Evidence for a Pro-oxidant Intermediate in the Assembly of Cytochrome Oxidase

Received for publication, March 20, 2007, and in revised form, April 11, 2007. Published, JBC Papers in Press, April 12, 2007, DOI 10.1074/jbc.M702379200

Oleh Khalimonchuk, Amanda Bird, and Dennis R. Winge
From the University of Utah Health Sciences Center, Departments of Medicine and Biochemistry, Salt Lake City, Utah 84132

The hydrogen peroxide sensitivity of cells lacking two proteins, Sco1 and Cox11, important in the assembly of cytochrome c oxidase (CcO), is shown to arise from the transient accumulation of a pro-oxidant heme A-Cox1 stalled intermediate. The peroxide sensitivity of these cells is abrogated by overexpression of a novel mitochondrial ATPase Afg1 that promotes the degradation of CcO mitochondrially encoded subunits. Studies on the hydrogen peroxide sensitivity in CcO assembly mutants reveal new aspects of the CcO assembly process.

Assembly of cytochrome c oxidase (CcO), the terminal enzyme of the mitochondrial respiratory chain, is a sophisticated, multistep process that involves a number of auxiliary proteins (1–3). Some of these proteins have a specific role, such as heme A biosynthesis or translation of mitochondrial-encoded subunits. Other assembly proteins act as chaperones or facilitators of the assembly process. Among them are a series of copper-binding proteins implicated in the assembly of the two copper centers of CcO. Copper metallation of CcO occurs within the mitochondrial intermembrane space (IMS) and is mediated by metallochaperone proteins. Cox17 is a key copper donor to two accessory proteins, Sco1 and Cox11, to form the Cu₄ and Cu₅ copper centers in the mature CcO complex (4).

Sco1 and Cox11 are each integral membrane proteins with C-terminal globular domains protruding into the IMS (5). The globular domain of Sco1 possesses a thioredoxin fold consisting of a central four-stranded β sheet covered with flanking helices (6–8). Two notable features of Sco proteins are a conserved pair of cysteinyl residues within a CXXC motif and a conserved histidyl residue that forms a Cu(I) binding site. Mutations of the Cys or His residues abrogate Cu(I) binding and lead to a nonfunctional CcO complex and respiratory-deficient cells (9). Thus, the in vivo function of Sco1 correlates with Cu(I) binding. The globular domain of Cox11 adopts an immunoglobulin-like β fold. Three conserved Cys residues are candidate ligands for the Cu(I) ion. Mutation of these Cys residues attenuates Cu(I) binding as well as CcO function (10). Thus, like Sco1, the residues in Cox11 important for Cu(I)-binding correlate with in vivo function, suggesting that Cu(I) binding is important in Cox11 function. These observations are consistent with the postulate that Cu(I) ions donated to Sco1 and Cox11 via Cox17 are subsequently transferred to Cox2 and Cox1 for assembly of the Cu₄ and Cu₅ sites, respectively. The function of both Sco1 and Cox11 was predicted to be solely attributable to formation of CcO copper centers (11).

Although recombinant Sco1 is isolated with bound Cu(I) ions in copper-supplemented bacterial cultures, attempts to crystallize the Cu(I)-Sco1 complex failed in a number of laboratories (7, 8, 12). The structure of the Cu(I)-Sco1 complex was subsequently achieved by NMR spectroscopy (13). The inability to crystallize Sco1 with a bound Cu(I) led some investigators to speculate that Sco1 might function in CcO assembly independent of Cu(I) binding (7, 12). The structural resemblance of Sco1 to peroxiredoxins and thioredoxins raised the possibility that Sco proteins may function as a thiol-disulfide oxidoreductase to maintain the Cu₄ site cysteines in the reduced state ready for metallation (14). Alternatively, Sco1 was suggested to function as a redox switch, in which oxidation of Cu(I) to Cu(II) induces release of the Cu(II) ion, thereby permitting the two thiolates to participate in a peroxidase reaction. In support of a redox role for Sco1, sco1Δ yeast cells were observed to be sensitive to hydrogen peroxide (7). In addition, cox11Δ cells, but not cox17Δ or cox4Δ cells, are also peroxide-sensitive (15, 16). The apparent selective peroxide sensitivity of sco1Δ and cox11Δ cells suggested that Sco1 and Cox11 have a mitochondrial function independent of copper metallation. To elucidate the mechanism of the peroxide sensitivity of sco1Δ and cox11Δ cells, we surveyed a wide array of yeast mutants and isolated genetic suppressors. We show that the hydrogen peroxide sensitivity of...
sco1Δ and cox11Δ cells arises from a pro-oxidant Cox1 assembly intermediate involving Cox1 and heme A. We demonstrate that both Sco1 and Cox11 have protein chaperone roles in stabilizing Cox1 and Cox2 chains in addition to their copper transfer functions. These studies on hydrogen peroxide sensitivity in CcO assembly shed further insight into the assembly process.

