Characterization of COX17, a Yeast Gene Involved in Copper Metabolism and Assembly of Cytochrome Oxidase*

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Mutations in the COX17 gene of Saccharomyces cerevisiae cause a respiratory deficiency due to a block in the production of a functional cytochrome oxidase complex. Because cox17 mutants are able to express both the mitochondrial and nuclearly encoded subunits of cytochrome oxidase, the Cox17p most likely affects some late posttranslational step of the assembly pathway. A fragment of yeast nuclear DNA capable of complementing the mutation has been cloned by transformation of the cox17 mutant with a library of genomic DNA. Subcloning and sequencing of the COX17 gene revealed that it codes for a cysteine-rich protein with a molecular weight of 8,057. Unlike other previously described accessory factors involved in cytochrome oxidase assembly, all of which are components of mitochondria, Cox17p is a cytoplasmic protein. The cytoplasmic location of Cox17p suggested that it might have a function in delivery of a prosthetic group to the holoenzyme. A requirement of Cox17p in providing the copper prosthetic group of cytochrome oxidase is supported by the finding that a cox17 mutant is rescued by the addition of copper to the growth medium. Evidence is presented indicating that Cox17p is not involved in general copper metabolism in yeast but rather has a more specific function in the delivery of copper to mitochondria.

Cytochrome c oxidase, the terminal complex of mitochondrial and bacterial respiratory chains (1), makes use of two different types of electron carriers. One is the central iron of the heme prosthetic groups of cytochromes a and a3. Each cytochrome has an associated copper center, composed either of one or two copper ions. The ligands for the two heme A groups are contributed exclusively by subunit 1 of the enzyme, and those for copper are shared by subunits 1 and 2 (2, 3).

Because the synthesis of heme A from protoheme occurs in the mitochondrial inner membrane (4, 5), this prosthetic group is made available at the site of its utilization during assembly of the complex. Copper, however, must be imported from the cytoplasm in amounts commensurate with its requirement in mitochondria; most of the metal is probably used for cytochrome oxidase. It is unlikely that intracellular copper, whether cytoplasmic or mitochondrial, exists as a free solute. Instead, copper and other trace heavy metals are more likely to be complexed to storage proteins, carriers, and metalloenzymes. The routes used for the intracellular distribution of copper in Saccharomyces cerevisiae are only now beginning to be discerned. External copper is imported into yeast by an ATP-dependent pump encoded by CTR1 (6). One of the better understood routes of internalized copper uses the products of CCC2 (7) and FET3 (8). CCC2 has been shown to code for a cytoplasmic protein of yeast that transfers copper to a membrane-bound ceruloplasmin-like oxidase encoded by FET3 (7, 8). Mature Fet3p is a constituent of the cytoplasmic membrane and is distinct from the high-affinity iron transporter but is essential for iron uptake in yeast (6–8). Even though mutations in CCC2 produce a respiratory defective phenotype, it is not clear whether this is because Cooc2 also functions on the pathway of copper transport to mitochondria or whether this is secondary to an iron deficiency. That copper delivery by Cooc2 is selective, rather than general, is supported by the observation that the total concentration of copper is not significantly different in ccc2 mutants and in wild-type cells, and that such mutants have normal cytoplasmic copper-dependent superoxide dismutase (7).

In the present study, we have characterized a putative nuclear respiratory-deficient mutant of yeast assigned previously to complementation group G74. The failure of the mutant to assemble functional cytochrome oxidase is corrected by high concentrations of exogenous copper, indicating that the lesion limits the availability of copper during assembly of the complex. The responsible gene, referred to as COX17, has been cloned and shown to code for a cysteine-rich cytoplasmic protein. Cox17p is not involved in copper uptake in yeast but rather appears to function in the pathway responsible for copper delivery to mitochondria.

MATERIALS AND METHODS

Yeast Strains and Media—The genotypes and sources of the strains of S. cerevisiae used in this study are listed in Table I. The media used for the growth of yeast have been described elsewhere (4).

