Identification of Cox20p, a Novel Protein Involved in the Maturation and Assembly of Cytochrome Oxidase Subunit 2*

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We have identified Cox20p, a 23.8-kDa protein of the mitochondrial inner membrane that is involved in the biogenesis of the yeast cytochrome oxidase complex. Cytochrome oxidase subunit 2 (Cox2p) accumulates as a precursor in cox20 mutants, suggesting a defect in biogenesis of this mitochondrial encoded protein. The inability of cox20 mutants to process the subunit 2 precursor (pCox2p) is not due to impaired export of the protein across the inner membrane or to an inactive Imp1p/Imp2p peptidase. Rather, Cox20p specifically binds the newly synthesized pCox2p, a step required to present the exported pCox2p as a substrate to the Imp1p peptidase. All of the endogenous pCox2p accumulated in an Δimp1 mutant, and a small fraction of Cox2p in wild type yeast, is detected in a complex with Cox20p. Following maturation Cox20p remained associated with Cox20p, prior to assembling into the cytochrome oxidase complex. We propose that Cox20p acts as a membrane-bound chaperone necessary for cleavage of pCox2p and for interaction of the mature protein with other subunits of cytochrome oxidase in a later step of the assembly process.

An important event in the biogenesis of cytochrome oxidase is the sorting and processing of the subunit 2 precursor (pCox2p) after its synthesis by mitochondrial ribosomes (1–5). Mature subunit 2 (Cox2p) is an integral membrane protein with two transmembrane segments and an orientation such that the copper binding domain with the Cu₆ center of the enzyme is in the intermembrane space (6). In Saccharomyces cerevisiae, Cox2p is synthesized with an N-terminal presequence of 15 amino acids, which is removed following its export to the intermembrane space but prior to assembly of the protein into the functional enzyme complex (47).

Export of pCox2p across the inner membrane is mediated by the Oxa1p machinery (5, 8, 9). Mutations in OXA1 induce a deficiency in cytochrome oxidase due to the failure of pCox2p to become exported across the inner membrane (5, 8–11). Another characteristic of oxa1 mutants is the reduced level of the cytochrome bc₁ complex and a gross deficiency in oligomycin-sensitive ATPase (12, 13). Exported pCox2p is processed to its mature form by the inner membrane Imp1p/Imp2p peptidase (14–16), which also promotes maturation of cytochrome b₄ (7) and cytochrome b₅ reductase (Mcr1p) (17). This protease consists of at least two subunits, Imp1p and Imp2p (16). Mutations in either subunit result in the accumulation of pCox2p. An other mitochondrial protein involved in processing of pCox2p, Som1p, was identified as a multicopy suppressor of temperature-sensitive imp1 mutants (18). The relationship of Som1p to the Imp1p-Imp2p complex, however, is not clear.

Respiratory-deficient pet mutants of S. cerevisiae are powerful tools for studying different aspects of mitochondrial function and biogenesis (for review see Ref. 19). Such mutants have been grouped into broad phenotypic classes based on their cytochrome spectra and the enzymatic activities of the respiratory chain complexes. An important class of pet mutants is defined by cytochrome oxidase-deficient strains (20). This phenotype is encountered not only in mutants with lesions in the structural genes of the enzyme but also in a large number of other strains that are defective in expression of the mitochondrial encoded cytochrome oxidase subunits 1–3 (for review see Refs. 21–24), in heme A biosynthesis (25), and in mitochondrial copper import (26, 27). The roles of other proteins proposed to be involved in late events of cytochrome oxidase assembly (28–31) remain poorly understood.

To ascertain whether there are still other factors required for the biogenesis of Cox2p, we have screened a group of cytochrome oxidase yeast mutants for defects in processing the Cox2p precursor. We report a new gene, designated COX20, whose product functions in Cox2p maturation. This gene is defined by the complementation group G92 of a previously described pet collection (19). The product of this gene, Cox20p, is an integral protein of the mitochondrial inner membrane. Accumulation of pCox2p in cox20 mutants is not a consequence of a defect in translocation of the Cox2p precursor across the inner membrane by the Oxa1p machinery. Cox20p is also not essential for the intrinsic proteolytic activity of the Imp1p-Imp2p complex. Instead, Cox20p functions at a step subsequent to export of the pCox2p. We present evidence that Cox20p binds to the exported pCox2p species and chaperones it through the Imp1p processing step and perhaps subsequent assembly into the cytochrome oxidase complex.