MATERIALS AND METHODS

Strains and Growth Media—The S. cerevisiae strains used in this study are listed in supplemental Table 1. S. cerevisiae cells were grown in YP (1% yeast extract, 2% peptone) with either 2% glucose, raffinose, galactose, lactate, or glycerol as a carbon source or synthetic minimal medium supplemented with respective amino acids and 2% glucose or glycerol. For cloning and plasmid amplification, Escherichia coli strain DH5α was used. The culture medium used was as described (17).

Plasmids and Constructs—For all manipulations with DNA fragments and plasmids, standard procedures were used (17). Yeast cells were transformed as described (18). Plasmids carrying either the COX11 gene or its mutated forms (COX11-C111A, COX11-C208A, COX11-C210A) (10) were used for complementation tests. Similarly, plasmids that carry the SCO1 gene or its mutated versions (SCO1-C148A, SCO1-C152A, SCO1-C208A, SCO1-C216A) were used (9). We also used the Sco1/Cox11 chimera, consisting of the N-terminal part of Cox11, including the transmembrane helix, fused with the C-terminal moiety of Sco1 (19) for complementation tests. The human SCO1 open reading frame was PCR-amplified from templates described previously (20) and cloned into pRS426 vector under the MET25 promoter (21). For overexpression of COX15, the open reading frame was subcloned into pRS426 under the MET25 promoter.

Hydrogen Peroxide Treatment of Yeast Cells—Cells of the respective strains or transformants were grown in full or selective liquid medium containing 2% glucose, at 30 °C. When cells reached midexponential growth, hydrogen peroxide was added to a final concentration of 0.5, 1, or 6 mM. After a 2-h incubation at 30 °C, cells were serially diluted and plated onto YPD plates. Heme synthesis was inhibited with 0.5 mM succinyl acetone (Sigma) for a 3-h period prior to the addition of hydrogen peroxide.

Hydrogen Peroxide Sensitivity Suppressor Screen—A high copy genomic library constructed using the total DNA isolated from S. cerevisiae with five times genome coverage was used to screen for high copy suppressors of peroxide sensitivity of cox11 null mutant. Each transformant was individually cultured in 96-well plates. Grown cells were treated as described above.
and stamped onto a YPD plate. Obtained colonies were analyzed and run through a second round of screening to exclude the false positives. Ten thousand transformants were analyzed. Retrieved AFG1 open reading frame was amplified from the total genomic DNA of wild-type BY4741 strain with the addition of a single Myc tag at the 3’-end and cloned into pRS426 vector under control of the MET25 promoter.

Mitochondrial Localization Studies—Mitochondria were isolated as described (22). CoQ activities were determined as described (23). Alkaline extraction of proteins was performed as described (24) using 0.1M Na2CO3, pH 10.5 or 11.5. Isolated mitochondria were treated with proteinase K (10 mg/ml) and analyzed as described (25).

Assessment of Carbonylation Levels—Cells of the respective strains were grown overnight at 30 °C to middle exponential phase. Each culture was split into halves; one was treated with H2O2 as described above, and the second half served as a control. Carbonylation levels were assessed using the OxyBlot™ protein oxidation detection kit (Serologicals Corp., Norcross, GA) according to the manufacturer’s manual.

