

Mitochondrial copper metabolism in yeast: interaction between Sco1p and Cox2p

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Abstract Yeast mitochondrial Sco1p is required for the formation of a functional cytochrome *c* oxidase (COX). It was suggested that Sco1p aids copper delivery to the catalytic center of COX. Here we show by affinity chromatography and coimmunoprecipitation that Sco1p interacts with subunit Cox2p. In addition we provide evidence that Sco1p can form homomeric complexes. Both homomer formation and binding of Cox2p are neither dependent on the presence of copper nor affected by mutations of His-239, Cys-148 or Cys-152. These amino acids, which are conserved among the members of the Sco1p family, have been suggested to act in the reduction of the cysteines in the copper binding center of Cox2p and are discussed as ligands for copper. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Sco1p; Yeast; Mitochondrion; Copper metabolism; Cytochrome *c* oxidase

1. Introduction

Cytochrome *c* oxidase (COX) catalyzes the electron transfer from cytochrome *c* to molecular oxygen. This terminal multi-subunit enzyme of the respiratory chain is localized in the inner membrane of mitochondria or of aerobic bacteria. The catalytic center of the enzyme is constituted by evolutionary conserved subunits: Cox1p, which carries two heme A moieties (cytochrome *a* and *a*₃) and one copper (Cu_B), and Cox2p with a binuclear copper center (Cu_A) [1]. A number of additional subunits, which are found in eukaryotic COX, seem to be involved in the assembly and regulation of the enzyme [2].

Study of the respiratory deficient mutants of the yeast *Saccharomyces cerevisiae* revealed that the assembly of COX requires a number of specific proteins, which are not subunits of the enzyme [3,4]. One of these factors is Sco1p, a 30 kDa protein of the mitochondrial membrane [5]. Topological studies show that Sco1p is anchored in the inner membrane by a transmembrane segment. The major carboxyl terminal portion of Sco1p protrudes into the intermembrane space (IMS) [6,7]. Yeast mutants lacking the *SCO1* gene are characterized by a rapid proteolytic degradation of newly synthesized mitochondrially encoded COX subunits, especially of Cox2p [8]. Sco1p

seems to act at a late step in the assembly of COX, possibly after incorporation of Cox2p into the assembling enzyme [9]. Sco1p is presumably involved in the incorporation of copper into COX: in the presence of elevated copper concentrations over-expression of Sco1p can suppress the COX deficiency of strains lacking Cox17p [10], which is discussed as a copper shuttle between the cytosol and the IMS [11]. It was proposed that Sco1p transfers copper ions directly from Cox17p to the COX subunit Cox2p [10], but a direct transfer of copper from Cox17p to Cox2p is also discussed [12]. In line with the former view is the presence of a CXXXC motif in Sco1p, which is similar to the copper binding site of Cox2p [10]. Mutational alterations of either of these two cysteines abolish the function of Sco1p [13].

The discovery of a significant similarity of the C-terminal portion of Sco1p to several peroxiredoxins and to bacterial thiol:disulfide oxidoreductases led to the suggestion of an alternative reaction mechanism, according to which Sco1p facilitates the coordinated reduction of cysteine residues in the copper binding center of Cox2p to allow insertion of copper from the associated Cox17p [14]. However, an arginine residue, which is conserved among peroxiredoxins (Arg-137 in the rat thioredoxin HBP23) and involved in the formation and stabilization of a reactive thiolate intermediate, is not present in the members of the Sco1p protein family. It was proposed that in this case activation of the catalytic group is performed by a conserved histidine residue (His-239 in *S. cerevisiae* Sco1p) at the respective position [14]. In the *Bacillus subtilis* homolog YpmQ this histidine is essential for the function of the protein [15].

Recent genome analyses revealed a number of genes with significant homology to *SCO1* in a number of organisms from bacteria to man [15–18], possibly reflecting similar mechanism of COX assembly. In the yeast *S. cerevisiae*, a further homologous mitochondrial protein (Sco2p) of unknown function was identified [19]. In humans so far two homologs have been detected. Both proteins are localized in the mitochondria [17,18]. At least one of these homologs, hSco2p, is essential for mitochondrial function: patients with compound heterozygous mutations in *hSCO2* suffer from a fatal cardiomyoencephalopathy, which is characterized by a severe COX deficiency in skeletal and cardiac muscle [20,21].