EXPERIMENTAL PROCEDURES

Yeast Strains—The genotypes and sources of the yeast strains used in this study are listed in Table I. The OXA1 disruption/deletion strain (aW303/OXA1 Δoxa1) was constructed by replacing the nucleotides 220–1038 of the open reading frame encoding Oxa1p, with the HIS3 gene, as described (32).

Cloning of COX20 and Construction of a cox20 Null Allele—C176/L6 (MATa leu2-3, 112, cox20-1) was transformed by the method of Beggs (33) with a yeast genomic library constructed in the shuttle plasmid

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The abbreviations used are: pCox2p, precursor protein of cytochrome oxidase subunit 2; Cox2p, cytochrome oxidase subunit 2; PAGE, polyacrylamide gel electrophoresis; SPDP, N-succinimidyl 3-[2-pyridyldithio]propionate; MPP, mitochondrial processing peptidase.
Identification of Cox20p

Antibodies Against Cox20p—Antibodies were obtained against Cox20p expressed from a trpE fusion gene. An E. coli-HindIII fragment containing the entire gene was synthesized by polymerase chain reaction amplification with primers 5'-GGGGGATCCGAATTCACCATG-3' and 5'-GGGCGGATCCC-CAGAACTTGTACCATTTC. Following digestion with EcoRI and HindIII and ligated in-frame to pATH20 (37). E. coli transformed with this plasmid, expressed a protein of approximately 60 kDa that co-fractionated quantitatively with the insoluble protein fraction. The fusion protein was used to immunize rabbits after solubilization with SDS and partial purification on a Bio-Gel A 0.5 sizing column (Bio-Rad).

Construction of COX20-BIO—Biotinylated Cox20p was expressed from a fusion gene consisting of COX20 fused at its 3' end to a sequence coding for the biotinylation site of bacterial transcarboxylase (38). Twenty-nucleotides of 5' sequence and its termination replaced with a BamHI site was polymerase chain reaction-amplified with the forward primer 5'-CACACTCCATGATCCAGCAGGACGTAGCCAATTCCAG-3' and reverse primer 5'-GGGGGATCCCGAAGATCTCACTCATAGAGG-3'. The fragment was digested with EcoRI and HindIII and ligated in-frame to the N-terminal coding region of trpE in pATH20 (37). E. coli transformed with this plasmid, expressed a protein specifically bound to 15 mg of protein A-Sepharose (Amersham Pharmacia Biotech) with the cross-linker dimethyl pimelidate, as described (39). Isolated mitochondria were lysed in SDS buffer (1% SDS, 10 mM phenylmethylsulfonyl fluoride, 100 mM Tris/HCl, pH 7.4) and were diluted 20-fold with TBS/Triton X-100 buffer (10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.5% (w/v) Triton X-100). Immunoprecipitation of cross-linked adducts, using preimmune or specific antisera against either Cox20p or Cox2p, was performed essentially as described before (9, 40). Samples were analyzed by SDS-PAGE and autoradiography.

Co-immunoprecipitation of Cox20p with Antibodies Against Cox20p—Antibodies against Cox20p and preimmune serum (100 μl each) were incubated in gentle shaking conditions for 1 h at 4 °C with protein A-Sepharose beads bearing either antibodies specific for Cox20p or preimmune serum. The beads were washed three times with the digitonin buffer lacking the protease inhibitors, except phenylmethylsulfonyl fluoride, and then were eluted with SDS-sample buffer. The co-immunoprecipitates were analyzed by SDS-PAGE and Western blotting, followed by immune decoration with Cox2p-specific antisera.