In Vivo Labeling of Yeast Cells—Cells of the respective strains or transformants were grown and pulse-labeled with [35S]methionine in the presence of cycloheximide as described (26) for 30 min. Following the labeling, cells were incubated at 37 °C, and samples were collected at time points 0, 10, 30, and 60 min, resolved by SDS-PAGE, and analyzed by autoradiography. Densitometric analysis was performed using Image J 1.35 software (available on the World Wide Web at image.bio.methods.free.fr/ijdoshell.html).

RESULTS

As shown previously, yeast cells lacking Sco1 or Cox11 are sensitive to hydrogen peroxide (15, 16). Cells were incubated in the presence or absence of hydrogen peroxide for 2 h at 30 °C prior to plating out the cultures on rich growth medium in the absence of peroxide. Both mutants are growth-compromised in a pretreatment with 1 or 6 mM hydrogen peroxide (Fig. 1, A and B). The mutants are not sensitive to paraquat (16). Cells lacking Cox11 are slightly, but reproducibly, more sensitive to peroxide than cells lacking Sco1 (Fig. 1, A and C). The peroxide sensitivity of sco1Δ and cox11Δ cells is manifest only in cells harvested in log phase, since stationary cultures are largely resistant to 6 mM hydrogen peroxide (data not shown). To establish that the

FIGURE 2. Hydrogen peroxide sensitivity of cells devoid of mitochondrially encoded CoQ subunits. Cells of the strains depleted for mitochondrially coded COX1, COX2, or COX3 genes as well as cells lacking translational activators Pet309 or Pet111 and CoQ assembly proteins Cox20 or Cox10 were treated and grown as described in Fig. 1. WT, wild type.

FIGURE 3. Changes in mitochondrial translation affect H2O2 sensitivity of yeast cells. A, cox11Δ, sco1Δ, and cox20Δ cells were grown to midexponential phase and incubated with (+ CAP) or without (− CAP) chloramphenicol (8 mg/ml) prior to incubation with hydrogen peroxide. Then cells were treated and grown as described in the legend to Fig. 1. B, cox11Δ cells were turned into the rho− state by intensive treatment with ethidium bromide. The obtained cells along with the cox11Δ strain with intact mitochondrial genome were incubated with (+) or without (−) 6 mM H2O2 and grown as described in the legend to Fig. 1. C, cox11Δ, cox14Δ, and double cox11Δ cox14Δ mutant cells were incubated with (+) or without (−) 0.5 mM H2O2 for 2 h at 30 °C, serially diluted, and spotted onto plates containing 2% glucose and incubated for 36–48 h at 30 °C. D, cox11Δ, pet309Δ, and double cox11Δ pet309Δ mutant cells were grown to midexponential phase and incubated with (+) or without (−) 1 or 6 mM H2O2 and grown as described in the legend to Fig. 1. WT, wild type.
peroxide sensitivity arose from the deletion of either SCO1 or COX11, transformants of the null strains with the wild-type gene were tested for hydrogen peroxide sensitivity. SCO1 transformants of sco1Δ cells and COX11 transformants of cox11Δ cells were insensitive to hydrogen peroxide (Fig. 1, A and B). Surprisingly, Sco1 mutants with substitutions of the two Cu(I)-binding Cys residues, Cys148 and Cys152, were also effective in suppressing the peroxide sensitivity of sco1Δ cells (Fig. 1A). Likewise, Cox11 mutants with substitutions of three Cu(I)-binding Cys residues also were effective in suppressing the peroxide sensitivity of cox11Δ cells (Fig. 1A). Cells harboring these mutant alleles of Sco1 and Cox11 are peroxide-resistant, the cells remain respiratory-deficient, since CcO assembly is impaired. The suppression of sco1Δ and cox11Δ cells is protein-specific. High copy SCO1 cannot suppress the peroxide sensitivity of cox11Δ cells and overexpression of COX11 fails to suppress the peroxide sensitivity of sco1Δ cells (Fig. 1B). However, a Sco1/Cox11 chimera consisting of the Sco1 N-terminal segment, including the transmembrane domain fused to the C-terminal globular domain of Cox11, was a weak suppressor of the peroxide sensitivity of both sco1Δ and cox11Δ cells (Fig. 1B).