In this paper we analyze the interaction of yeast Sco1p. We show that it can form homomers in vitro and binds to the carboxyl terminal portion of Cox2p. These interactions are also observed in the absence of copper and with mutant forms of Sco1p, in which the cysteines of the CXXXC motif or His-239 are replaced by alanines or threonine, respectively.

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2. Materials and methods

2.1. Strains and media

Escherichia coli strains used in this work were DH5 α (BRL) and BL21 [22]. *S. cerevisiae* strains W303 (MATa, *ade2-1*, *his3-1*, *his3-15*, *leu2-3*, *leu2-112*, *trp1-1*, *ura3-1*) [23], AR2 (MATa, *his3-1*, *his3-15*, *leu2-3*, *leu2-112*, *ura3-228*, *ura3-251*, *ura3-379*, *sco1::URA3*, *sco2::Sphis5+*) (this work), GR20 (MATa, *his3-1*, *his3-15*, *leu2-3*, *leu2-112*, *ura3-228*, *ura3-251*, *ura3-379*, *sco1::URA3*) [24] and CEN.PK2 (MATa/ α , *his3-1*, *leu2-3*, *leu2-112*, *trp1-289*, *ura3-52*) (kindly provided by R.J. Schweyen, Vienna, Austria) were used.

2.2. Construction of the *S. cerevisiae* Δ *sco1*/ Δ *sco2* double null mutant strain AR2

Plasmid pFA6a-HIS3MX6 was used as a template for the amplification of the *his5+* gene of *Schizosaccharomyces pombe* with primers 5'-CCAAGTTATAGAAGACTGCCCTTCATCCCGGGGTAGTCGTACGCTGCAGGTCGAC-3' and 5'-ACGAAAGGAAAAAAGCAATCTCGCGAGTGGATGGCATCGGATCGATGAATTCGAGTCG-3' by short flanking homology-polymerase chain reaction (PCR) [25]. The resulting deletion cassette consists of the *Sphis5+* gene flanked by 40 bp long DNA sequences identical to the 5'- and 3'-flanking regions of *SCO2*, respectively. In a one step gene disruption [26] the deletion cassette was used to replace the *SCO2* orf in the genome of GR20. The replacement was verified by Southern blot hybridization (data not shown).

2.3. Site directed mutagenesis of *Sco1p* H239

Overlap extension PCR [27] was performed to construct the *Sco1p*[H239T] derivative using mutagenic primers 5'-GAAGATGGAAGTGTCTACCAATAATC-3' and 5'-GATTATTTGGTAGACACTTCCATCTTC-3' and flanking primers 5'-GTCCTCGAGCTCCCAATTGAACTAAATTG-3' and 5'-CTTCCAAGCTTCACATAGCCTC-3'. Bold letters indicate the altered codon. The resulting fragment was cloned into the *SacI*/*HindIII* sites of vectors YEp351 [28] and pRS415 [29].

