Cox17p was previously shown to be essential for the expression of cytochrome oxidase in Saccharomyces cerevisiae. In the present study COX17 has been placed under the control of the GAL10 promoter in an autonomously replicating plasmid. A yeast transformant harboring the high copy construct was used to purify Cox17p to homogeneity. Purified Cox17p contains 0.2–0.3 mol of copper per mol of protein. The molar copper content is increased to 1.8 after incubation of Cox17p in the presence of a 6-fold molar excess of cuprous chloride under reduced conditions. An antibody against Cox17p was obtained by immunization of rabbits with a carboxyl-terminal peptide coupled to bovine serum albumin. The antiserum detects Cox17p in both the mitochondrial and soluble protein fractions of wild type yeast and of the transformant overexpressing Cox17p. Exposure of intact mitochondria to hypotonic conditions causes most of Cox17p to be released as a soluble protein indicating that the mitochondrial fraction of Cox17p is localized in the intermembrane space. These results are consistent with the previously proposed function of Cox17p, namely in providing cytoplasmic copper for mitochondrial utilization.

Cox17p is a low molecular weight protein of yeast (1) and mammalian cells (2). In Saccharomyces cerevisiae, mutations in COX17 cause a respiratory defect stemming from a specific deficiency in cytochrome oxidase, the activity of which depends on the presence of copper (1). The ability of high concentrations of copper in the growth medium to rescue the mutant phenotype indicated that the COX17 gene product is involved in mitochondrial copper metabolism, most likely by targeting cytoplasmic copper to the organelle (1). A role of Cox17p in copper homeostasis is also supported by its small size and high cysteine content, both features being common to other copper proteins such as metallothioneins (3) and Atx1p (4). The latter has recently been shown to mediate the transfer copper to an intact mitochondria to hypotonic conditions causes most of Cox17p to be released as a soluble protein indicating that the mitochondrial fraction of Cox17p is localized in the intermembrane space. These results are consistent with the previously proposed function of Cox17p, namely in providing cytoplasmic copper for mitochondrial utilization.

§ To whom correspondence should be addressed. Tel.: 212-854-2920; Fax: 212-865-8246.
FIG. 1. Physical map of pG74/ST31. The LEU2 gene and GAL10 promoter (GAL10p) in YEp52 are indicated by solid arcs. The solid bar denotes the location of COX17 in the HindIII fragment of the insert. The ADH promoter (ADH3), and terminator (ADH2) flanking the GAL4 gene are located in the BclI fragment downstream of COX17. COX17 and GAL4 transcription, indicated by the arrows, is from the same strand. The restriction sites for HindIII (H) and EcoRI (E) and the hybrid BclI/BamHI sites (B/C) are marked on the inset.

FIG. 2. Separation of Cox17p on Sephadex G-50. The 55–80% ammonium sulfate precipitate (2 g of protein) was applied to a column of Sephadex G-50 (fine) with a bed volume of 480 ml. The column was equilibrated and eluted with 20 mM Tris-HCl, pH 7.5, and 0.05 M NaCl. Fractions of 8 ml were collected at a flow rate of 1 ml/min and were analyzed for Cox17p as described in the legend to Fig. 3. The elution of Cox17p is shown in the inset. The fractions pooled for further fractionation on Mono Q are indicated by the bar. The second peak absorbing at 280 nm corresponds to low molecular weight compounds retained by the column.

Copper Assay

Copper was assayed by a modification of the method of Felsenfeld (9). Samples of protein adjusted to 2.1 ml with water were treated with 0.23 ml of 50% trichloroacetic acid and 50 μl of 30% hydrogen peroxide and heated at 90 °C for 3 min. Precipitated protein was removed by centrifugation at 100,000 g for 5 min. The following reagents were added sequentially with mixing following transfer of 2 ml of the clarified solution to a fresh tube: 1) 0.5 ml of 0.25% 2,2′-biquinoline in glacial acetic acid, 2) 0.1 ml of 10% trichloroacetic acid, 3) 0.5 ml of saturated sodium acetate. The copper biquinoline complex was extracted into 0.6 ml of isomyl alcohol by vigorous mixing for 15 s. The upper phase was separated by centrifugation at 400 × gav, transferred to a spectrophotometer cuvette, and clarified by addition of 30 μl of ethanol. Absorbance was read at 555 nm against a reagent blank treated as above. The concentration of copper was estimated from a standard curve obtained from a known solution of cupric sulfate.

Preparation of Mitochondria

For the localization and distribution of Cox17p, yeast mitochondria with intact outer membranes were prepared by the method of Glick and Pon (10). The supernatant fraction after sedimentation of mitochondria was centrifuged at 100,000 × gav for 10 min. The 100,000 × g supernatant is referred to as the post-mitochondrial supernatant.

