Cox17 Is Functional When Tethered to the Mitochondrial Inner Membrane*

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Cox17 is an essential protein in the assembly of cytochrome c oxidase within the mitochondrion. Cox17 is implicated in providing copper ions for formation of Cu₄ and Cu₆ sites in the oxidase complex. To address whether Cox17 is functional in shuttling copper ions to the mitochondrion, Cox17 was tethered to the mitochondrial inner membrane by a fusion to the transmembrane domain of the inner membrane protein, Sco2. The copper-binding domain of Sco2 that projects into the intermembrane space was replaced with Cox17. The Sco2/Cox17 fusion protein containing the mitochondrial import sequence and transmembrane segment of Sco2 is exclusively localized within the mitochondrion. The Sco2/Cox17 protein restores respiratory growth and normal cytochrome oxidase activity in cells harboring the cox17–1 mutant. These studies suggest that the function of Cox17 is confined to the mitochondrial intermembrane space. Domain mapping of yeast Cox17 reveals that the carboxyl-terminal segment of the protein has a function within the intermembrane space that is independent of copper ion binding. The essential C-terminal function of Cox17 maps to a candidate amphipathic helix that is important for mitochondrial uptake and retention of the Cox17 protein. This motif can be spatially separated from the N-terminal copper-binding functional motif. Possible roles of the C-terminal motif are discussed.

Copper plays an essential role in the biochemistry of all aerobic organisms (1). This metal functions as a cofactor permitting the facile transfer of electrons in key enzymes including Cu/Zn superoxide dismutase for antioxidant defense, tyrosinase for melanin synthesis, and cytochrome c oxidase for electron transport in the mitochondrial respiratory chain. When copper homeostasis is perturbed, the reactivity of copper with dioxygen may also lead to toxicity (2, 3). For this reason, specific mechanisms have evolved for the compartmentalization and trafficking of copper within cells (4).

The mechanism of copper ion routing to cytochrome c oxidase (CcO)¹ within the mitochondrion is unknown. Bovine CcO consists of 13 polypeptide subunits, three of which (Cox1–Cox3) are encoded by the mitochondrial genome, and the remaining subunits are encoded by the nuclear genome (5). The three mitochondrial copies of these subunits form the catalytic core of the enzyme. Cox2 requires two copper ions in the binuclear Cu₄ site, and Cox1 requires one copper ion in the Cu₆ site (6). CcO is localized within the inner mitochondrial membrane. The inner membrane (IM) differs from the outer membrane (OM) in being highly convoluted and folding into tubular cristae. Three internal spaces are created by the double membrane structure. The volume enclosed within the inner membrane is the matrix, which represents about 80% of the total mitochondrial space (7). The space between the inner and outer membranes is called the intermembrane space (IMS) and is interrupted by junction points in which the IM and OM are in contact. The IMS is very narrow and is separated from the third space, intracristae space, by tubular cristal junctions (8, 9). The bulk of the respiratory complexes, including CcO, exist within the cristae (10). Thus, assembly of functional CcO requires transport of nuclear-encoded subunits across both mitochondrial membranes. It is unclear whether the assembly of newly synthesized subunits occurs within the cristae or on the peripheral surface of the IM.

In yeast, several metallochaperones have been identified that shuttle copper ions to sites of utilization (4). Copper insertion into Cu/Zn-superoxide dismutase (Sod1) in yeast requires the function of the Lys7 (CCS) metallochaperone (11–14). Likewise, Ccc2, a P-type ATPase copper ion transporter, receives copper ions from the Atx1 metallochaperone (15, 16). Based on the necessity of Lys7 and Atx1 for shuttling copper ions to sites of utilization, the prediction is that CcO would require a specific protein involved in copper shuttling to the mitochondrion.