Miscellaneous—Growth of yeast strains and isolation of mitochondria was performed, as described previously (39, 43). Outer membrane vesicle preparation, alkaline extraction, and mitoplasting were performed as described (32, 44). Protein was estimated according to published methods (45, 46). Enzymatic measurement of respiratory chain activities was performed essentially as described before (47). Mitochondrial translation products were labeled with [35S]methionine in total yeast cells, as previously published (48, 49). Detection of blotted proteins following SDS-PAGE was performed using the ECL detection system (Amersham Pharmacia Biotech).

RESULTS

Cloning and Identification of COX20—C176/L6 is one of 11 independent isolates previously assigned to complementation group G92 of a collection of respiratory-defective pet mutants (19). This strain is deficient in cytochrome oxidase activity (Table I) and lacks the absorption bands of cytochromes a and a3 (Fig. 1). Other segments of the respiratory chain, measured as NADH-cytochrome c reductase, are normal or even some-
what elevated. Oligomycin-sensitive ATPase activity is also comparable with that of wild type yeast.

Transformation of C176/L6 with a yeast genomic library yielded a clone whose respiratory competence was ascertained to depend on a segregating plasmid (pG92/T1). The DNA insert of pG92/T1 was approximately 3 kilobase pairs in length and spanned nucleotides 925,368 to 928,408 of chromosome IV. This region contains three entire reading frames (Fig. 2A), the longest of which (YDR229w) was unlikely to be the gene since the subclone pG92/ST3 lacking the N-terminal coding region of the YDR229w reading frame was able to restore respiration in C176/L6. The other two open reading frames YDR230w and YDR231c contained a substantial degree of sequence overlap. The identity of YDR231c as the gene responsible for conferring respiration to C176/L6 was established in several ways. The polymerase chain reaction-amplified reading frames corresponding to YDR231c and YDR230w were cloned in the CEN plasmid pYX122 (Novagen) behind the triose-phosphate isomerase promoter. Only the construct containing reading frame YDR231c was able to restore growth of the mutant on non-fermentable substrates. This was also true of a plasmid construct expressing YDR231c fused at its C terminus to an 8-kDa peptide containing the biotinylation site of bacterial transcarrboxylase (38). In contrast, the open reading frame YDR230w was not able to restore growth using both types of assays. In keeping with the previously used convention for designating genes affecting cytochrome oxidase, the gene YDR231c defined by complementation group G92 has been named COX20.

COX20 was partially deleted in a respiratory competent strain of yeast, W303-1A. The resulting null mutant w303ΔCOX20 (Δcox20) had a phenotype similar to that observed in C176/L6 (Fig. 1 and Table II). It lacked cytochromes α and α3 (Fig. 1) and had reduced cytochrome oxidase but not NADH-cytochrome c reductase activity (Table II). Oligomycin-sensitive ATPase activity was also not affected.

Allelism tests provided additional evidence that C176/L6 carries a mutation in COX20. An integrative plasmid containing the sequence of COX20 from the HindIII site upstream of the initiation codon and ending immediately after the termination codon (pG92/ST18) was linearized at the unique SphI site inside the gene (Fig. 2A). Leucine prototrophic clones obtained by transformation of C176/L6 with the linear plasmid were respiratory-competent consistent with integration of the linear plasmid at the cox20 locus thereby creating one wild type

### Table II

Respiratory and ATPase activities of mitochondria from wild type and cox20 mutants refer to mol of ATP hydrolyzed or of cytochrome c reduced or oxidized per min per mg of mitochondrial protein as described under "Experimental Procedures."

| Strain          | ATPase -Oligomycin | NADH cytochrome c reductase | Cytochrome oxidase |
|-----------------|--------------------|-----------------------------|-------------------|
| WT              | 3.50               | 1.13                        | 0.78              |
| Δcox20          | 3.63               | 1.09                        | 0.65              |
| C176/L6         | 3.68               | 0.91                        | 0.74              |

**FIG. 1. Absorption spectra of mitochondrial cytochromes.** Mitochondria prepared from the wild type strain W303–1A (WT) and the two cox20 mutants, Δcox20 and C176/L6, were extracted with 1% potassium deoxycholate, as described previously (47). Difference spectra of sodium hydrosulfite-reduced and potassium ferricyanide-oxidized extracts were recorded at room temperature. The extractions were done at final protein concentrations of 5 mg/ml except C176/L6 whose concentration was 7.1 mg/ml.