Human SCO1 fails to complement the respiratory defect of yeast sco1Δ cells, yet human SCO1 does reverse the peroxide sensitivity of sco1Δ cells (Fig. 1B). Taken together, these data demonstrate that peroxide resistance requires one aspect of the Sco1 and Cox11 proteins but not the copper binding aspect.

To uncover the source of the peroxide sensitivity in CcO assembly mutants, we screened a variety of additional CcO mutants for peroxide sensitivity. Cells lacking Cox1 are respiratory-deficient but resistant to 6 mM peroxide (Figs. 1C and 2). Cells lacking Cox2 or Cox3 are resistant to 1 mM peroxide but show sensitivity at 6 mM (Figs. 1C and 2). Cells lacking the...
Heme-Cox1 Intermediate

translational activator Pet309 or Pet111 required for Cox1 and Cox2 translation, respectively, are resistant to hydrogen peroxide. Cells lacking Cox10, which functions in heme A formation, are resistant to peroxide, but cells lacking the Cox20 chaperone for Cox2 proteolytic maturation are partially sensitive to exposure to hydrogen peroxide (Figs. 1C and 2). One scenario is that cells lacking Cox20, Cox11, or Sco1 accumulate a transient intermediate in CcO assembly that may confer hydrogen peroxide sensitivity.

Studies were undertaken to determine whether the pro-oxidant contains a CcO mitochondrial-encoded subunit. The peroxide sensitivity of sco1Δ, cox11Δ, and cox20Δ cells is partially reversed by preincubation of those cultures with chloramphenicol for 10 min prior to exposure to peroxide (Fig. 3A). The presence of chloramphenicol inhibits mitochondrial protein synthesis. Chloramphenicol failed to suppress the peroxide sensitivity of ctt1Δ cells lacking the cytosolic Ctt1 catalase. In addition, the peroxide sensitivity of cox11Δ cells is abrogated by conversion of cox11Δ cells to a rho- state (Fig. 3B).

Cox1 is implicated as a component of the pro-oxidant, since cells lacking Cox2 or Cox3 are partially sensitive, whereas cells lacking Cox1 are peroxide-resistant (Figs. 1C and 2). To substantiate the role of Cox1 in the peroxide sensitivity, COX14 was deleted, creating the cox11Δcox14Δ double null strain. Cells lacking Cox14 hyperaccumulate nascent Cox1 chains (27). Cells lacking both Cox11 and Cox14 are hypersensitive to hydrogen peroxide, showing growth impairment with 0.5 mM H2O2 (Fig. 3C). The role of Cox1 was substantiated by the demonstration that the peroxide sensitivity of cox11Δ cells is attenuated by disruption of the Cox1 translational activator Pet309. The cox11Δpet309Δ double mutant was peroxide-resistant, consistent with Cox1 being a component of the pro-oxidant (Fig. 3D).

The observed peroxide sensitivity may arise with the generation of a pro-oxidant or the disruption of a redox system. One candidate pro-oxidant would be the heme A3 moiety in Cox1. Heme A3 has an open coordination site that may yield peroxidase activity. Heme A is probably inserted into Cox1 prior to the addition of Cox2 in the assembly pathway (28). The structure of Cox1 has an open channel from the IMS side of the IM, where heme A insertion may occur (Fig. 4A). The peroxide sensitivity of cox11Δ cells may arise from the transient accumulation of a heme A-Cox1 intermediate (Fig. 4C, 1). The solvent accessibility of this putative intermediate may enable the heme to catalyze formation of the hydroxyl radical pro-oxidant. Residual heme A is observed in cells lacking Cox11 or Sco1 (29).