Cox17, a 8-kDa protein conserved in eukaryotes, has several characteristics consistent with a role in copper ion trafficking to the mitochondrion (17). The initial observation implicating Cox17 in metallation of CcO was the demonstration that the respiratory defect of cells harboring a non-functional cox17–1 mutant was suppressed by the addition of near toxic levels of copper ions in the growth medium (18). Two subsequent observations consistent with this postulate were the dual localization of Cox17 in both the cytosol and mitochondrial intermembrane space compartments and the Cu(I) binding ability of Cox17 (19, 20). Three cysteinyI residues present in a Cys-Cys-Xaa-Cys sequence motif are critical for in vivo Cox17 function and Cu(I) ion binding (21).

Although these observations suggest that Cox17 plays an important role in copper ion trafficking, the direct role of Cox17 in copper delivery to the mitochondria is unresolved. Cox17 may deliver Cu(I) ions to a mitochondrial OM transporter in analogy to the Atx1 routing of Cu(I) ions to the Ccc2 trans-Golgi translocase. Alternatively, Cox17 may ferry Cu(I) ions directly across the semi-porous mitochondrial OM. If the former is the case, Cox17 may have a secondary function within

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1 The abbreviations used are: CcO, cytochrome c oxidase; IM, inner membrane; OM, outer membrane; IMS, intermembrane space; Sod1, superoxide dismutase; WT, wild-type.

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Tethered Cox17

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MTETDKKQEQENHAECDKPKCCVKPKEEERDT

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59

CILFNGQDSKCKKEIKYKCMKGCYGFEPVSAN

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Fig. 1. Sequence of yeast Cox17. Residues that are identical with Cox17 molecules from other eukaryotes have an asterisk below; those residues having similar chemical functionalities have a dot below. The positions of the two truncates are labeled with the residue position.

chondria was performed by diluting intact mitochondria (150–250 μg) into 7 volumes of 20 mM Hepes-KOH, pH 6.7, buffer yielding intact mitoplasts. Membrane-associated proteins were solubilized by treatment of the mitoplasts in unbuffered 0.1 mM Na3C03. Inner membrane proteins were solubilized with 1% Triton X-100. Membranes were removed by centrifugation at 12,000 × g for 10 min.

Cytochrome c Mitochondrial Assays—Respiratory function was assayed by growth tests on synthetic complete selective plates (Ura, complete medium) using the non-fermentable carbon source, 3% glycerol. Cytochrome c enzymatic activity in isolated mitochondria was quantified by monitoring the oxidation of 32 μM reduced bovine cytochrome c at 550 nm by mitochondrial samples (5 to 10 μg protein) in 40 mM KH2PO4, pH 6.7, 0.5% Tween 80.

Western Analysis—Protein (10–50 μg) from the mitochondrial or post-mitochondrial (cytosolic) fraction was electrophoresed on a 15% SDS-PAGE gel system and transferred to nitrocellulose (Bio-Rad Laboratories). The membrane was subsequently probed with appropriate antibodies and proteins were visualized with ECL reagents (Pierce) following the addition of an horseradish peroxidase-conjugated, secondary antibody. The antisera against Cytb (soluble IMS mitochondrial protein) was kindly provided by Dr. Rosemary Stuart. Antisera to the mitochondrial OM porin (Porl) and cytosolic phosphoglycerol kinase (Pgk1) were obtained from Molecular Probes. Antiserum to the mitochondrial IM Sco1 and Cox17 were prepared as described previously (21, 29). To ensure equal loading of mitochondrial or post-mitochondrial protein into the gel wells, the membranes were stripped by 3 washes in stripping buffer (25 mM glycine, pH 2, 100 mM NaCl, and 0.5% Tween 20) and regreased with either an anti-Porl or an anti-Pgk1 antibody.

Purification and Analysis of Cox17—Cox17 was expressed as a soluble protein in Escherichia coli and purified as described previously (20). To evaluate the quaternary structure of Cox17, analytical gel filtration was performed using a 10/30 G-75 Superdex size exclusion column equilibrated with 20 mM phosphate, pH 7, 100 mM NaCl, and 1 mM dithiothreitol. Data were recorded using Unico software. Protein luminescence was monitored on a PerkinElmer LS55 luminescence spectrometer and analyzed using PerkinElmer Life Sciences F11 software. Protein was quantified by amino acid analysis on a Beckman 6300 analyzer after hydrolysis in 5.7N HCl containing 0.1% phenol in vacuo at 110 °C. The copper concentration of the protein samples was measured using a PerkinElmer Life Sciences Analyst 100 spectrophotometer.

