Mutational Analysis of the Mitochondrial Copper Metallochaperone Cox17*

The copper metallochaperone Cox17 is proposed to shuttle Cu(I) ions to the mitochondrial for the assembly of cytochrome c oxidase. The Cu(I) ions are liganded by cysteinyl thiolates. Mutational analysis on the yeast Cox17 reveals three of the seven cysteinyl residues to be critical for Cox17 function, and these three residues are present in a Cys-Cys-Xaa-Cys sequence motif. Single substitution of any of these three cysteines with serines results in a nonfunctional cytochrome oxidase complex. Cells harboring such a mutation fail to grow on nonfermentable carbon sources and have no cytochrome c oxidase activity in isolated mitochondria. Wild-type Cox17 purified as untagged protein binds three Cu(I) ions/molecule. Mutant proteins lacking only one of these critical Cys residues retain the ability to bind three Cu(I) ions and are imported within the mitochondria. In contrast, Cox17 molecules with a double Cys→Ser mutation exhibit no Cu(I) binding but are still localized to the mitochondria. Thus, mitochondrial uptake of Cox17 is not restricted to the Cu(I) conformer of Cox17. COX17 was originally cloned by virtue of complementation of a mutant containing a nonfunctional Cys→Tyr substitution at codon 57. The mutant C57Y Cox17 fails to accumulate within the mitochondria but retains the ability to bind three Cu(I) ions. A C57S Cox17 variant is functional, and a quadruple Cox17 mutant with C16S/C36S/C47S/C57S substitutions binds three Cu(I) ions. Thus, only three cysteinyl residues are important for the ligation of three Cu(I) ions. A novel mode of Cu(I) binding is predicted.

Functional cytochrome c oxidase requires the assembly of a macromolecular complex involving nuclearly and mitochondrially encoded subunits (1–3). The assembly is dependent on the insertion of cofactors, including two heme A groups, three copper ions, one zinc ion, and one magnesium ion (2). A series of nucleurally encoded accessory proteins mediate formation of the oxidase complex, although the function of only a few is understood (3–7). Two accessory proteins are enzymes that modify protoheme to heme A (8). Delivery and insertion of Cu ions into the oxidase appear to require at least three proteins, Cox17, Sco1, and Cox11 (9–12). Two Cu ions are inserted into cytochrome oxidase subunit II forming the binuclear Cu₄ site, and a single Cu ion into subunit I forming the binuclear Cu₆-heme a₃ reaction center (2). Because subunits I and II are mitochondrially synthesized, Cu ions must be delivered from the cytoplasm and inserted within the mitochondrion.

Cells harboring a mutant COX17 gene are respiratory-deficient but synthesize both mitochondrially and nuclearily encoded cytochrome oxidase subunits (9). Cox17 was implicated in copper ion delivery to mitochondria by the observation that the respiratory defect of cox17Δ cells was suppressed by high exogenous Cu(II) levels (9). Exogenous Cu(II) did not rescue a subset of other cytochrome oxidase mutants (9). Two observations are consistent with the predicted role of Cox17 as a Cu shuttle protein. First, Cox17 is an 8057-dalton polypeptide localized in the cytosol and intermitochondrial membrane space (13). Second, Cox17 is a Cu(I)-binding protein with at least two Cu(I) ions bound in a polycopper cluster (14). Cox17 has been proposed to shuttle Cu ions to the mitochondria and transfer Cu to Sco1 as an intermediate step in Cu donation to cytochrome oxidase (10). Sco1 was implicated in copper ion delivery by the observation that the respiratory-deficient phenotype of cox17-1 cells harboring a Cys→Tyr substitution in Cox17 was suppressed by high copy SCO1 or the homologous SCO2 gene (10). Sco1 is an inner mitochondrial membrane protein important specifically in cytochrome oxidase assembly (15). Cells lacking a functional Sco1 are defective in cytochrome oxidase activity; this phenotype is not reversed by high exogenous copper (10). In contrast, cells lacking a functional Sco2 are respiratory competent (10, 16). The proposed transfer of Cu(I) ions from Cox17 to Sco1 is supported by the mutational analysis of Sco1 in which substitutions in the potential metal binding motif, CXXXC, abolish Sco1 function (11). Human Sco1 homologs are known, and mutations in human SCO2 result in cytochrome oxidase deficiency and a fatal cardioencephalomyopathy (17, 18).