Miscellaneous Procedures

Standard procedures were used for the preparation and ligation of DNA fragments and for transformation and recovery of plasmid DNA from Escherichia coli (11). Proteins were analyzed by PAGE with the buffer system of Laemmli (12). The concentration of acrylamide used was generally 15% with 15% glycerol added to the separation gel.
Western blots were treated with antibodies against the Cox17p carboxyl-terminal peptide followed by a second reaction with 125I-protein A.

RESULTS AND DISCUSSION

Subcellular Localization of Cox17p in Wild Type and \( \Delta \)COX17/ST31—In earlier experiments, Cox17p was detected as a biotinylated protein with a 7-kDa carboxyl-terminal extension containing a bacterial biotinylation signal (15). The introduction of the biotin tag was necessitated by the lack of an antibody against Cox17p. Most of the biotinylated Cox17p was found in the post-mitochondrial supernatant fraction, corresponding to the soluble cytosolic proteins of yeast. This was true irrespective of whether the COX17/BIO fusion gene was expressed from a multicopy plasmid or from a single chromosomally integrated copy of the gene (1).

The availability of an antibody against Cox17p made it possible to re-examine the subcellular localization of the native protein. These studies were done with the parental wild type strain W303-1B and with W303ΔCOX17/ST31, a \( \alpha \)cox17 null mutant transformed with COX17 under GAL10 regulation. The two fractions (70 \( \mu \)g of wild type post-mitochondrial supernatant and mitochondrial proteins) were loaded per lane. In the case of the transformant, 7 \( \mu \)g of each fraction was separated on a 15% polyacrylamide gel containing 15% glycerol, transferred to nitrocellulose, and probed with a 1:100 dilution of antiserum against Cox17p. Cross-reacting proteins were visualized by a second reaction with 125I-protein A.

The recoveries are based on cells obtained from 32 liters of YPGal medium.

| Fraction | Total protein | Cox17p | Total Cox17p | Recovery | Fold purification |
|----------|---------------|--------|--------------|----------|------------------|
| PMS      | 14,304 mg     | 0.0041 mg | 59 mg       |          |                  |
| 55–80% AS | 3,744 mg     | 0.0105 mg | 40 mg       | 68       | 2.6              |
| Sephadex G-50 pool | 100 mg | 0.224 mg    | 25 mg       | 37       | 55               |
| Mono Q  | 33 mg         | ND     | ND           |          |                  |
| Hydroxyapatite | 22 mg | 0.545 mg    | 12 mg       | 20       | 133              |
| Red Sepharose | 16 mg | 0.66 mg    | 11 mg       | 19       | 161              |
| C4 reverse-phase | 5.4 mg | 1 mg    | 5.4 mg      | 9        | 244              |

The concentration of Cox17p was determined by quantitative Western blot analysis. Purified Cox17p was used to obtain a standard curve relating amounts of protein to the density of the signals obtained with the antiserum against the carboxyl-terminal peptide. The density of the signals was quantitated with a Visage 110 Bioimager (Millipore Corp.). To increase the accuracy of the measurement, only a 3-fold range in the concentrations of the Cox17p was used for the standard curve, and the amount of the different fractions applied to the gel was adjusted so that the signals would fall within this range.

In the absence of a functional assay for Cox17 it was necessary to use an immunological method of detection during the purification. As indicated in Table I, size separation of the proteins excluded by the sieving column, some loss of Cox17p is incurred (Fig. 2). Chromatography of the pool from Sephadex...
ST31 transformant also had a copper content of approximately 1.8 mol of copper per mol of protein. This would suggest that the real stoichiometry of the metal. This would suggest that the real stoichiometry of Cox17p purified from the mitochondrial fraction of the reconstituted protein on the sieving column leads to a release of most of the bound copper, indicating a low binding constant for reconstitution of dithiothreitol-reduced protein with cuprous chloride under limiting oxygen to prevent reoxidation. Under these conditions 1.8 mol of copper was bound per mol of protein.

Based on its cysteine content Cox17p is expected to bind 2–3 mol of copper with an apparent molecular mass of 14 kDa even though its real mass is 7,926 daltons. Properties of Purified Cox17p—The purity of Cox17p obtained either from the post-mitochondrial supernatant or mitochondria was confirmed by protein sequencing. The amino-terminal 5 residues matched the sequence encoded by COX17 starting from the second residue (threonine). The molar yield of amino acids was consistent with the amount of protein sequenced. There was no evidence of secondary contaminating sequences. The absence of the initiator methionine in the protein indicates that this residue is processed. Cox17p migrates anomalously with an apparent molecular mass of 14 kDa in SDS-polyacrylamide gels (see Fig. 4). The discrepancy in size is unlikely to be due to modifications or dimerization of the protein because the mass measured by mass spectrometry is in accord with the molecular weight calculated from the translated gene sequence.