2.4. Cloning, expression and purification of glutathione-S-transferase (GST) fusion proteins

For expression of the C-terminal portion of *Sco1p* fused to GST in yeast the coding region for amino acids 93–295 was amplified by PCR using primers 5'-TATATAGGATCCAGGGAGAAACGCAGATTGG-3' and 5'-CTTCCAAGCTTCACATAGCCTC-3' and cloned into the *Bam*HI/*Hind*III sites of GST expression vector pEG-KT [30] yielding plasmid *SCO1*(C)/pEG-KT. The plasmids *SCO1*(C)/pEG-KT and pEG-KT (control) were transformed in yeast CEN.PK2 cells. Transformants were cultivated at 30°C on minimal medium containing 2% sucrose as carbon source until an OD₆₀₀ of 1 was reached, and protein expression was induced by addition of galactose to a final concentration of 4%. Cells were harvested after 16 h, washed twice in buffer A (150 mM KCl, 10 mM Tris, 10 mM sodium molybdate dihydrate, pH 7.4) and resuspended in 4 volumes of ice-cold buffer A containing 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1% Triton X-100. One ml of glass beads (ϕ 0.45 mm) per g cells were added and cells were disrupted by vortexing for 5 \times 1 min. Cell lysate was centrifuged at 6000 \times g and 4°C for 10 min, followed by a ultracentrifugation step at 100 000 \times g and 4°C for 1 h. The supernatant was incubated with glutathione (GSH)-Sepharose 4B (Amersham Pharmacia Biotech) at 4°C with gentle rotation for 2 h. Sepharose beads were washed three times with 10 volumes of 1 \times phosphate buffered saline (PBS) containing 1 mM DTT, 1 mM PMSF and 0.5% Triton X-100. Expression and purification was confirmed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and subsequent Coomassie blue staining of the gel. 15–20 μ g protein bound to the Sepharose beads were used for in vitro binding assays.

For expression of the C-terminal portion of Cox2p (amino acids (aa) 108–351) fused to GST in *E. coli*, the mitochondrial genetic code was adjusted to the general genetic code by site directed mutagenesis using overlap extension PCR. The first three subfragments were amplified using yeast DNA prepared according to [31] as template and primers 5'-TATATAGGATCCGATGAAGTTATTTCCACCAGCTATGACT-3' and 5'-TTCATATTTCCAATACCAATGATATCC-3'; primers 5'-GGATATCAATGGTATTGGAAATATGAATATT-CAG-3' and 5'-TGTATCTACAGGTACAACCATAGAAGTATC-

3' as well as primers 5'-GATACTTCTATGGTTGTACCTGTAGAT-ACA-3' and 5'-TATATAGTCGACTTATTGTTTCATTTAACCATCCCAA-3'. Bold letters indicate the altered bases. In a second step the three subfragments were used as template for a PCR with primers 5'-TATATAGGATCCGATGAAGTTATTTCCACCAGCTATGACT-3' and 5'-TATATAGTCGACTTATTGTTTCATTTAACCATCCCAA-3'. The resulting fragment was purified by gel extraction, cut with *Bam*HI/*Sall* and cloned into vector pGEX-4T-3 (Amersham Pharmacia Biotech) to obtain plasmid COX2(C)/pGEX. Plasmids COX2(C)/pGEX and pGEX-4T-3 were transformed into *E. coli* strain BL21, and heterologous protein expression was induced with either 0.05 or 0.1 mM isopropyl β -D-thiogalactopyranoside for 2 h. Harvested cells were resuspended in 1 \times PBS containing 10% glycerol, 1 mM PMSF and 1% Triton X-100, kept for 30 min on ice and lysed by sonication. After centrifugation at 14 000 \times g and 4°C for 20 min the supernatant was incubated with GSH-Sepharose 4B (Amersham Pharmacia Biotech) at 4°C with gentle rotation for 1 h. Sepharose beads were washed in 1 \times PBS containing 1 mM PMSF. Expression and purification was confirmed by SDS-PAGE and subsequent Coomassie blue staining of the gel. 15–20 μ g protein bound to the Sepharose beads were used for in vitro binding assays.

2.5. Isolation of mitochondria

Yeast cells were grown to stationary phase in 50 ml minimal medium glucose. Mitochondria were isolated as described [32].

2.6. In vitro binding assay

300–500 μ g of isolated mitochondria from *S. cerevisiae* strains containing the test-proteins were lysed on ice for 1 h in 200 μ l of lysis buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% Nonidet P 40, 1 mM PMSF). After centrifugation at 20 000 \times g and 4°C for 15 min the supernatant was incubated with the GST fusion proteins bound to the GSH-Sepharose beads. All incubations were carried out at 4°C with gentle rotation for 2 h in the presence of 1 mM DTT. In some cases 3 mM of the copper chelator bathocuproinedisulfonic acid (BCS) were added or the GST fusion protein was pretreated with 3 mM BCS. After incubation the Sepharose beads were washed three times in at least 10 volumes of 1 \times PBS containing 0.5% Triton X-100, 1 mM PMSF and 1 mM DTT. Bound proteins were eluted by boiling for 5 min in the presence of 15 μ l of 2 \times SDS sample buffer containing DTT and separated on a 12% SDS-polyacrylamide gel.