**FIG. 2. Cox20p is encoded by the open reading frame YDR231c.** A. maps of the nuclear DNA insert in pG92/T1 and derivative subclones. The locations of the restriction sites for EcoRI (E), HindIII (H), KpnI (K), SpI (Sp), StuI (St), NheI (N), and BglII (G) are marked on the pG92/T1 insert. The fragments of DNA subcloned in YEp351 (ST1, ST2, and ST4), YEp351 (ST18), YEp352 (ST3), YX122 (ST13) are denoted by the bars in the upper part of the figure. Complementation and lack thereof of cox20 mutants by different subclones are indicated by the plus and minus signs, respectively. B, amino acid sequence of Cox20p. C, hydropathy profile of Cox20p according to Kyte and Doolittle (55).
Cox20p Is a Mitochondrial Protein—The mitochondrial localization of Cox20p was determined in two ways. A polyclonal antiserum was raised against a protein expressed from a \textit{trpE}/COX20 fusion gene. Using this antiserum, a protein of \(23\) kDa was detected in mitochondria and not in the cytoplasm of wild type yeast. This protein was absent in the \(\Delta\)cox20 mitochondria (Fig. 3A).

Independent evidence that Cox20p is a constituent of mitochondria was obtained with a biotin-tagged form of the protein. COX20-BIO coding for Cox20p with a C-terminal biotinylation signal fully complemented the \(\Delta\)cox20 mutant, either on a multicopy plasmid or chromosomally integrated COX20-BIO gene. The protein has two putative transmembrane domains between residues 91–109 and 116–132 (Fig. 2B). The protein possesses an N-terminal sequence of Cox20p that does not resemble a typical mitochondrial import signal, which characteristically are rich in basic and hydroxylated residues (50). Neither is it possible to discern an internal (40, 51) or C-terminal sequence (52) which would clearly qualify as an import signal. Cox20p does not exhibit any sequence similarity to other known proteins in the current data bases.

Cox20p Is an Integral Protein of the Inner Membrane—The submitochondrial localization of Cox20p was examined in wild type yeast and in the \(\Delta\)cox20 mutant with a chromosomally integrated COX20-BIO gene. Cox20p is an intrinsic membrane protein as it was resistant to extraction with sodium carbonate (Fig. 4A). Cox20p was not present in purified mitochondrial outer membranes (Fig. 4B). Both native and biotinylated Cox20p were protected against proteinase K in intact mitochondria but not in mitoplasts prepared by hypotonic swelling of mitochondria (Fig. 4, C and D). No C-terminal fragment of the Cox20p with the biotin tag was observed following proteolytic treatment of mitoplasts, suggesting this domain is accessible to protease in the intermembrane space (Fig. 4D).

The apparent protease sensitivity of the C terminus in the intermembrane space and the presence of two predicted transmembrane segments would suggest Cox20p has an N\textsubscript{out}–C\textsubscript{out} orientation in the membrane, with a short hydrophilic loop in the matrix.

Cox20p Is Required for the Maturation of pCox2p—\textit{In vivo} labeling of the mitochondrial translation products indicated that \(\Delta\)cox20 mutants are capable of synthesizing subunits 1–3 of the cytochrome oxidase (Fig. 5A). In contrast to wild type cells, newly synthesized cytochrome oxidase subunit 2 (Cox2p) accumulated as the precursor form (pCox2p) in the C176/L6 and \(\Delta\)cox20 strains (Fig. 5A). The precursor form was also evident in the control \(\Delta\)oxa1 mitochondria (aW303/ST20), a null mutant of \textit{OXA1}, see Table I (Fig. 5A) but not in other cytochrome oxidase mutants analyzed in parallel (results not shown). Thus accumulation of pCox2p is not a general phenomenon for all cytochrome oxidase mutants but rather indicates a specific requirement of Cox20p for processing of the precursor. In addition, the level of subunit 1 (Cox1p), which accumulated in the \(\Delta\)cox20 mutant, was reduced. Similar reductions in the level of Cox1p were also observed in other cytochrome oxidase complex mutants and hence is not a specific phenotype associated with the loss of
Identification of Cox20p