Cu metallation of the Cu₉₈ site may require the Cox11 protein. Cox11 was recently shown to be important for Cu₉₈ site formation in Rhodobacter sphaeroides (12). Saccharomyces cerevisiae Cox11 is a 28-kDa mitochondrial membrane polypeptide essential for the accumulation of oxidase subunit I (8). If yeast Cox11 is a metallochaperone for the Cu₉₈ site of cytochrome oxidase, the possibility exists that CuCox17 delivers Cu(I) to both Sco1 and Cox11 for subsequent donation to the Cu₄ and Cu₉₈ sites, respectively.

Previously, we demonstrated that Cox17 bound Cu(I) within a polycopper cluster with predominantly thiolate ligands (14). If Cox17 donates Cu ions, the prediction is that mutations of the Cox17 Cu-binding thiolates would abrogate function. Here, we test this prediction by mutagenesis of the seven cysteinyl residues in Cox17. We show that only three of the seven cysteinyl residues are essential for Cox17 function and that sub-

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stition of multiple essential cytochrome c proteins with serines abolishes copper ion binding.

MATERIALS AND METHODS

Yeast Strains and Culture Conditions—Strain W303 and the isogenic COX17 strain (MATa, ade2-1, his3-15, leu2-3,112, trp1-1, ura3-1, Δcox17::TRP1) were used in all studies. The COX17 strain was generously provided by Dr. A. Tzagoloff (9). Yeast strains were cultured in complete medium (SC) lacking uracil to ensure maintenance of plasmids. Culture plates contained 2.5% bacto agar (Difco Laboratories) and either 3% glycerol or 2% glucose were used to assess the respiratory competency of cells harboring COX17 mutants. DNA transformations were performed using a lithium acetate protocol.

Vectors—The use of PCR fragment of YEp-pRS426 containing the MET25 promoter and CYC1 terminator was excised and cloned into the YEp-based vector pRS316 with URA3 selection (19). COX17 and mutants were cloned into the modified pRS316, designated YCpCOX17, to yield low copy expression plasmids. Methionine levels were altered in the growth medium to modulate expression levels. Cells were cultured in medium containing 134 μM methionine to partially repress expression of the MET25/COX17 fusions. Cells cultured in this medium exhibit approximately 60% of the maximal expression observed in cells cultured in medium without methionine (19). For Esherichia coli expression, COX17 or mutant genes were PCR-amplified to include 5’ NdeI and 3’ BamHI sites. The first five codons of each PCR product were optimized for E. coli expression (20). The resulting DNA products were subcloned into the E. coli (BL21) expression vector pAED4, which is a derivative of the T7-based expression vector, pET-3a (21).

Mutagenesis—COX17 point mutations were constructed using PCR primers containing mismatches at the appropriate codon. Because of the small size of the gene, each of the mutants could be constructed by varying the 5’ or the 3’ oligonucleotide PCR primer. Each of the COX17 gene variants was verified by sequencing.

Mitochondrial Isolation and Oxidase Activity—Mitochondria were isolated from S. cerevisiae as described previously (22). Mitochondrial protein concentration was determined by the Bradford assay (23). Five μg of total mitochondrial protein were added to 100 μl of cytochrome c reactions (45 μM cytochrome c, 50 mM phosphate, 0.1% deoxycholate). Oxidation of reduced cytochrome c (preincubated with sodium dithionite) was measured by monitoring the absorbance at 550 nm for 3 min. Reaction rates were normalized to total mitochondrial protein. Similar data were obtained if cytochrome c oxidase activity was normalized to total mitochondrial protein. Similar data were obtained.