Preparation of Yeast Mitochondria and Enzyme Assays—Wild-type and mutant yeast were grown to stationary phase in YPGal (2% galactose, 1% yeast extract, and 2% peptone), and mitochondria were prepared by the procedure of Faye et al. (12), except that Glusulase was replaced by Zymolase 20,000 (ICN Biomedicals, Inc.) to prepare spheroplasts. Mitochondrial translation products were labeled with [35S]methionine in the presence of cycloheximide, as described previously (13). Cytochrome spectra of mitochondrial extracts were obtained at room temperature. Cytochrome oxidase activity was measured by following oxidation of ferricytochrome c at 550 nm (13).

Cloning of the COX17 Gene—The wild-type COX17 gene was cloned by transformation of the cox17 mutant C129/U1 by the method of Schiestl and Gietz (14). The library used for the transformation was constructed from partial Sau3A1 fragments of nuclear DNA (averaging 7–10 kb) cloned into the BamHI site of the shuttle vector YEp24 (15). This library was kindly provided by Dr. Marian Carlson (Department of Genetics and Development, Columbia University). Approximately 5 × 106 transformants were screened with the appropriate primers.

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RESULTS

Phenotype of cox17 Mutants—C129 is a single mutant of complementation group G74 of our pet mutant collection (10). The phenotype of this mutant suggests that the respiratory deficiency stems from a specific defect in cytochrome oxidase. The mutant shows a substantial decrease in cytochrome oxidase activity (data not shown). Western blot analysis of the subunits synthesized in the mitochondria also precludes an involvement of the gene in expression or import of these proteins (Fig. 1B). These results indicated that Cox17p was likely to be involved in the addition/synthesis of a prosthetic group or in the assembly of the holoenzyme (11). Right, subunits 1–8 of cytochrome oxidase.

FIG. 1. Phenotype of cox17 mutants. A, spectra of W303-1B (Wild-Type), the cox17 mutants C129 (cox17-1) and W303Δcox17 (cox17::TRP1). Mitochondria from the wild-type and mutant cells were extracted with deoxycholate to solubilize all the cytochromes (13). Difference spectra of reduced versus oxidized extracts were recorded at room temperature. The absorption α-bands of cytochromes a, a₃, and cytochrome b, and cytochromes c, c₁, and c₅ are indicated by the arrows. B, Western blot analysis of cytochrome oxidase subunits in mitochondria of W303-1B (Wild-Type) and the cox17 mutants C129 (cox17::TRP1) and W303Δcox17 (cox17::TRP1 + COX17-BIO). Total mitochondrial proteins (20 μg of protein) were separated on a 15% polyacrylamide gel containing 15% glycerol (31). After transfer to nitrocellulose, cytochrome oxidase subunits were detected either with subunit-specific antisera or with antiserum to the holoenzyme (11). Right, subunits 1–8 of cytochrome oxidase.

in the COX17 gene, which lacks any detectable a-type cytochromes (Fig. 1A) as well as cytochrome oxidase activity. In vivo labeling of the mitochondrially encoded subunits indicated that their synthesis is not affected in the mutants (data not shown). Western blot analysis of the subunits synthesized in the cytoplasm also precludes an involvement of the gene in expression or import of these proteins (Fig. 1B). These results indicated that Cox17p was likely to be involved in the addition/synthesis of a prosthetic group or in the assembly of the subunits.