The failure of \( \text{cox20} \) mutants to process pCox2p could stem from a defect in the Imp1p peptidase. Like pCox2p, cytochrome \( b_2 \) and cytochrome \( b_6 \) reductase (Mcr1p) are substrates of Imp1p (7, 17). Cytochrome \( b_2 \), a soluble protein of the intermembrane space, is synthesized as a precursor, which is cleaved twice upon import. The mitochondrial processing peptidase (MPP) cleaves the matrix-targeting signal to generate an intermediate size species, which subsequently is proteolytically matured by Imp1p. The Mcr1p protein displays a dual submitchondrial localization. A non-processed (34 kDa) form of Mcr1p is present in the outer mitochondrial membrane. Another soluble form (32 kDa), derived from the 34-kDa form, is matured by Imp1p and is located in the intermembrane space (17).

The efficiency of processing both of cytochrome \( b_2 \) and Mcr1p, as well as that of Cox2p, was analyzed in mitochondria isolated from the yeast strains \( \Delta \text{cox20}, \Delta \text{cox4} \) (aw303\( \Delta \text{COX4} \), a null mutant in subunit 4 of the cytochrome oxidase, see Table I), and \( \Delta \text{imp1} \) (pet2858::LEU2, a null mutant of the Imp1p, see Table I) (Fig. 5B). Processing of cytochrome \( b_2 \) and Mcr1p in \( \Delta \text{cox20} \) and \( \Delta \text{cox4} \) mitochondria was comparable to those in the wild type mitochondria. In contrast, only the intermediate and the 34-kDa forms of cytochrome \( b_2 \) and Mcr1p, respectively, were observed in the control \( \Delta \text{imp1} \) mitochondria. Cox2p was present as its precursor species in \( \Delta \text{cox20} \) and in the control \( \Delta \text{imp1} \) mitochondria. Only mature Cox2p was observed in wild type and \( \Delta \text{cox4} \) mitochondria, confirming that the processing defect observed in \( \text{cox20} \) mutants is not a general property of strains defective in cytochrome oxidase assembly. The steady state levels of pCox2p were significantly lower in the \( \Delta \text{cox20}, \Delta \text{imp1}, \) and \( \Delta \text{cox4} \) mitochondria than that of the mature protein in wild type mitochondria. This is most likely due to high turnover of non-assembled Cox2p in cytochrome oxidase-deficient strains.

A further indication that the function of the Imp1p peptidase was not compromised in the \( \text{cox20} \) mutants came from the analysis of the rate of processing of the cytochrome \( b_2 \) precursor following its import into isolated \( \Delta \text{cox20} \) mitochondria. Radiolabeled cytochrome \( b_2 \) was processed to its mature form in \( \Delta \text{cox20} \) and \( \Delta \text{cox4} \) mitochondria, with kinetics indistinguishable from those observed in the wild type control mitochondria (Fig. 5C).

\( \text{Cox20p Is Not Required for the Export of the N Terminus of pCox2p} \)—In order to test whether Cox20p plays a role in the export of pCox2p, we analyzed the topology of newly synthesized pCox2p accumulated in the absence of a functional Cox20p. Export of the \( N \) and \( C \) termini of Cox2p to the intermembrane space was assessed by accessibility of these domains to added protease in hypotonically swollen mitochondria following synthesis of Cox2p in \textit{vitro} (Fig. 6). Newly synthesized Cox2p was accessible to added protease in mitoplasts derived from the \( \Delta \text{cox20} \) strain, as was the mature Cox2p in wild type mitoplasts. Thus the export of the \( N \) terminus apparently does not require the function of the Cox20p. Some of the newly synthesized Cox2p failed to become completely sorted to its \( N_{\text{out}}-C_{\text{out}} \) orientation following synthesis in the \( \Delta \text{cox20} \) mitoplasts, as indicated by the generation of a protease-protected 31-kDa fragment. This fragment is generated from the proteolytic treatment of a partially exported Cox2p species whose \( N \) terminus has reached the intermembrane space but whose \( C \) terminus has not.