RESULTS

Mutational Analysis of COX17

Yeast COX17 contains seven cysteinyl residues as shown in Fig. 1, yet only six of these are conserved between species. Cys16 is the one cysteinyl residue in yeast COX17 not conserved in other species. Yeast COX17 was mutated to generate variants with single Cys → Ser substitutions. The substitution at Cys27 was made to a Tyr as that was the original mutation in the COX17-1 mutant that led to the cloning of the gene (9). Mutant COX17 genes were reintroduced into S. cerevisiae cells on a YEp plasmid under the inducible MET25 promoter and terminator. The MET25 promoter was used to enable evaluation of COX17 function under both low expression (high methionine levels) and high expression (no added methionine) conditions. Cells lacking a functional COX17 are respiratory-deficient because of the lack of cytochrome c oxidase activity but grow well on fermentable carbon sources (9). The respiratory defect of COX17 cells was reversed in cells harboring episomal wild-type COX17 cultured in methionine-containing medium to limit expression of the MET25 promoter (Fig. 2). In addition, cells harboring mutant COX17 with Cys → Ser substitutions at codons 16, 36, and 47 were also respiratory competent, suggesting that these three cysteinyl residues are not critical for in...
**Mutational Analysis of Cox17**

**Fig. 2. Complementation of Δcox17 cells by mutant cox17 genes.** W303Δcox17 cells were transformed with low copy plasmids containing cox17 variants under the control of the MET25 promoter. Transformants were cultured on synthetic medium containing methionine but lacking uracil and containing 2% glucose or 3% glycerol. Serial dilutions of cells are shown from left to right.

**vivo** Cox17 function. Although the C36S Cox17 mutant grew on glycerol, the growth was reproducibly impaired relative to cells containing the wild-type protein. In contrast, cells harboring COX17 with Cys → Ser substitutions at codons 23, 24, and 26 failed to grow with glycerol as the sole carbon source. It was reported previously that cells with the C57Y substitution fail to grow on glycerol (9). Thus, Cys23, Cys34, Cys28, and Cys57 appear essential for Cox17 function. Mutant cox17 cells with mutations in these four essential cysteine also failed to grow when the mutant genes were overexpressed (minus methionine), so failure to grow was not due to expression levels. The mutant proteins accumulated to approximately similar levels as determined by Western analysis (Fig. 3).

Wild-type cells were transformed with the mutant cox17 genes to ensure that mutations did not have a dominant negative effect on growth. All transformants of wild-type cells grew well on glycerol in either medium with high methionine or no added methionine (Fig. 4).

Cytochrome c oxidase activity was quantified in mitochondria isolated from each transformant (Fig. 5). As expected, cox17Δ cells with wild-type, C16S, C36S, and C47S Cox17 exhibit near normal levels of cytochrome c oxidase activity. Consistent with the lack of growth on glycerol, cells with C23S, C24S, C26S, and C57Y show greatly reduced oxidase activity with respect to the wild-type control. The remaining cytochrome c oxidase activity seen with C57Y Cox17 cells was inhibited by KCN and was not augmented by overexpression of the mutant (data not shown).

Mutations resulting in the substitution of the two nonessential conserved cysteines (Cys36 and Cys47) or all three nonconserved cysteine (Cys16, Cys36, and Cys47) or all three nonessential cysteine (Cys16, Cys36, and Cys47) to serines were engineered to determine whether these cysteines had any synergistic effect in Cox17. Cells harboring the double or triple cox17 mutants grew well on glycerol (Fig. 1) and had appreciable cytochrome c oxidase activity (Fig. 5). The weak glycerol growth of the C36S Cox17 single mutant was not observed with the triple mutant.

The inability of C57Y mutant Cox17 to support growth of cells on glycerol may arise from the nonconservative substitution. A C57S substitution was engineered in Cox17, and cells harboring this mutant Cox17 were glycerol prototrophs, unlike the glycerol auxotrophy for the C57Y mutant (Fig. 6A). C57S Cox17 cells exhibited wild-type cytochrome oxidase activity in isolated mitochondria (Fig. 6B). Thus, Cox17 is functional with either a Cys or Ser at position 57. Only Cys23, Cys34, and Cys28 are essential cysteinyI residues for Cox17 function.

