried within subunit I of the aa_3 or cbb_3 Cox [29,56], and it was therefore proposed that Cu_B might be inserted co-translationally [57]. A requirement for SenC during co-translational Cu insertion into CcoN would probably limit SenC–CcoN contacts to early stages of cbb_3 Cox assembly, which might further reduce the chances for detecting SenC–CcoN cross-linking products. Nevertheless, a role of SenC in copper delivery to cbb_3 Cox is supported by three observations: (1) SenC binds copper. (2) Copper rescues the cbb_3 Cox-deficiency of a Δ*senC* strain. (3) *SenC* expression is affected by copper in the absence of the Cu export ATPase CcoI. The membrane-bound copper chaperone Cox11 is required for Cu_B centre assembly of aa_3 Cox [24,58] and Cox11 probably inserts Cu_B from the periplasmic side of the membrane into a not yet completely folded subunit I [24]. A Cox11 homologue is absent in the *R. capsulatus* genome, which suggests that Cu_B assembly into aa_3 Cox and into cbb_3 Cox follow different pathways [15,58]. However, both Cox11 and SenC carry a copper binding site in their respective periplasmic domains [24] and thus, it is possible that SenC functionally replaces Cox11 during Cu_B insertion into cbb_3 Cox. For the Cu_A centre of *T. thermophilus* ba_3 Cox, it was proposed that Sco is required for reducing the Cu_A-ligating cysteine residues prior to Cu insertion [16]. Such a function does not seem to be required for Cu_B insertion, as Cu_B in cbb_3 Cox is coordinated via three conserved histidine residues, of which one is covalently linked to a tyrosine residue [29]. Although histidine residues can be oxidized to 2-oxo histidines, this mainly occurs during oxidative stress and serves as a marker for oxidative protein damage [59].

Although we favor a role of SenC in Cu_B insertion into CcoN, it is possible that SenC delivers copper not directly to CcoN but rather to another protein that inserts it into the active site of CcoN. This would also explain why we were unable to detect any SenC–CcoN interaction via cross-linking. Several *R. capsulatus* proteins like CcoA [34] or CcoI [30] have been implicated in copper trafficking and in the formation of the binuclear centre, but their exact function is largely speculative, mainly because direct evidence for Cu binding or transfer is missing [28]. We now show that *R. capsulatus senC* expression is up-regulated in the absence of CcoI. CcoI is essential for cbb_3 Cox assembly [30], and available information indicate that CcoI belongs to the CopA2-like P_{1B}-class ATPases [48,60], which are not required for general Cu homeostasis, but rather involved in cupro-protein assembly. This is related to their low turnover rates, which are not sufficient to provide Cu tolerance [60]. Our experiments show that the intracellular Cu concentration in the Δ*ccoI* strain is similar to that of a wild type, and that absence of CcoI does not affect the growth of *R. capsulatus* cells at different Cu concentrations (data not shown). The up-regulation of *senC* in the absence of CcoI therefore suggests that Cu availability for cbb_3 Cox assembly is specifically monitored. The overall data suggest that SenC and CcoI are functioning in the same pathway, although the phenotypes of the Δ*senC* and the Δ*ccoI* strains are different, as copper supplementation does not rescue the cbb_3 Cox deficiency in the Δ*ccoI* strain. Whether CcoI interacts directly with SenC or whether additional proteins like PCu_AC are involved in

Cu-delivery to cbb_3 Cox in *R. capsulatus* is currently unknown. Attempts to identify interactions between SenC and CcoI or PCu_AC via cross-linking failed so far, mainly due to the poor quality of the available antibodies.

In summary, our data show for the first time that the *R. capsulatus* Sco homologue SenC interacts directly with cbb_3 Cox and that it is linked to the Cu delivery pathway to the catalytic CcoN subunit. The high affinity of ScoI homologues for Cu (K_D approx 3×10^{-15} M) [61] supports the observation that SenC is specifically required at low Cu concentrations and becomes dispensable at high copper concentrations. Future experiments are required for determining which proteins are directly involved in copper loading of SenC and how copper is then further transferred to CcoN.

Acknowledgements

This work was supported by grants from the Deutsche Forschungsgemeinschaft (DFG-GRK1478 to HGK and DFG-FOR 929 to HGK), from the German–French-University (DFH) PhD College on "Membranes and Membrane Proteins" to HGK and in parts by grants from the NIH (GM 38237) and the Division of Chemical Sciences, Geosciences and Biosciences, Office of Basic Energy Sciences of the U.S. Department of Energy Grant DE-FG02-91ER20052 to F.D. for providing materials and for the preparation of the manuscript. PIT was supported by a fellowship from the Erasmus program of the European Union.

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