\( \Delta \text{cox20} \) and \( \Delta \text{cox4} \) strains, at 25 °C, for times indicated. Following import samples were proteinase K-treated (50 \( \mu \)g/ml) and analyzed by SDS-PAGE, Western blotting, and autoradiography. The percentage of imported and MPP-processed cytochrome \( b_2 \), which had been processed by Imp1p, is given.
plex between these two proteins. For Cox2p, confirming that it represented a cross-linked adduct could also be immunoprecipitated by antibodies specific consistent with the size of Cox2p covalently linked to Cox20p. The apparent molecular weight of the cross-linked product is con-
ned from the detergent extract and its full recovery on the iner membrane (4, 5). This fragment of Cox2p was also generated in other respiratory-de cient mutants analyzed (Fig. 6). Thus, the 31-kDa proteolytic fragment probably indicates a less effective export of the C terminus due to a lowered membrane potential in these mutants.

Cox20p Associates with Cox2p during Its Assembly Path-
way—Does Cox20p interact directly with the Cox2p during its biogenesis or does it have an indirect effect on the maturation of Cox2p? In a ﬁrst approach we asked if newly synthesized Cox2p physically associates with Cox20p. The chemical cross-linker SPDP was added to isolated mitochondria following the synthesis of mitochondrial translation products in the presence of [35S]methionine (Fig. 7A). In wild type mitochondria a cross-linked product of 51 kDa was observed that was immunoprecipitated with antisera speciﬁc for Cox20p. This adduct was absent in mitochondria isolated from the cox20 null mutant. No cross-linked products were immunoprecipitated using preimmun serum or Cox20p antiserum when the translation was performed in the absence of added cross-linker (Fig. 7A). The apparent molecular weight of the cross-linked product is consistent with the size of Cox2p covalently linked to Cox20p. The adduct could also be immunoprecipitated by antibodies speciﬁc for Cox2p, conﬁrming that it represented a cross-linked complex between these two proteins.

Interaction of newly synthesized Cox2p with Cox20p was also observed in Δimp1 mitochondria (Fig. 7B). In this case the cross-linked adduct was slightly larger (53 kDa) than that observed in wild type mitochondria, indicating the non-pro-
cessed precursor form of Cox2p was associated with Cox20p. Thus, the interaction of Cox2p with Cox20p may occur prior to, and/or independent of, processing by Imp1p. Furthermore, Cox2p remains in association with Cox20p after the Imp1p-processing event, as indicated by the cross-linking observed in the wild type and Δcox4 mitochondria (Fig. 7B). No interaction between Cox2p and Cox20p was detected in mitochondria from the Δoxa1 mutant. These data suggest that the interaction of Cox2p with Cox20p occurs following Oxa1p-dependent export across the inner membrane.

In a second approach, physical interaction of Cox20p with endogenous Cox2p was demonstrated by co-immunoprecipitation. Association of the two proteins in mitochondria isolated from wild type and also the Δcox20, Δimp1, and Δcox4 mutants was examined by co-immunoprecipitation with Cox20p-speciﬁc antiserum following solubilization of mitochondria with digitonin (Fig. 7C). In the Δimp1 mitochondria all the pCox2p was found in association with Cox20p, as indicated by depletion of Cox2p from the detergent extract and its full recovery on the protein A-Sepharose beads. Mature size Cox2p was associated with Cox20p in the Δcox4 mutant mitochondria. Similar to the results with the Δimp1 mutant, all of the mature size Cox2p in the Δcox4 mitochondria was associated with Cox20p. In addi-