Western analysis was carried out to verify that the mutant Cox17 proteins that failed to rescue nonfermentable growth of transformed cox17Δ cells were expressed (Fig. 3). The analysis was carried out in cells cultured in methionine medium to limit COX17 expression. Cox17 was detected in the cytosol of cox17Δ cells transformed with each of the mutants, although Cox17 protein levels were slightly attenuated for the three essential Cys mutants. Cox17 is co-localized in the cytosol and the intermitochondrial membrane space with 60% of the protein localized to the intermitochondrial membrane space (13). Overexpression of COX17 results in an increase in the total cellular content of COX17 but a lesser elevation in the mitochondrial content (13). Thus, the mitochondrial content of Cox17 is limited. In the present experiments with COX17 expressed episomally, total Cox17 levels are elevated at least 4-fold relative to Cox17 levels in wild-type cells expressing COX17 chromosomally, and the percentage of total Cox17 within mitochondria is about 10%, a percentage similar to that seen previously with overexpressed Cox17 (13). Mutant Cox17 molecules with individual Cys → Ser substitutions at the three essential Cys positions (positions 23, 24, and 26) were also co-localized within the mitochondria, although the mitochondrial level of C26S Cox17 was partially attenuated relative to the wild-type protein. The only Cox17 mutant that failed to accumulate significantly within the mitochondria was the C57Y variant (Fig. 3). The C57Y mutant protein showed consistently attenuated mi-
Mutational Analysis of Cox17

Yeast Cox17 contains seven cysteinyl residues, only six of which are conserved in fungi and animals. Three of the conserved cysteinyl residues (Cys\(^{36}\), Cys\(^{47}\), and Cys\(^{57}\)) are nonessential in that substitution of any one of these cysteines with serines results in a functional cytochrome oxidase complex. Likewise, replacement of the nonconserved Cys\(^{16}\) with a Ser does not perturb cytochrome oxidase function. The nonessential Cys residues do not appear to have a synergistic effect in that cells containing a mutant Cox17 with a triple C16S/C36S/C47S substitution have a functional cytochrome oxidase. Only three cysteinyl residues in Cox17 are essential, and these three residues are present in a Cys-Cys-Xaa-Cys sequence motif. Single substitution of any of these three cysteines with serines

collection tags bound 2.8 Cu ions/monomer (Table I). The Cu binding stoichiometry was not significantly altered in any of the single mutants (Table I). Mutant Cox17 molecules with one of the three essential Cys residues mutated to Ser bound the same quantity of Cu as the wild-type protein. However, Cu binding was abolished in Cox17 with a double C23S/C24S substitution.

Because Cu binding was abolished with the double C23S/C24S mutant Cox17, the question arose whether the mitochondrial localization of Cox17 was related to the Cu-binding property of Cox17. Mitochondria were isolated from cells harboring the non-Cu-binding mutant Cox17 with the double C23S/C24S substitution. Western analysis of the mitochondrial extract and post-mitochondrial supernatant revealed the mutant protein was present in both compartments (Fig. 7). Thus, mitochondrial localization of Cox17 is not dependent on Cu loading of Cox17.

**DISCUSSION**

Yeast Cox17 contains seven cysteinyl residues, only six of which are conserved in fungi and animals. Three of the conserved cysteinyl residues (Cys\(^{36}\), Cys\(^{47}\), and Cys\(^{57}\)) are nonessential in that substitution of any one of these cysteines with serines results in a functional cytochrome oxidase complex. Likewise, replacement of the nonconserved Cys\(^{16}\) with a Ser does not perturb cytochrome oxidase function. The nonessential Cys residues do not appear to have a synergistic effect in that cells containing a mutant Cox17 with a triple C16S/C36S/C47S substitution have a functional cytochrome oxidase. Only three cysteinyl residues in Cox17 are essential, and these three residues are present in a Cys-Cys-Xaa-Cys sequence motif. Single substitution of any of these three cysteines with serines

![Fig. 5. Cytochrome c oxidase activity in isolated mitochondria from cells harboring various cox17 mutants.](Image)

**FIG. 5.** Cytochrome c oxidase activity in isolated mitochondria from cells harboring various cox17 mutants. Mitochondria were isolated from Δcox17 cells transformed with vectors containing cox17 variants. The oxidation of reduced horse heart cytochrome c was monitored at 550 nm. The data represent at least four separate isolates of purified mitochondria. Rates were determined by taking the initial slope of the reaction and normalizing to the mitochondrial protein concentration. WT, wild type.