RESULTS

The Cox17 polypeptide sequence is conserved in eukaryotes except for the chain termini (Fig. 1). If one of these segments is important for mitochondrial import and not for other functions of the protein, sequence diversity may exist, because mitochondrial targeting sequences do not show high sequence conservation. Amino- and carboxyl-truncation mutations were constructed to test for mitochondrial targeting. The N-terminal truncate lacked the N-terminal nine residues of Cox17 (designated ΔN), whereas the C-terminal truncate lacked the C-terminal ten residues (designated 59Δ) (Fig. 1). The truncated mutants of Cox17 under the control of the MET25 promoter were transformed into cox17Δ cells, and the transformants were tested for respiratory growth and cytochrome c oxidase activity. The ΔN truncate restored wild-type growth on glycerol-containing medium and wild-type cytochrome c oxidase activity (Fig. 2, A and B). In addition, the localization of ΔN Cox17 in
both the mitochondria and cytosolic fractions was similar to that of wild-type Cox17 (Fig. 2C). Thus, the N-terminal segment of Cox17 is not required for mitochondrial targeting.

In contrast, cells containing the 59Δ truncate were compromised for growth on glycerol-containing medium. Glycerol growth was improved when the 59Δ truncate was overexpressed in medium lacking methionine (Fig. 2A). As expected from the glycerol growth results, CcO activity was minimal under low expression conditions (Fig. 2B). The 59Δ truncate was abundant within the cytosol, but accumulated to a lesser extent in the IMS compared with the WT Cox17 or the 9Δ truncate (Fig. 2C). Under conditions of overexpression the 59Δ truncate accumulates predominantly within the cytosol (data not shown). Consistent with the 59Δ truncate data, the non-functional C57Y mutant of Cox17 also fails to accumulate within the mitochondrial IMS (21).

To test whether defective mitochondrial uptake of the 59Δ truncate was responsible for the slow growth on glycerol medium, DNA encoding the presequence of Cyb2 was fused to the 59Δ truncate. The presequence of Cyb2 is known to target heterologous molecules into the mitochondrial IMS (33). Cells with the Cyb2/59Δ Cox17 fusion showed no growth regardless of the expression levels and no oxidase activity (Fig. 3A). In contrast, cells harboring the Cyb2 presequence fused to the N terminus of full-length Cox17 and the Δ9 truncate had wild-type CcO activity (Fig. 3A). The addition of the Cyb2 presequence failed to increase quantities of 59Δ Cox17 in the IMS, even though the fusion of the Cyb2 presequence to WT Cox17 increased the IMS levels of Cox17 considerably (Fig. 3B). As expected, the addition of the Cyb2 presequence to the 59Δ Cox17 did significantly reduce the amount of Cox17 in the cytoplasm. Thus, the failure of the Cyb2/59Δ Cox17 fusion to accumulate within the IMS suggests that the C-terminal motif in Cox17 is important for IMS retention and/or stability.

Stability of the CuCox17 protein may be impaired if the C-terminal truncation compromised Cu(I) binding. The 59Δ truncate was expressed in E. coli and purified to evaluate its copper binding properties. The truncate bound Cu(I) normally and exhibited near wild-type Cu(I) luminescence (Fig. 4, inset). The luminescence of CuCox17 arises from the binding of Cu(I) ions within a polycopper cluster (20). Our previous mutational studies suggested that Cox17 function was dependent on the tetrameric structure (20). Cox17 is predicted to exist as a tetrameric molecule within the IMS based on the established equilibrium constant of dimeric verses tetrameric states of wild-type Cox17 and the quantitation of Cox17 levels within the IMS (20). In contrast to wild-type Cox17, the truncate showed an abnormal quaternary structure (Fig. 4). The isolated 59Δ truncate was predominantly monomeric. We demonstrated previously that Cox17 mutants that failed to oligomerize to the tetrameric state were inactive (20). However, not all C-terminal mutants are compromised in oligomerization. The C57Y mutant protein forms predominantly tetrameric species and binds Cu(I) normally (data not shown). Thus, lack of retention within the IMS in C-terminal Cox17 mutants is not primarily due to defective oligomerization or reduced Cu(I) binding. These data are consistent with the C-terminal segment of Cox17 having a role in mitochondrial retention and/or stability.