Cloning and Sequencing of COX17—To clone the gene carrying the mutation in C129, a derivative strain, C129/U1, with a ura3 mutation, was transformed with a yeast genomic library as described under “Materials and Methods.” Three of the ura3-independent and respiratory-competent clones obtained from the transformation were found to have plasmid with related inserts of yeast nuclear DNA. The region responsible for complementation of C129/U1 was mapped to a 700-base pair
Characterization of COX17

**Fig. 2. Restriction map of pG74/T8 and subclones.** The location of BamHI (B), XbaI (X), SstI (S), HindIII (H), EcoRI (E), KpnI (K), Nhel (N), and PstI (P) sites are shown in the nuclear insert of pG74/T8. The unique Sphi site (Sp) of YEp24 is shown to indicate the orientation of the insert. The fragments of DNA subcloned in the shuttle vector YEp352 are denoted by the horizontal lines above the pG74/T8 insert. The 1.4-kb HindIII fragment of pG74/ST4, however, was cloned into YEp352H, a modified version of YEp352 containing only the COX17 allele is present in C129 mutants. The Cys→Tyr mutation present in C129 is indicated in brackets above the amino acid sequence. This sequence has been deposited with GenBank under accession number L75948.

**Fig. 3. Sequence of the COX17 gene obtained from pG74/ST6.** A partial sequence of the insert subcloned in pG74/ST6 is presented. The sense strand is shown, and the reading frame proposed to code for Cox17p has been translated. The EcoRI site used to create the disrupted cox17::TRP1 allele is underlined. The Cys→Tyr mutation present in C129 is indicated in brackets above the amino acid sequence. This sequence has been deposited with GenBank under accession number L75948.

**Fig. 4. Locations of orfs overlapping with COX17 and regions of DNA amplified by PCR.** The reading frame coding for Cox17p (frame 3) and the three other overlapping open reading frames (frames 1, 2, and 4) are indicated by the arrows (solid in the case of COX17) below the restriction map. The spans of DNA subcloned either directly (pG74/ST2) or by PCR amplification (pG74/ST7 and ST8) are indicated by the lines above the restriction map. The locations of the restriction sites for HindIII (H), EcoRI (E), XbaI (X), and Nhel (N) are marked on the map. Of the three subclones indicated in the figure, only pG74/ST8 complemented the C129 mutant.

derepressed conditions, and both the mitochondria and postmitochondrial supernatant fractions were assayed for the presence of a novel biotinylated protein (Fig. 5). The results of these analyses failed to show the presence of a new biotinylated protein in either fraction of the transformant containing orf2 fused to the biotinylation signal sequence. The transformant harboring the orf3 fusion, however, had an abundant novel biotinylated protein of M, 16,000. This product was found exclusively in the postmicrosomal supernatant fraction. The size
of the new biotinylated protein is in agreement with the expected size of the protein expected to be translated from the orf3 fusion gene. The finding that only the orf3 fusion was capable of being expressed in vivo provided strong evidence for the identity of this frame as the COX17 gene.

COX17 encodes an acidic protein of 69 amino acids with a high content of cysteine (10%) residues (Fig. 3). The sequence is very hydrophilic, consistent with its properties. The mutation in C129 was determined by sequence analysis of two independent clones obtained by PCR amplification of nuclear DNA prepared from this mutant. A comparison of the PCR-generated sequences with that of the wild-type gene indicated a single mutation at codon 57 of the gene.

In situ Disruption of COX17—A null allele of COX17 was created by insertion of the yeast TRP1 gene at the EcoRI site internal to the coding sequence (Fig. 6). The disrupted allele was isolated on a linear fragment and was used to transform the respiratory-competent haploid strains W303-1A and W303-1B. Two respiratory-deficient and trypophan prototrophic clones (W303ΔCOX17 and aw303ΔCOX17) obtained from the transformation were verified by genomic Southern analysis to have acquired the disrupted cox17::TRP1 allele (Fig. 6). The respiratory-deficient phenotype of the two transformants was complemented by μ0 strains but not by C129. W303ΔCOX17 strains exhibit a specific cytochrome oxidase deficiency similar to C129 (Fig. 1).