![Fig. 6. Functionality of the C57S Cox17 mutant.](Image)

**FIG. 6.** Functionality of the C57S Cox17 mutant. W303Δcox17 cells were transformed with plasmids encoding C57S, C57Y, and C23S/C24S (C\(^{23,24S}\)) Cox17 mutants. A, cells were plated on synthetic complete medium lacking uracil and containing 2% glucose or 3% glycerol. B, mitochondria were isolated from W303Δcox17 cells harboring C57Y, C57S, and C23S/C24S Cox17 mutants. Cytochrome c oxidase activity was measured by following the oxidation of reduced cytochrome c oxidase.

![Fig. 7. Western analysis of Cox17 comparing presence of Cox17 variants in the mitochondria (mito) and the post-mitochondrial supernatant (PMS) representing the cytoplasmic fraction.](Image)

**FIG. 7.** Western analysis of Cox17 comparing presence of Cox17 variants in the mitochondria (mito) and the post-mitochondrial supernatant (PMS) representing the cytoplasmic fraction. Mitochondria were isolated from W303Δcox17 cells transformed with cox17 variants. Protein gels were blotted onto nitrocellulose membrane and probed with a polyclonal anti-Cox17 antibody or monoclonal antibodies to either porin, a mitochondrial outer membrane protein, or cytosolic phosphoglycerate kinase (PGK). Western analysis of the mitochondrial C57S mutant protein is not shown as the content of the C57S mutant protein was reproducibly less than that observed for the C57Y protein (Fig. 3), making visualization difficult. WT, wild type.

| Protein            | Cu:protein ratio |
|--------------------|------------------|
| Wild-type Cox17    | 3.0 ± 0.5 (n = 8) |
| C23S              | 3.2 ± 0.3 (n = 3) |
| C24S              | 3.2 ± 0.3 (n = 2) |
| C26S              | 2.7 ± 0.3 (n = 2) |
| C16S/C36S/C47S    | 3.0 ± 0.2 (n = 3) |
| C16S/C36S/C47S/C57S | 0.1 ± 0.0 (n = 2) |
| C23S/C24S        | 2.6 ± 0.5 (n = 1) |
| C57Y              | 2.5 ± 0.3 (n = 3) |

Table I

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results in a nonfunctional cytochrome oxidase complex. Cells harboring these mutant Cox17 molecules exhibit no cytochrome c oxidase activity in purified mitochondria and fail to grow on medium with nonfermentable carbon sources. These mutants likely represent a loss of function, as wild-type cells containing one of the Cox17 mutants at varying expression levels failed to exhibit any dominant negative effects on cytochrome oxidase function.

Although a Cys → Ser substitution at sequence position 57 is functional, a Cys → Tyr substitution in Cox17 was shown previously to result in non-respiratory cells (9). Cells harboring C57Y Cox17 exhibit residual cytochrome c oxidase activity, and therefore partial functionality exists in the mutant protein. As Tyr is less chemically related to Cys than Ser, the Tyr substitution likely perturbs some function of Cox17. The C57Y Cox17 mutant binds the same number of Cu ions, yet fails to accumulate within the mitochondria. The low mitochondrial content of the C57Y Cox17 may arise from impeded mitochondrial entry or reduced stability. The C-terminal segment of Cox17, including Cys57, may be part of an important mitochondrial targeting sequence. Most mitochondrial proteins are localized to the mitochondria through an N-terminal target sequence (24), although a series of molecules are known to contain internal or C-terminal targeting sequences (25, 26). A second metabolite transporter, ATP/ADP translocase, which functions as an ADP/ATP carrier, is targeted for mitochondrial uptake via the TOM (translocation of the outer membrane) translocation complex by an internal signal motif (27). However, it is unlikely that the nonfunctional status of the C57Y Cox17 relates primarily to low mitochondrial uptake or stability, as only minimal quantities of the functional C57S are found in mitochondria. Episomal expression of COX17 in these studies led to a 4-fold increase in total cellular Cox17, and therefore only a fraction of Cox17 in the mitochondria in the present studies is necessary for its function. An additional function of Cox17 is likely to be impaired in the C57Y Cox17 mutant. A second candidate function of Cox17 is the donation of Cu(I) ions in the mitochondrial intermembrane mitochondria membrane space, presumably resulting from docking to the Sco1 accessory protein. The C-terminal portion of Cox17 containing Cys57 may be important in docking. Two observations are consistent with this prediction. First, overexpression of the SCO1 homolog, SCO2, efficiently suppresses the respiratory defects of cox7-1 cells harboring the C57Y Cox17 mutation but not cox7Δ cells (10). The residual cytochrome oxidase activity in cox17-1 cells harboring the C57Y Cox17 mutation but not cox7Δ cells (10). The residual cytochrome oxidase activity in cox17-1 cells may relate to limited functional interactions with Sco1/Sco2. It is conceivable that the C57S mutant Cox17 interacts better with Sco1 than the C57Y protein. Weak interactions of Cox17 with Sco1 may result in Cox17 instability and degradation. Second, a hemaglutinin epitope tag at the C-terminal end of Cox17 impairs Cox17 function, although mitochondrial uptake and Cu(I) binding are normal (data not shown). In contrast, an N-terminal HA epitope-tagged Cox17 is functional and accumulates normally within the mitochondria.