The copper content of purified Cox17p was determined colorimetrically with 2,2-biquinoline as a copper-specific chelator and by atomic emission. Both methods yielded comparable values of 0.3 mol of copper per mol of protein. The latter method also failed to detect any cobalt, nickel, or zinc in the protein. Based on its cysteine content Cox17p is expected to bind 2–3 mol eq of copper. The substoichiometric recovery of copper in the final protein suggests that the metal is lost during the purification procedure. The copper binding capacity of Cox17p was assessed by reconstitution of dithiothreitol-reduced protein with cuprous chloride under limiting oxygen to prevent reoxidation. Under these conditions 1.8 mol of copper was bound per mol of protein. No binding occurred when cupric sulfate was used under aerobic conditions. The Sephadex G-25 chromatography step used in the reconstitution achieves a complete separation of free from protein-bound copper (Fig. 5). Chromatography of the reconstituted protein on the sieving column leads to a release of most of the bound copper, indicating a low binding constant for the metal. This would suggest that the real stoichiometry of copper binding is probably greater than that measured by this method. Cox17p purified from the mitochondrial fraction of the ST31 transformant also had a copper content of approximately 0.3 mol/mol of protein.

Localization of Cox17p in the Intermembrane Space—As indicated above, 60% of Cox17p in wild type yeast is detected in mitochondria. It was of interest to determine whether the protein is located in the matrix or intermembrane space. Mitochondria prepared under conditions favoring maximal integrity of the outer membrane (10) were diluted either with buffered sorbitol (Fig. 6). Approximately 60% of Cox17p, cytochrome b2, and α-ketoglutarate dehydrogenase were assayed by Western blot analysis. Almost all of Cox17p, cytochrome b2, and α-ketoglutarate dehydrogenase remained associated with mitochondria diluted in the presence of an osmotically stabilizing concentration of sorbitol (Fig. 6). Approximately 60% of Cox17p, however, was released into the soluble protein fraction when mitochondria were diluted in the absence of sorbitol. In contrast virtually all of cytochrome b2 was recovered in the supernatant indicating quantitative lysis of the outer membrane. As expected all of α-ketoglutarate, the matrix marker, remained associated with the mitoplast fraction. The solubilization of most of Cox17p by the hypotonic treatment indicates that the
protein is located in the intermembrane space of mitochondria (10). The complete release of cytochrome $b_2$ by the osmotic shock of mitochondria indicates that the Cox17p recovered in the membrane fraction is not due to partial lysis of the outer membrane. The fraction of Cox17p present in the mitoplasts is not located in the matrix compartment because of its susceptibility to proteinase K. An alternative explanation for its incomplete release is that a fraction of Cox17p may be physically complexed to another component of mitochondria. It is unlikely that Cox17p is bound to Sco1p since its relative distribution is not altered in yeast harboring SCO1 on a multicopy plasmid (data not shown).

In addition to high concentrations of copper in the medium, the cytochrome oxidase deficiency of $cox17$ mutants can be partially suppressed by SCO1 when present on a high copy plasmid (24). SCO1 has been shown by Schulze and Rodel (25) to be essential for expression of cytochrome oxidase. These observations were taken to indicate that Sco1p functions in the pathway of copper addition to apocytochrome oxidase (24). A role of Sco1p in mitochondrial copper metabolism was also supported by the presence in the protein of a domain with sequence similarity to subunit 2 of cytochrome oxidase (24). This domain includes two conserved cysteine residues that are known to be copper ligands of subunit 2 (26). Sco1p is an insoluble protein of mitochondria that was localized in the outer membrane (27). The extra membrane domain of Sco1p probably faces the intermembrane space. This is supported by the sensitivity of Sco1p to proteinase K in mitoplasts but not in intact mitochondria (Fig. 7). This suggests that the proposed copper binding site of Sco1p and the copper site of subunit 2 of cytochrome oxidase (26) face the same mitochondrial compartment as Cox17p. Such a localization would be in accord with our previous model in which Cox17p targets copper specifically to mitochondria by transferring the metal to Sco1p. At present it is still not clear whether Sco1p is a carrier that transports copper to the matrix or whether it acts as a transferase that adds copper directly to cytochrome oxidase. If copper addition occurs after membrane insertion of subunit 2, a role of Sco1p as a copper transferase would be more attractive of the two models (Fig. 8). Since cytochrome oxidase is the only known copper-containing protein of yeast mitochondria, it is not clear whether the presence of copper in the matrix is required.
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