FIG. 6. Newly synthesized pCox2 is exported in Δcox20 mito-
chondria. Mitochondria isolated from wild type W303-1A (WT) and isogenic Δcox20, Δcox4 and Δcor1 strains were converted to mitoplasts by hypotonic swelling for 30 min on ice. Translation of proteins in mitoplasts was performed in the presence of [35S]methionine for 20 min at 25 °C. All samples were divided and were either mock-treated or treated with 50 μg/ml proteinase K (PK), as indicated. Mitoplasts were re-isolated and analyzed by SDS-PAGE and autoradiography. For the abbreviations used see Fig. 5, and f, C-terminal 31-kDa fragment of Cox2p, which indicates an N-out–C-in-topology of Cox2p.

FIG. 7. Cox20p interacts with Cox2p. A, mitochondria were isolated from the indicated strains. Labeling of mitochondrial translation products was performed for 20 min at 25 °C in the presence of [35S]methionine. After the addition of cold methionine and puromycin, mitochondria were reisolated, washed, and incubated with cross-linker SPDP (+) or were mock-treated (−). Immunoprecipitation of cross-linked adducts was performed using antiserum speciﬁc for Cox20p (α-20), Cox2p (α-2), and preimmune serum (PI). B, labeling and cross-linking reactions were performed as in A, except that the cross-linker, SPDP, was added during translation, 15 min after the start of the translation reaction. Mitochondria were lysed as described (9), and the supernatants were used for immunoprecipitation with speciﬁc anti- serum against Cox20p. A and B, immunoprecipitates were analyzed by SDS-PAGE and autoradiography. Abbreviations used are as follows: 53 kDa, cross-link of the precursor of Cox2p to Cox20p; 51 kDa, cross-link of mature Cox2p to Cox20p. C, co-immunoprecipitation of endogenous Cox2p with antisera speciﬁc for Cox20p. Mitochondria isolated from the strains Δcox4, Δimp1, Δcox20, and W303-1A (WT) were solubilized in buffer containing 1% digitonin. After a clarifying spin, the supernatant was divided into three portions. One portion (equivalent to 50 μg of solubilized protein) was precipitated with trichloroacetic acid (T), and the remaining portions (equivalent to 250 μg of protein) were incubated with antisera speciﬁc for Cox20p (α-20) or preimmune serum (PI), covalently bound to protein A-Sepharose beads. After centriﬁgation, the supernatant (Sup) was removed, and a portion (equivalent to 50 μg of protein) was trichloroacetic acid-precipitated. The beads were washed, and the bound material (IP) was eluted with SDS-containing sample buffer. All samples were subjected to SDS-PAGE and Western blotting. The blots were decorated with antisera speciﬁc for Cox2p. The amounts of Cox2p were strongly reduced in the mutants as compared with wild type (Fig. 5B), therefore the blots of the mutant strains were exposed on ﬁlms for longer periods.
tion, a smaller but significant portion of mature Cox2p could be co-immunoprecipitated with Cox20p in wild type mitochondria. The specificity of co-immunoprecipitation was confirmed in each case by the failure to recover Cox2p on the protein A-Sepharose beads when preimmune serum was used. Furthermore, Cox2p could not be immunoprecipitated with antibodies against Cox20p in extracts from the Δcox20 mitochondria, thus excluding artifactual cross-reactivity of the antisera (Fig. 7C).

The observed interaction of mature sized Cox2p with Cox20p in wild type and Δcox20 mitochondria suggests that in addition to its function in presenting pCox2p as an efficient substrate for Imp1p peptidase, Cox20p may also play a role in the assembly steps that follow the cleavage event. The further involvement of Cox20p in the assembly pathway of the cytochrome oxidase is also supported by the finding that in wild type a smaller percentage of total Cox2p was co-immunoprecipitated with Cox20p. This may correspond to the fraction of still unassembled Cox2p. The majority of the Cox2p which is assembled protein into the functional cytochrome oxidase complex, however, is not associated with Cox20p.