The mitochondrial localization of Cox17 is consistent with the postulate that Cox17 functions in copper ion insertion into cytochrome oxidase. Curiously, only minimal quantities of the functional C57S mutant Cox17 are found in mitochondria. The interpretation of this result is unclear. Cox17 may be functional without mitochondrial targeting. Alternatively, Cox17 levels in the intermembrane mitochondrial membrane space may be in excess of what is required for function; therefore, only a fraction of the protein in this compartment is critical for function. Nothing is known of the fate of mitochondrially localized Cox17. If Cox17 shuttles Cu(I) ions to the mitochondria, it is unclear whether the apo-protein shuttles out of mitochondria or is degraded. Low mitochondrial accumulation of C57S Cox17 may relate to reduced stability of the protein in the intermembrane mitochondrial membrane space.

The prediction that Cox17 functions as the mitochondrial copper metallochaperone rests largely on the observed suppression of the glycerol auxotrophy of cox7 cells by exogenous copper salts (9). The proposed model for Cox17 function involves Cu ion shuttling to the mitochondrial intermembrane space and subsequent transfer of bound Cu ions to subunit II of cytochrome oxidase, perhaps via Sco1 (13). The mitochondrial uptake of Cox17 is not restricted to the Cu(I) conformation of Cox17, as the non-copper binding mutant Cox17 with the double C23S/C24S substitution localizes normally to mitochondria. This important result negates a model that Cu(I) binding induces a conformer in Cox17 competent for mitochondrial entry.

The copper content of Cox17 purified as an untagged molecule (3 mol eq) is higher than the 2 mol eq stoichiometry observed previously for GST-Cox17 fusion (14). The biophysical properties of CuCox17 purified as a GST fusion differ from those of the untagged CuCox17 complex even when the GST moiety is removed.2 We had proposed that the two Cu(I) ions bound to Cox17 purified as a GST fusion exist within a binuclear thiolate cluster with either four or five thiolate ligands (14). It is now clear that the copper thiolate center in Cox17 is more complex than that proposed originally for the GST fusion protein.2 Evidence will be presented elsewhere that the three Cu(I) ions in CuCox17 are bound within a polycopper cluster. The present mutagenesis data are consistent with only three cysteinyl residues being important for ligation of three Cu(I) ions. Additional studies are necessary to determine the Cu(I) binding geometry and whether other non-thiolate ligands are involved in Cu coordination. Because the copper content is not altered with single Cys substitutions in the three critical residues, Cys23, Cys24, and Cys26, the prediction is that Cu(I) binding in the single mutant occurs in an altered center with coordination by one or more of the nonessential Cys residues for ligation. The nonessential Cys residues may be spatially close to the essential three residues and may facilitate Cu release through ligand exchange reactions, as proposed for the transfer of Cu(I) from the Atx1 metallochaperone to Ccc2 (28). Biophysical characterization of the Cu(I) complexes of the single Cox17 mutants with changes at Cys23, Cys24, and Cys26 reveals a distinct change in the polycopper center.2

It is likely that Cys23, Cys24, and Cys26 are essential because of Cu(I) ligation, as the C23S/C24S double mutant fails to bind Cu(I). However, the possibility remains that the three cysteine residues are important in other aspects of Cox17 function, such as protein docking within the intermembrane space. As details emerge on the interactions of Cox17 within the intermembrane space, additional studies with the mutants may be insightful in elucidating the physiological significance of the three important cysteine residues.

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