2.7. Epitope tagging of *Sco1p* and *Sco1p* derivatives

HA tagged versions of *Sco1p* were created by PCR amplification with primers 5'-GTCCTCGAGCTCCCAATTGAACTAAATTG-3' and 5'-TATATAGTCGACGTTTGAATAAGAAGGAGTA-3' using plasmids *SCO1*/YEp351, *SCO1*[C148A]/YEp351, *SCO1*[C152A]/YEp351, *SCO1*[C148A;C152A]/YEp351 [13] and *SCO1*[H239T]/YEp351 as templates. The PCR products were cloned into the *SacI*/*Sall* digested YEp351-3HA (kind gift of R.J. Schweyen, Vienna, Austria), which contains three tandem copies of the HA epitope for C-terminal fusions. To yield the HA tagged *Sco1p* in yeast from a single copy vector plasmid *SCO1*-3HA/YEp351 was cut with *SacI*/*Hind*III, the 1.2 kb fragment containing the *SCO1*-3HA fusion was isolated and cloned into vector pRS415 to yield plasmid *SCO1*-3HA/pRS415.

2.8. Coimmunoprecipitation

300 μ g of isolated mitochondria from strain GR20 transformed with plasmid *SCO1*-3HA/pRS415 or *SCO1*/pRS415 were lysed on ice for 1 h in 1 ml of lysis buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% Nonidet P 40, 1 mM DTT, 1 mM PMSF). After centrifugation at 20 000 \times g and 4°C for 15 min the supernatant was added to 50 μ l of protein A-agarose (Santa Cruz Biotechnology), which was preincubated with 8 μ g anti-HA antibody (Boehringer Mannheim). Incubation was carried out with gentle rotation at 4°C for 4 h followed by precipitation of protein A-agarose at 500 \times g and two washing steps for 20 min each using 1 ml lysis buffer. Bound proteins were eluted by boiling for 5 min in the presence of 20 μ l of 2 \times SDS sample buffer containing DTT and separated on a 15% SDS-polyacrylamide gel.

2.9. Western blot analysis

Protein electrophoresis in the presence of SDS was carried out according to Laemmli [33]. Proteins were transferred onto a polyvinylidene difluoride membrane (Millipore) and probed with antibody directed against HA (Boehringer Mannheim), *Sco1p* [34] or Cox2p (Molecular

Probes). The detection of bound antibodies was performed with horseradish peroxidase conjugated secondary antibodies and the ECL-Plus Kit (Amersham Pharmacia Biotech).

2.10. Miscellaneous procedures

Standard DNA techniques were as described [35]. Yeast cells were transformed by the lithium acetate method [36]. GENOMED[®] columns were used for the isolation of DNA fragments from agarose gels. DNA sequencing was carried out with the dideoxy chain termination method [37] using 5' IRD800 labeled primers (MWG-Biotech) and the 'Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP' (Amersham Pharmacia Biotech) and the LI-COR DNA sequencer, model 4000 (MWG-Biotech). Protein concentrations were determined by the Lowry method (Bio-Rad).

3. Results

3.1. Interaction of Sco1p and Cox2p

As outlined in Section 1, the current model for Sco1p action predicts an interaction between Sco1p and Cox2p. To test this interaction mitochondrial lysate of the wild type strain W303 was incubated with GSH–Sepharose coupled GST–Sco1p(C). (This fusion protein contains the carboxyl terminal portion of Sco1p (aa 93–295), which has been shown to protrude into the IMS.) Bound material was separated by SDS–PAGE, blotted and analyzed with antibody against Cox2p. As shown in Fig. 1A, Cox2p is bound by GST–Sco1p(C), but not by GST alone. This result shows that Sco1p interacts with Cox2p in this *in vitro* assay.