To determine whether the Cu(I) binding motif and the C-terminal retention motif can function independently, we constructed a head-to-tail dimer of Cox17 such that each half was compromised for one of these functions (Fig. 5A). Previously, we demonstrated that Cu(I) binding was confined to the

![Fig. 2. Complementation of cox17Δ cells by plasmid-borne COX17 (designated WT), or truncate mutants encoding the Δ9 or 59Δ proteins. Wild-type W303 cells are designated 303. The COX17 genes were under the control of the MET25 promoter enabling low expression (5× methionine levels (0.67 mM)) or high expression (no added methionine). A, growth test of transformants cultured with either glucose or glycerol as carbon source. B, cytochrome c oxidase activity of transformants cultured in medium containing 0.67 mM methionine. Vec, vector. C, Western analysis of Cox17 protein levels in isolated mitochondria or cytosol from transformants cultured in 0.67 mM methionine. Pgk1 is used as a cytosolic marker and Cyb2 as a mitochondrial IMS marker.](image-url)
C23C sequence motif near the central segment of the molecule. A C23S/C24S double substitution abolishes Cu(I) binding, but the mutant protein is taken into the mitochondrion normally (21). The non-functional C57Y allele forms a wild-type Cu(I) thiolate cluster but is defective in mitochondrial retention and/or stability (21). DNA encoding the non-functional C57Y allele was fused to the 5′/H11032 end of a mutant COX17 encoding the C23S/C24S double substitution that fails to bind Cu(I). The resulting head-to-tail fusion protein consisted of a N-terminal half containing a functional Cu(I) binding motif and the C-terminal half containing a functional C-terminal retention motif. Transformants of the chimeric gene, under the control of the MET25 promoter in cox17Δ cells, were cultured under low expression conditions were tested for respiratory growth on glycerol-containing medium (Fig. 5B). Whereas neither the mutant C57Y allele nor the mutant C23S/C24S allele was able to support glycerol growth, cells containing the fusion gene were competent for glycerol growth (Fig. 5B). The C57Y mutant is non-functional even if the Cyb2 presequence is fused to its N terminus to direct mitochondrial uptake (Fig. 5B, pC57Y on right). The head-to-tail chimeric protein restores CcO activity to 50% of WT levels (Fig. 5C). Western analysis revealed that the fusion protein was stable within the IMS, although a cleavage product was apparent that exhibited an electrophoretic mobility similar to the WT Cox17 (Fig. 5D).

Because neither the C57Y nor the C23S/C24S protein was functional by itself, the functional species must be the fusion molecule. Multiple secondary structure algorithms predict that the C-terminal segment of Cox17 contains an helical segment between residues 45 and 59. Projecting this sequence on a helical wheel reveals that most residues conserved in eukaryotic Cox17 sequences lie on one face of the putative helix (Fig. 6A). A number of these residues are hydrophobic, whereas the other face of the putative helix is highly hydrophilic. To determine the functional significance of these conserved residues, three double mutants were constructed in COX17 under its own promoter and terminator. The conserved hydrophobic Phe and Ile residues at positions 50 and 51 lie within the putative helix and were changed to aspartates. The conserved sulfur residues Cys-57 and Met-58 were changed to alanines. Outside the putative helix are two conserved residues Tyr-61 and Gly-62 that were changed to aspartates. COX17 genes encoding the double mutants were transformed into cox17Δ/1 cells and evaluated for function. Cells with the Y61D/G62D mutant showed growth on glycerol medium (Fig. 6B) and CcO activity. Cells with the C57A/M58A or F50D/I51D mutants showed minimal glycerol growth, yet Western analysis revealed that the mutant proteins were unstable in both the IMS and cytoplasm (data not shown). The lack of detection of the F50D/I51D mu-
tant Cox17 did not arise from a failure of the Cox17 polyclonal antisera to react with the protein. Expression of the F50D/I51D mutant in *E. coli* resulted in recovery of a stable Cu(I)-binding protein that was detected by Western analysis (data not shown). The purified Cu(I) mutant protein bound Cu(I) normally and exhibited near wild-type Cu(I) luminescence (Fig. 6C, inset). The F50D/I51D mutant showed a monomer/dimer distribution. Thus, instability is a property of the F50D/I51D mutant within yeast and not a general property of the mutant protein. Thus, the mutation appears to abrogate a function within yeast.