Localization of a Biotinylated COX17 Fusion Protein—As indicated above, the biotinylated product was detected in the postmicrosomal supernatant fraction but not in mitochondria, suggesting that Cox17p is a cytoplasmic protein. Since the localization of Cox17p was done in a transformant containing the fusion gene on a multicopy plasmid, the possibility existed that overproduction of the protein interfered with its import into mitochondria. This was excluded by transformation of a COX17 null mutant with the same construct in an integrative vector (pG74/ST17). The presence in the transformant W303ΔCOX17ST17 of the COX17-BIO gene, in a single copy at the URA3 locus, restored normal growth on nonfermentable carbon sources, indicating that the presence of the carboxyl-terminal extension with biotin does not significantly affect the activity of the protein (see also the spectrum in Fig. 1A).

Analysis of the distribution of biotinylated Cox17p expressed from the chromosomally integrated COX17-B1O fusion also showed the protein to be present exclusively in the postmicrosomal supernatant fraction (Fig. 5). This fraction consists not only of the soluble cytoplasmic proteins of yeast but also contains proteins released from nuclei as a result of the fractionation procedure. A nuclear localization of the Cox17p was excluded since purified nuclei did not contain any biotinylated Cox17p (data not shown).

Rescue of cox17 Mutants by High Concentrations of Exogenous Copper—The cytoplasmic localization of Cox17p precluded a chaperone-like function for this protein. The presence of near normal levels of the mature, cytoplasmically synthesized proteins in the mutant also strongly argue against a role of Cox17p in expression of this set of cytochrome oxidase subunit polypeptides. A cytoplasmic protein, however, could affect production of the functional complex if it were required for the synthesis or mitochondrial import of a prosthetic group or cofactor. The two known electron carriers of cytochrome oxidase are heme a and copper (1–3). Since heme a is synthesized in mitochondria (5), it is unlikely that Cox17p is involved in this process. The alternative possibility that Cox17p might be involved in copper metabolism, was tested by examining the effect of copper on growth of cox17 mutants on nonfermentable substrates as carbon sources. As shown in Fig. 7A, growth of the null strain W303ΔCOX17 was restored on ethanol/glycerol when the medium was supplemented with 0.4% copper. This effect was also seen at lower copper concentrations (0.1%), but the cells grew more slowly. Higher concentrations of copper (0.8%) were lethal to both the mutant and the wild-type strain. Even though CTR1 (structural gene for the copper transporter) on a high copy plasmid does not suppress the respiratory defect of the cox17 null mutant, it does substantially lower the con-
centration of copper (0.01%) needed to support growth on nonfermentable substrates (Fig. 7B). The ctr1 mutant and the cox17 mutant transformed with CTR1 on a high copy plasmid (W303ΔCOX17/ST19) both acquire respiratory competency in the presence of 0.01% copper in the medium (Ref. 6; Fig. 7). The cox17 transformant, however, grows more slowly.

The ability of copper to rescue the deficiency of the cox17 mutation is not general to cytochrome oxidase mutants. Copper supplemented media failed to elicit growth of a large number of different cytochrome oxidase mutants, including the sco1 mutant shown in Fig. 7. The SCO1 gene has been shown to be required for a posttranslational step during cytochrome oxidase assembly (27).

Is Cox17p Required for Maturation of Cytoplasmic Superoxide Dismutase?—The ability of copper to reverse the cytochrome oxidase defect in cox17 mutants strongly supported the idea that the encoded protein played an important role in delivery of copper to mitochondria. It was not excluded, however, that Cox17p might have a more general function in making copper available to other copper-bearing proteins of yeast such as the cytoplasmic superoxide dismutase. The cytoplasmic enzyme is known to use copper as its prosthetic group (28). The superoxide dismutase activity was compared in wild-type and in several different pet strains, including the cox17 and ctr1 null mutants (Fig. 8). The ctr1 mutant, because of its impaired copper transport, fails to make active cytoplasmic superoxide dismutase (30). The absence of cytoplasmic superoxide dismutase in the ctr1 strain is confirmed by the results shown in Fig. 8. In contrast, the cox17 mutant shows the presence of superoxide dismutase activity, although it is somewhat lower than in the wild-type parent or in the mutant transformed with COX17. This partial decrease, also observed in another cytochrome oxidase deficient mutant (cox14), is probably a secondary effect of decreased cellular ATP in respiratory-defective mutants.