The interaction between both proteins *in vivo* was confirmed by co-immunoprecipitation of Sco1p and Cox2p. To this end a Sco1p derivative with a triple HA-tag (Sco1p-3HA)

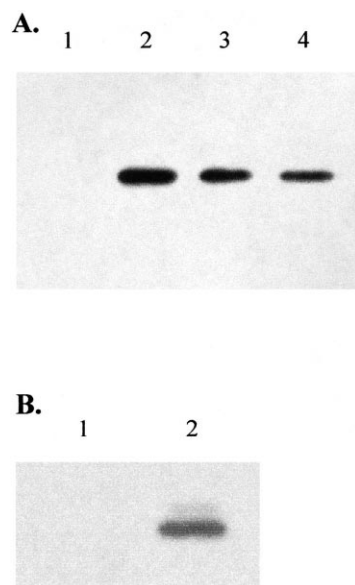


Fig. 1. *In vitro* interaction of Sco1p and Cox2p. A: GST (lane 1) and GST–Sco1p(C) (lanes 2–4) were isolated from transformants of strain CEN.PK2, bound to GSH–Sepharose beads and incubated with lysate of mitochondria prepared from yeast wild type strain W303. Incubation was carried out in the absence (lanes 1 and 2) or presence of the copper chelator BCS (lane 3). In lane 4 the GST–Sco1p(C) fusion protein was pretreated with BCS after binding to GSH–Sepharose. The blot was probed with Cox2p specific antibody. B: GST (lane 1) and GST–Cox2p(C) (lane 2) were isolated from *E. coli* transformants and coupled to GSH–Sepharose. Incubation was carried out with mitochondrial lysates of strain AR2 expressing Sco1p-3HA. The Western blot was probed with anti-HA antibody.

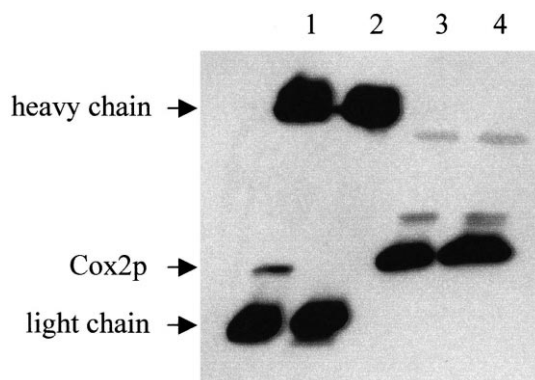


Fig. 2. Coimmunoprecipitation of Sco1p and Cox2p. Mitochondria containing HA tagged Sco1p (lanes 1 and 3) or untagged Sco1p (lanes 2 and 4) were lysed and coimmunoprecipitation was carried out using anti-HA antibody. Western blot analysis of the precipitates was done with anti Cox2p-antibody. Immunoprecipitates (lanes 1 and 2) and mitochondrial lysates (lanes 3 and 4) are shown. The secondary antibody used for detection of Cox2p antibody also recognizes the heavy and light chains of the anti-HA antibody. The respective positions are indicated.

was constructed as described in Section 2. The tagged version of Sco1p can be detected in mitochondrial preparations of strain GR20 transformed with plasmid SCO1-3HA/pRS415 (data not shown). As these transformants are able to grow on non-fermentable glycerol medium, Sco1p-3HA can substitute for the authentic Sco1p. Sco1p-3HA was precipitated with antibody against HA from GR20 transformants and the precipitate was analyzed in a Western blot with antibody directed against Cox2p. In a control experiment, an identical procedure was performed with strain GR20 transformed with plasmid SCO1/pRS415. Fig. 2 shows the result of this experiment: Cox2p can be exclusively detected in the precipitate of Sco1p-3HA expressing cells (lane 1), but not in the control (lane 2). This result shows that Sco1p and Cox2p interact *in vivo*.

We next tested whether the binding of Cox2p by Sco1p is dependent on the presence of copper. To this end GSH–Sepharose beads with bound GST–Sco1p(C) were incubated with mitochondrial lysate from wild type strain W303 in the presence of 3 mM of the copper chelator BCS. As can be seen in Fig. 1A (lane 3) the interaction of Sco1p and Cox2p is unaffected by the presence of this chelator. An identical result was obtained when the GST–Sco1p(C) fusion protein was pretreated with BCS after binding to GSH–Sepharose prior to incubation with mitochondrial lysate (lane 4). These results demonstrate that binding between Cox2p and Sco1p does not require copper ions.