If Cox17 is a functional Cu(I) metallochaperone for the mitochondrion, the dual localization of Cox17 may be essential for function. To address this, we tethered Cox17 within the mitochondrial IM. The coding sequence of either COX17 or the Δ9 truncate was fused to the segment of SCO2 encoding the mitochondrial targeting sequence and its transmembrane domain (Fig. 7A). Sco2 is not essential for assembly of CcO, unlike its homolog Sco1. If Cox17 cycling between the cytosol and IMS is an essential function of Cox17, we predict that tethering Cox17 to the IM will compromise function. Mutant cox17Δ cells containing the two different SCO2/COX17 chimeras grew on glycerol medium under low expression conditions (Fig. 7B, shows SCO2/Δ9COX17 chimera) and in copper-limiting medium (data not shown). CcO oxidase activity was restored to 80% of WT levels with SCO2/COX17 (data not shown). Western analysis revealed a chimeric molecule of the expected size present within the mitochondrial (Fig. 7C). Fractionation of the mitochondria by hypotonic lysis demonstrated that Sco2/Cox17 remained associated with the mitoplasts and behaved similarly to the inner membrane Sco1 protein in its solubilization properties with carbonate buffer and detergent (Fig. 8). These studies confirm that the Sco2/Cox17 chimera to be properly inserted within the mitochondrion. Although Sco2/Cox17 was effective in restoring glycerol growth of cox17Δ cells, the chimera failed to complement sco1Δ cells.

To ensure that the complementation of cox17Δ cells by the SCO2/COX17 chimera was from the fusion protein and not from low levels of a Cox17 cleavage product, the expression level of SCO2/COX17 chimera was reduced by expressing the fusion gene from the GAL1 promoter in cells cultured in glycerol, a growth condition in which the GAL1 promoter is minimally expressed. The cox17Δ cells containing either the pGAL1-SCO2/COX17 chimera or pGAL1-COX17 grew normally in glycerol-containing medium despite very low levels of the Cox17 proteins (data not shown). The likelihood that complementation arose from a cleavage product is also min.
imal because no cleavage of the Sco2/Cox17 fusion protein was observed when the fusion was highly overexpressed (cells cultured in glycerol/H110010.1% galactose) (Fig. 7D).

**DISCUSSION**

Cox17 has been implicated as the mitochondrial copper metallochaperone responsible for shuttling Cu(I) ions to the mitochondrion for assembly of cytochrome c oxidase. This postulate is based on the known dual localization of Cox17 in the cytoplasm and mitochondrial IMS and that the respiratory defect of cox17Δ cells can be suppressed by high exogenous copper salts. The goal of the present studies was to map the mitochondrial targeting sequence of Cox17 and to determine whether cycling between the mitochondrion and cytoplasm is necessary for Cox17 function. These studies demonstrate that the C-terminal segment of Cox17 is an important determinant in mitochondrion and cytoplasm localization.
drial uptake and/or retention and also show that Cox17 is functional when exclusively localized to the IMS. Tethering Cox17 to the N-terminal segment of Sco2 containing the mitochondrial import sequence and transmembrane segment results in exclusive mitochondrial localization.