**Fig. 7.** Restoration by copper of growth of cox17 mutants on nonfermentable substrates. A, the wild-type strain W303-1B (WT), respiratory-defective mutants W303ΔCOX17 (ΔCOX17) and W303ΔSCO1 (ΔSCO1), and the transformant W303ΔCOX17/ST8 (ΔCOX17/ST8) were streaked on YPD (glucose medium containing yeast extract and peptone). The YPD master was replicated on YEPG (glycerol plus ethanol medium containing yeast extract and peptone) and on YEPG supplemented with 0.4% copper sulfate. The growth of W303ΔCOX17 on copper-supplemented YEPG required at least 4 days of incubation at 30°C. B, the wild-type strain W303-1B (WT), the mutants W303ΔCOX17 (ΔCOX17) and YSC542 (ΔCTR1), and the transformant W303ΔCOX17/ST19 (ΔCOX17/ST19) were replicated on YEPG and YEPG supplemented with 0.01% copper sulfate. Growth of YSC542 on the copper-supplemented medium was discerned after 1 day incubation at 30°C, whereas growth of the transformant required an additional 2-3 days of incubation.

**Fig. 8.** Presence of cytoplasmic superoxide dismutase in cox17 mutants. The wild-type strains W303-1B (lane 1) and YPH252 (lane 5), the mutants W303ΔCOX17 (lane 2), W303ΔCOX14 (lane 4), and YSC542 (lane 6), and the transformant W303ΔCOX17/ST8 (lane 3) were grown in 2% galactose medium. Cells were fractionated into mitochondrial and postmitochondrial supernatant fractions. The postmitochondrial supernatant fractions (20 μg of protein) were separated on a nondenaturing 10% polyacrylamide gel prepared exactly as described in Laemmli (22), except for the omission of sodium dodecyl sulfate. Superoxide dismutase in the gel was assayed by the method of Beauchamp and Fridovich (29).

**DISCUSSION**

The synthesis of cytochrome oxidase in *S. cerevisiae* is a complex process requiring the expression not only of the nuclearly and mitochondrially encoded subunits of the enzyme but also numerous other nuclear genes that code for factors involved in the processing of mitochondrial cytochrome oxidase-specific mRNAs (31, 32), translation of the mitochondrial mRNAs for subunits 2 and 3 (33, 34), and the assembly process itself, which to date remains poorly understood (11, 27, 35, 36). The COX17 gene reported here does not appear to be important for either synthesis, import, or processing of the subunits but rather fits into the category of genes whose products intercede during the late stages of cytochrome oxidase assembly. There are currently half a dozen examples of such proteins (11, 27, 35, 36). The COX17 gene described here is unusual because its
Recent studies indicate that mutations in the copper ligands of assembled intermediates against proteolytic degradation (4) suggests that heme A protects the holoenzyme, or partially stabilization in the steady-state levels of some subunits in such mutants have a profound effect on cytochrome oxidase (4, 5). The reduction in the steady-state levels of some subunits in such mutants suggests that heme A protects the holoenzyme, or partially assembled intermediates, against proteolytic degradation (4). Recent studies indicate that mutations in the copper ligands of subunit 2 also destabilize the enzyme (37). This implies that mutations affecting the availability of copper for cytochrome oxidase synthesis should also increase turnover of the less stable subunits. Western analysis has revealed cox17 mutants to have severely reduced steady-state concentrations of subunits 1 and 2, a hallmark of mutants arrested in cytochrome oxidase assembly (11, 27, 34–36). This further supports the notion that copper contributes to the structural stability of the holoenzyme.

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