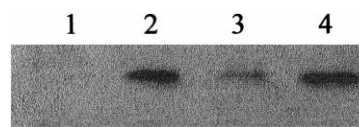


Fig. 3. *In vitro* homomerization of Sco1p. GST (lane 1) and GST–Sco1p(C) (lanes 2–4) were isolated from transformants of strain CEN.PK2 and bound to GSH–Sepharose. *In vitro* binding assay was carried out with mitochondrial lysate of strain AR2 expressing Sco1p-3HA in the absence (lanes 1 and 2) or presence of the copper chelator BCS (lane 3). In lane 4 the GST–Sco1p(C) fusion protein was pretreated with BCS after binding to GSH–Sepharose. The blot was probed with anti HA-antibody.

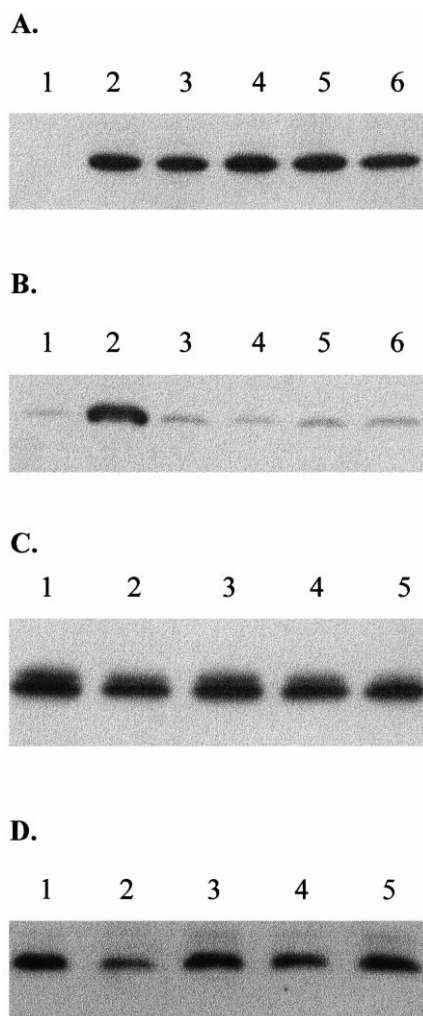


Fig. 4. Analysis of the Sco1p mutant proteins. A and B: Mitochondria were prepared from $\Delta sco1$ strain GR20 carrying no plasmid (lane 1) or plasmids SCO1/pRS415 (lane 2), SCO1[C148A]/pRS415 (lane 3), SCO1[C152A]/pRS415 (lane 4), SCO1[C148A;C152A]/pRS415 (lane 5) or SCO1[H239T]/pRS415 (lane 6). 30 μ g of mitochondrial protein were separated on a 12% PAA gel, blotted and probed with antibodies against Sco1p (A) or Cox2p (B). C and D: GST-Cox2p(C) isolated from *E. coli* (C) and GST-Sco1p(C) isolated from yeast strain CEN.PK2 (D), respectively, were bound to GSH-Sepharose and incubated with mitochondrial lysates of strain AR2 expressing Sco1p-3HA (lane 1), Sco1p[C148A]-3HA (lane 2), Sco1p[C152]-3HA (lane 3), Sco1p[C148A;C152A]-3HA (lane 4) or Sco1p[H239T]-3HA (lane 5). The Western blots were probed with anti HA-antibody.

The copper binding site (Cu_A) in the C-terminal portion of Cox2p is exposed to the mitochondrial IMS. To test whether this part of Cox2p interacts with Sco1p, we adjusted the sequence of the C-terminal portion of Cox2p (aa 108–351) to the general genetic code and fused it to GST as described in Section 2. The fusion protein was expressed in *E. coli* and coupled to GSH-Sepharose. Incubation of the GSH-Sepharose coupled protein was performed with mitochondrial lysate from strain AR2 ($\Delta sco1 \Delta sco2$), expressing Sco1p-3HA. As expected, Sco1p-3HA is bound by Cox2p(C)-GST (Fig. 1B). Due to the lack of Sco2p in the mitochondrial lysate of the AR2 transformant we conclude that the interaction of Sco1p and Cox2p is not mediated by Sco2p.