Two motifs in Cox17 are important for function. We previously demonstrated that the CCX sequence motif between residues 23 and 26 is essential for Cu(I) binding (21). We show presently that the C-terminal segment of Cox17 is also important for a function independent of Cu(I) binding. The various C-terminal mutants tested showed normal Cu(I) binding within a polycopper cluster as indicated by their luminescence. Structural prediction programs suggest that a C-terminal segment of Cox17 encompassing residues 45–59 folds into an α helix. Mutational analyses of conserved residues within this region are consistent with the predicted C-terminal helix. Two double mutants within the putative helix (F50D/I51D mutant and C57A/M58A mutant) display compromised function and protein stability within the IMS. A number of candidate functions exist for the C-terminal motif including oligomerization, mitochondrial import, protein stability and IMS retention. Although data are presented that suggest the C-terminal motif may contribute to each of these putative functions, an analysis of the entire data set eliminates several of these candidate functions.

Our previous studies showed a strong correlation between oligomerization of Cox17 and function (20). Mutants that fail to oligomerize to a tetrameric state are non-functional and tend to be unstable. The non-functionality of the C-terminal mutants cannot be completely due to impaired oligomerization as the C57Y mutant forms stable tetrameric complexes yet is inactive. Mutations of the Cu(I) binding Cys residues within the CCXC motif diminish tetramerization, yet the mutant proteins accumulate to reasonable levels within the IMS.

**FIG. 7**. Complementation studies of **cox17Δ** cells by plasmid-borne **COX17** fused to **SCO2**. The constructs were under the control of the **MET25** promoter and **CYC1** terminator. A, schematic of the Sco2/Cox17 fusion in which the mitochondrial target sequence (MTS) and transmembrane domain (TM) of Sco2 are fused to Cox17 or the Δ9 Cox17 truncate. B, growth test of Δ9 **COX17** truncate transformants cultured in medium containing 0.67 mM methionine with either glucose or glycerol as carbon sources. C, Western analysis of Sco2/Cox17 and Sco2/Δ9 Cox17 in both mitochondrial and cytosolic fractions. The arrow shows the electrophoretic position of pure Cox17. D, Western analysis of mitochondria purified from mitochondria from cells harboring pGAL1-**COX17** or pGAL1-**SCO2**/**COX17** cultured either in glycerol or glycerol + 0.1% galactose.
IMS (21). Thus, the impaired oligomerization is not strictly related to IMS stability.

Three facts suggest that mitochondrial import is also not the dominant function of the C-terminal motif. First, although mitochondrial IMS accumulation of the 59Δ truncate is decreased, attenuation of wild-type Cox17 levels in the IMS to levels less than that observed for the 59Δ protein does not abolish function. We reported previously that Cox17 with a C57S substitution unlike the C57Y substitution is functional but only minimal levels of protein were found in the IMS (21). Thus, only minimal quantities of Cox17 are needed for function (21, 37). Second, although the C-terminal motif may contribute to mitochondrial uptake, it cannot be the only determinant of mitochondrial uptake. A C-terminal truncation expressing only the N-terminal 40 residues was able to support limited glycerol growth when overexpressed (37). Third, insertion of hemagglutinin epitope tag at the C terminus of Cox17 results in efficient mitochondrial IMS accumulation of the 59Δ/59Δ truncate is decreased, attenuation of wild-type Cox17 levels in the IMS to levels less than that observed for the 59Δ protein does not abolish function. We reported previously that Cox17 with a C57S substitution unlike the C57Y substitution is functional but only minimal levels of protein were found in the IMS (21). Thus, only minimal quantities of Cox17 are needed for function (21, 37). Second, although the C-terminal motif may contribute to mitochondrial uptake, it cannot be the only determinant of mitochondrial uptake (21). A C-terminal truncation expressing only the N-terminal 40 residues was able to support limited glycerol growth when overexpressed (37). Third, insertion of hemagglutinin epitope tag at the C terminus of Cox17 results in efficient IMS Cox17 accumulation. The diminished accumulation of the C-terminal mutants within the IMS does not arise from general protein instability as cytoplasmic levels of the 59Δ mutant protein were normal and the F50D/I51D mutant was a stable Cu(I) protein when expressed in E. coli. However, instability within the IMS is apparent, because fusion of the Cyb2 presequence to either the C-terminal helix can influence the oligomerization state of Cox17, the possibility exists that this represents an interface for protein/protein interaction. This motif may be responsible for a docking with another protein during the metallation of CcO. Because Cox17 appears to function upstream of Sco1, the C-terminal segment of Cox17 may transiently dock with Sco1 during Cu(I) transfer to Sco1 prior to transfer to the CuA site of Cox2. Mutations that interfere with a docking interface may destabilize the interaction and result in Cox17 turnover.