**DISCUSSION**

We report here the identification of a novel protein of the mitochondrial inner membrane involved in the assembly of the cytochrome oxidase complex. Cox20p is essential for the maturation and for subsequent assembly of the mitochondrially encoded pCox2p, the precursor of subunit 2 of cytochrome oxidase. Despite the inability of Cox20 mutants to process pCox2p, the newly synthesized precursor is correctly sorted across the inner membrane as it is accessible to added proteases from the intermembrane space. Thus the Oxa1p-mediated export events appear not to be compromised in Cox20 mutant mitochondria. Indeed, export of other unrelated, nuclear-encoded substrates of Oxa1p was also not inhibited in the cox20 mutants (results not shown).

The activity of the Imp1p/Imp2p peptidase *per se* is not compromised in the cox20 mutants. Processing of cytochrome *b*₂ and Mnr1p proteins was indistinguishable in Δcox20 and wild type mitochondria. Cytochrome *c*₁, another substrate of the Imp1p-Imp2p complex, whose targeting sequence is cleaved by the Imp2p component, was also processed normally (results not shown). Hence the processing defect observed in cox20 mutants is highly specific for pCox2p. This phenotype is distinct from that of som1 mutants, which are defective in the processing of all substrates of Imp1p (18).²

Processing of cytochrome *b*₂ and cytochrome *c*₁ by Imp1p and Imp2p is coupled to the addition of the heme prosthetic groups (53, 54). Since Cox2p contains a binuclear Cuₐ center, it is conceivable that exported pCox2p may have to acquire its copper in order to be processed by Imp1p. This is highly unlikely in view of our observations that mutations in *COX17* and *SCO1*, both of which are required for addition of copper to Cox2p (26, 27), do not affect removal of the N-terminal extension from pCox2p (results not shown). Furthermore, Cox2p-derived proteins lacking the copper-binding site can also be processed by Imp1p (4, 5). Therefore, we conclude that the inability of cox20 mutants to mature pCox2p is not secondary to a defect in insertion of the copper moiety.

Exported Cox2p interacts directly with Cox20p prior to its maturation. We propose this interaction is crucial for the recognition of pCox2p as a substrate by the Imp1p-Imp2p peptidase. Failing to do so, exported Cox2p is unprocessed, as observed in the cox20 mutants. The complex between Cox2p and Cox20p is maintained after the Imp1p-processing event. This is supported by cross-linking of newly synthesized and matured Cox2p to Cox20p and detection of endogenous Cox2p complexed to Cox20p by co-immunoprecipitation.

A number of proteins proposed to be involved in the assembly of the cytochrome oxidase complex have been reported. Association of these putative assembly factors with specific assembly intermediates of the cytochrome oxidase complex has not yet been demonstrated. Hence their molecular function in the assembly process remains unclear. Our results indicate that the non-assembled Cox2p in cytochrome oxidase mutants accumulates exclusively in association with Cox20p. Thus, Cox2p remains firmly bound to Cox20p subsequent to processing by Imp1p, perhaps until it assembles further into the cytochrome oxidase complex. Cox20p is not a constituent of the isolated cytochrome oxidase nor are the levels of Cox20p discernibly different in mutants defective in cytochrome oxidase assembly.² In wild type mitochondria, the bulk of Cox2p is assembled into the enzymatically active cytochrome oxidase. A small fraction of the Cox2p, presumably the non-assembled species, however, is associated with Cox20p.

In conclusion, Cox20p binds directly to Cox2p during cytochrome oxidase assembly. The interaction with Cox20p occurs after pCox2p is inserted into the inner membrane but precedes the proteolytic cleavage by the peptidase Imp1p. We propose that Cox20p holds pCox2p in a processing competent conformation. The ability of Cox20p to bind mature, unassembled Cox2p raises the further possibility that Cox20p may also chaperone the Cox2p during its subsequent assembly into the cytochrome oxidase complex.

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² K. Hell, A. Tzagoloff, W. Neupert, and R. A. Stuart, unpublished results.

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