3.2. Homomer formation of Sco1p

The potential of Sco1p to form homomeric complexes in vitro was tested by affinity chromatography. GST-Sco1p(C) was expressed in yeast strain CEN.PK2 and coupled to GSH-Sepharose as described in Section 2. The bound GST-Sco1p(C) was incubated with a mitochondrial lysate which was isolated from the $\Delta sco1 \Delta sco2$ double null mutant AR2 expressing Sco1p-3HA. In a control, the lysate was incubated with GST-Sepharose. After extensive washing the Sepharose bound material was analyzed in a Western blot with antibody directed against HA. While the HA tagged Sco1p is bound by GST-Sco1p(C)-Sepharose, it is not by GST-Sepharose (Fig. 3, lanes 1 and 2). This result shows that the C-terminal portion of Sco1p can act as an interacting domain leading to the formation of homomers.

The interacting C-terminal portion of Sco1p contains the CXXXC motif, which is discussed as a copper binding site. To test whether the homomer formation requires bound copper ions, we performed the in vitro binding assay with Sepharose coupled GST-Sco1p in the presence of BCS, a copper specific chelator. As shown in Fig. 3 (lanes 3 and 4), homomer formation is also observed in the absence of copper.

3.3. Interactions of mutant forms of Sco1p

His-239, which is conserved among the members of the Sco1p family, was proposed to be engaged in the reduction of the copper binding center of Cox2p. We constructed a mutant allele, in which His-239 was replaced by threonine and transformed it into the $\Delta sco1$ null mutant GR20. The transformants proved to remain respiratory deficient. This result shows that His-239 plays an essential role for Sco1p function. An identical result was recently reported for mutant proteins, in which either one or both of the two essential cysteine residues of the conserved CXXXC motif were replaced by alanines. In all cases the mutations do not affect the stability of the mutant proteins as shown by the Western blot analysis of mitochondrial preparations from respective transformants with anti-Sco1p antiserum (Fig. 4A). To test the effects of the various mutations on the accumulation of Cox2p, we analyzed the mitochondrial proteins in a Western blot with antibody against Cox2p. Fig. 4B shows that none of the mutants differ significantly in its phenotype from the $\Delta sco1$ null mutant GR20: in all strains Cox2p is detectable at a concentration which is dramatically lower compared to strain GR20 transformed with SCO1/pRS415 (Fig. 4B, lane 2).

We next addressed the question whether these mutations interfere with the interaction of Sco1p and Cox2p. The wild type SCO1 and all four mutant alleles were tagged with 3HA and transformed into the $\Delta sco1 \Delta sco2$ double null mutant AR2. Mitochondrial lysates of the respective transformants were prepared and incubated with Sepharose coupled GST-Cox2p(C). As shown in Fig. 4C, all mutant proteins are able to interact with Cox2p. We conclude that neither the two cysteines nor His-239 are important for Sco1p/Cox2p interaction. The non-functionality of the proteins is not a consequence of a failure to interact with Cox2p.

Next we tested the ability of the mutant proteins to form homomeric complexes in vitro. GST-Sco1p(C) was coupled to GSH-Sepharose and incubated with mitochondrial lysates of AR2 transformants containing the HA tagged mutant proteins. As shown in Fig. 4D, all mutant proteins interact

with Sco1p. Therefore, neither His-239 nor Cys-148 nor Cys-152 are required for homomerization.