An important question concerning the function of Cox17 is whether cycling between the cytoplasm and IMS is important for function. The most direct experiment testing this postulate is the tethering of Cox17 to the IM domain of Sco2. Although the tethered Sco2/Cox17 was functional, the important concern is whether any Cox17 cleavage product existed that would be competent to cycle. The Sco2Δ9 fusion construct exhibited no detectable cleavage products. This fusion gene was functional even when it was expressed from the GAL1 promoter in glycerol-grown cells in which the expression level was significantly reduced. Thus, it is likely that the functional state of the Sco2/Cox17 chimera arises from the fusion molecule and not a cleavage product.

The functionality of the tethered Sco2/Cox17 suggests that Cox17 does not shuttle Cu(I) ions across the OM for CcO assembly. Because the fusion is functional in cells cultured in low copper medium, cells do not even require Cox17 to shuttle Cu(I) ions under Cu-deficient conditions. Cu(I) delivery may occur through a permease within the OM. The only other known mitochondrial copper protein is Sod1 which exhibits a dual localization within the cytosol and mitochondrial IMS (39). Copper ion mettallation of Sod1 likely occurs within the IMS because mitochondrial Sod1 import is dependent on its apo-conformer (40). Cu insertion into Sod1 is mediated by the CCS metallochaperone. A common pathway of Cu ion delivery to the IMS may exist to provide Cu ions for CcO assembly and mettallation of Sod1. We are currently screening for such a mitochondrial Cu ion transporter.

FIG. 8. Fractionation of Sco2/Cox17 in purified mitochondria. Mitochondria were incubated in either isotonic or hypotonic buffer.