4. Discussion

The binuclear Cu_A center of Cox2p, which accepts electrons from cytochrome *c*, is located in the C-terminal portion of the protein, which is exposed to the mitochondrial IMS. The exact mode of how copper is incorporated into Cox2p is currently unknown. As outlined in Section 1, there is convincing evidence that Sco1p is essential for this process. Two alternative models have been suggested for the role of Sco1p in copper delivery to COX: either it directly transfers copper ions from Cox17p to COX [10] or it is required for reduction of the cysteines in the Cox2p copper binding center [14]. In both cases a transient direct interaction between Sco1p and Cox2p is expected. Our results obtained by affinity chromatography and by coimmunoprecipitation show that Sco1p interacts with Cox2p. The interaction is not dependent on copper ions, because the presence of the copper specific chelator BCS does not interfere with complex formation. It remains to be elucidated whether the Cox2p molecules which bind in the absence of BCS, represent the copper-free cellular fraction of Cox2p or whether binding can also occur with copper bearing Cox2p. Western blot analysis of blue native gels of mitochondrial proteins gives no indication for a permanent association of Sco1p with COX (Nijtmans, personal communication).

Mutations of the cysteines in the conserved CXXXX motif, which are essential for Sco1p function, have no effect on the interaction with Cox2p. If Sco1p would bind copper via this motif, this result would confirm that copper binding is not necessary for interaction. In the case of the copper enzyme superoxide dismutase (SOD) it was shown that mutant SOD forms devoid of copper still interact with its chaperone CCS [38]. In case of Sco1p being a thioreductase this result means that the binding is not or at least not exclusively mediated by a transient heterodisulfide formation between the copper binding center of Cox2p and the CXXXX motif of Sco1p. The involvement of electrostatic interactions in complex formation between thioreductases and their target proteins, mediated by amino acids surrounding the active site, has been recently described for some variants of *E. coli* thioredoxin [39].

Substitution of His-239 of Sco1p by a threonine abolishes the function of Sco1p without having an effect on the interaction with Cox2p. While this result shows that His-239 is not engaged in complex formation, the molecular role of this amino acid remains to be elucidated. His-239 has been proposed to be involved in the formation and stabilization of a reactive thiolate intermediate [14], whereas in the case of the *B. subtilis* homolog YpmQ it was suggested that this conserved histidine may be involved in copper binding [15].

According to topological studies, Sco1p is anchored by a single transmembrane segment in the inner mitochondrial membrane with its major C-terminal portion exposed to the mitochondrial IMS [6,7]. On the basis of this topology only the IMS domain of Sco1p is able to interact with the C-terminal portion of Cox2p. In line with this expectation, we found that Sco1p(C) (starting next to the transmembrane segment), bound to GSH–Sepharose via the fused GST protein, is able to bind Cox2p.

In addition to the cysteines of the CXXXX motif, Sco1p possesses two additional cysteine residues (Cys-181 and Cys-

216, respectively) in its IMS exposed portion. Substitution of these cysteines, which are not conserved among all members of the Sco1p protein family, by valine and serine, respectively ([C181V]; [C216S]; [C181V, C216S]), does not impair the function of Sco1p (Lode, unpublished results). Therefore we conclude that the interaction with Cox2p does not require the formation of intermolecular disulfide bridges.

Our data show that Sco1p can form homomeric complexes. This homomer formation was suspected from two-hybrid analyses (data not shown) and confirmed by affinity chromatographic studies. Homomeric complexes have been reported for copper chaperones [40] as well as for thioredoxins [41]. As in the case of Cox2p binding the IMS exposed C-terminal portion of Sco1p participates in this interaction, but an additional involvement of the N-terminal portion and/or the transmembrane segment cannot be excluded. The formation of Sco1p homomers is neither dependent on copper nor on the conserved His-239, Cys-148 or Cys-152. Combined with the finding that Cys-181 and Cys-216 are not essential for Sco1p function, this result indicates that disulfide bridges are not involved in Sco1p/Sco1p interaction. It remains to be elucidated whether in vivo the interacting partner of Cox2p is Sco1p in its monomeric and/or homomeric form. SOD and its chaperone CCS have been shown to form homo- and heterodimers. In the case of CCS homodimer formation is stimulated upon copper binding [42]. Our data give no evidence for a significant influence of the copper concentration on Sco1p homomerization. However, in vivo the efficiency of homo- or heteromer formation may vary depending on the physiological conditions.

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