REFERENCES

1. Linder, M. C., and Hazegh-Azam, M. (1996) Am. J. Clin. Nutr. 63, 7975–8115
2. Henle, E. S., and Linn, S. (1997) J. Biol. Chem. 272, 19095–19098
3. Valentine, J. S., Wertz, D. L., Lyons, T. J., Liou, L. L., Goto, J. J., and Gralla, E. B. (1998) Curr. Opin. Chem. Biol. 2, 253–262
4. Huffman, D. L., and O’Halloran, T. V. (2001) Annu. Rev. Biochem. 70, 677–701
5. Capaldi, R. A. (1990) Annu. Rev. Biochem. 59, 569–596
6. Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Hakamata, R., Yasui, R., and Yoshikawa, S. (1995) Science 269, 1069–1074
7. Scorrano, L., Ishiya, M., Butele, K., Weiler, S., Oakes, S. A., Mannella, C. A., and Korosmeyer, S. J. (2002) Develop. Cell 2, 55–67
8. Frey, T. G., and Mannella, C. A. (2000) Science 284, 319–324
9. Mannella, C. A., Pfeiffer, D. R., Bradshaw, P. C., Moraru, I. I., Slepenko, B., Loew, L. M., Hsieh, C., Butele, K., and Marko, M. (2002) UMBB Life 52, 1–7
10. Peretti, M. E., Anderson, W. A., and Swift, H. (1983) J. Histochem. Cytochem. 31, 351–365
11. Caillet, V., Loew, L. M., Strain, J., Casareno, R. L. B., Krems, B., and Gitlin, J. D. (1997) J. Biol. Chem. 272, 23469–23472
12. Gasnet, F., and Lauquin, G. J. M. (1998) Eur. J. Biochem. 251, 716–723
13. Casareno, R. L. B., Waggoner, D., and Gitlin, J. D. (1998) J. Biol. Chem. 273, 23625–23628
14. Rae, R. D., Schmidt, P. J., Pufahl, R. A., Caillet, V. C., and O’Halloran, T. V. (1999) Science 286, 805–807
15. Lin, S.-J., Pufahl, R. A., Dancis, A., O’Halloran, T. V. O., and Caillet, V. C. (1997) J. Biol. Chem. 272, 9215–9220
16. Huffman, D. L., and O’Halloran, T. V. (2000) J. Biol. Chem. 275, 18611–18614
17. Amaravadi, R., Glerum, D. M., and Traughoff, A. (1997) Human Genetics 99, 329–333
18. Glerum, D. M., Shtanko, A., and Tsagkoff, A. (1996) J. Biol. Chem. 271,
19. Beers, J., Glerum, D. M., and Tzagoloff, A. (1997) J. Biol. Chem. 272, 33191–33196.
20. Heaton, D. N., George, G. N., Garrison, G., and Winge, D. R. (2001) Biochem. 40, 743–751.
21. Heaton, D., Nittis, T., Srinivasan, C., and Winge, D. R. (2000) J. Biol. Chem. 275, 37582–37587.
22. Gaume, B., Klaus, C., Ungermann, C., Guiard, B., Neupert, W., and Brunner, M. (1998) EMBO J. 17, 6497–6507.
23. Pfanner, N., and Wiedemann, N. (2002) Curr. Opin. Cell Biol. 14, 490–491.
24. Roise, D., Theiler, F., Horvath, S. J., Tomich, J. M., Richards, J. H., Allison, D. S., and Schatz, G. (1988) EMBO J. 7, 649–653.
25. Diekert, K., Kispal, G., Guiard, B., and Lill, R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 11752–11757.
26. Schulze, M., and Rodel, G. (1988) Mol. Gen. Genet. 211, 492–498.
27. Rentzsch, N., Krumm-Bloch, G., Hofer, A., Bartuschka, A., Ostermann, K., and Rodel, G. (1999) Curr. Genet. 35, 103–108.
28. Glerum, D. M., Shtanko, A., and Tzagoloff, A. (1996) J. Biol. Chem. 271, 20531–20535.
29. Nittis, T., George, G. N., and Winge, D. R. (2001) J. Biol. Chem. 276, 42520–42526.
30. Carr, H. S., George, G. N., and Winge, D. R. (2002) J. Biol. Chem. 277, 31237–31242.
31. Buchwald, P., Krumm-Bloch, G., and Rodel, G. (1991) Mol. Gen. Genet. 229, 413–420.
32. Mumberg, D., Muller, R., and Funk, M. (1994) Nucleic Acids Res. 22, 5767–5768.
33. Beasley, E. M., Muller, S., and Schatz, G. (1993) EMBO J. 12, 2303–2311.
34. Glick, B. S., and Pon, L. A. (1995) Methods Enzymol. 260, 213–223.
35. Bradford, N. M. (1976) Anal. Biochem. 72, 248–254.
36. Deleted in proof.
37. Rentzsch, N., Krumm-Bloch, G., Hofer, A., Bartuschka, A., Ostermann, K., and Rodel, G. (1999) Curr. Genet. 35, 103–108.
38. Glerum, D. M., Shtanko, A., and Tzagoloff, A. (1996) J. Biol. Chem. 271, 20531–20535.
39. Sass, E., Karniely, S., and Pines, O. (2003) J. Biol. Chem. 278, 45109–45116.
40. Field, L. S., Furukawa, Y., O’Halloran, T. V., and Culotta, V. C. (2003) J. Biol. Chem. 278, 28052–28059.
41. Huh, W. K., Falvo, J. V., Gerke, L. C., Carroll, A. S., Howson, R. W., Weissman, J. S., and O’Shea, E. K. (2003) Nature 425, 686–691.