If a heme A-Cox1 intermediate is the pro-oxidant, the observed peroxide sensitivity of cox11Δ cells should be reversed if heme A formation is blocked and exacerbated if heme A production is increased. The inhibition of heme synthesis in cox11Δ cells by the addition of 0.5 mM succinyl acetone abrogated the sensitivity to 1 mM hydrogen peroxide (Fig. 5A). Furthermore, overexpression of COX15, which is known to significantly increase the levels of heme A in CcO assembly mutants (30), exacerbated the hydrogen peroxide sensitivity of cox11Δ and sco1Δ cells (Fig. 5B). A growth defect was observed with 0.5 mM H2O2 in the overexpressor cells. Overexpression of COX15 also induced mild sensitivity in wild-type cells to 0.5 mM H2O2 but curiously no sensitivity in shy1Δ cells. The lack of peroxide sensitivity in shy1Δ cells is consistent with a defect in heme A3 insertion in these cells. Rhodobacter cells lacking Shy1 are compromised in heme A3 insertion (31).

The accumulation of a heme A-Cox1 pro-oxidant intermediate in cox11Δ cells may result in generalized reactive oxygen damage. Wild-type and cox11Δ cells were cultured in the presence of 1 mM H2O2 for 2 h at 30 °C for isolation of mitochondria. The accumulation of carbonylated proteins was assessed by immunoblotting after dinitrophenyl hydrazone derivatization. cox11Δ cells showed nearly 2-fold enhancement in carbonylation, consistent with enhanced reactive oxygen sensitivity (Fig. 6).

Multicopy suppressors of the peroxide sensitivity of cox11Δ cells were isolated. Null cells transformed with a high copy DNA library were screened for clones able to propagate in 6 mM hydrogen peroxide. Two plasmid-borne suppressors

FIGURE 7. AFG1 and STR2 are specific high copy suppressors of the H2O2 sensitivity of sco1Δ and cox11Δ mutants. A, sco1Δ or cox11Δ cells containing empty vector or plasmids expressing high copy suppressor AFG1 or STR2 were treated with H2O2, and serial dilutions were spotted as described in the legend to Fig. 1. B, serial dilutions of sco1Δ cells transformed with either an empty vector or plasmids expressing SCO1, AFG1, or STR2 genes were spotted onto plates containing 2% glucose or 2% lactate, glycerol. C, cells lacking cytochrome c peroxidase (cpp1Δ) and cytosolic catalase (ctt1Δ) transformed with an empty vector or plasmids expressing AFG1 or STR2 were treated with hydrogen peroxide and grown as described above. WT, wild type.
mitochondria of a strain expressing C-terminally Myc-tagged Afg1 (Fig. 7A) were treated with 0.1M Na2CO3 and fractionated by high speed centrifugation. Obtained pellet (P, lanes 2 and 4) and supernatant (S, lanes 3 and 5) fractions were analyzed by immunoblotting using antibodies against Myc epitope, matrix protein Sod2, and IM-anchored protein Cox2. B, intact mitochondria (lanes 1, 2, and 4) or mitoplasts (Swelling; lane 3) isolated from a strain expressing Afg1-Myc were incubated with (lanes 2, 3, and 5) or without (lanes 1 and 4) proteinase K (PK). In lanes 4 and 5, mitochondria were lysed with 1% Triton X-100 (TX-100) prior to incubation with proteinase K. Proteins were separated by SDS-PAGE and analyzed by Western blot using antibodies specific for Myc-tag, matrix protein Sod2, and intramitochondrial space protein Ccp1.

were isolated (Fig. 7A). Overexpression of STR2 or AFG1 was found to suppress the peroxide sensitivity but not the respiratory deficiency of both sco1Δ and cox11Δ cells (Fig. 7B). Str2 is a cystathionine γ-synthase that converts cysteine into cystathionine (32). A C-terminal green fluorescent protein fusion of Str2 was reported to localize to the cytoplasm and nucleus (33). Afg1 is a putative mitochondrial ATPase (34). STR2 but not AFG1 was found to strongly suppress the peroxide sensitivity of ccp1Δ cells lacking cytochrome c peroxidase localized to the IMS. Neither gene suppressed the peroxide sensitivity of cells lacking the cytosolic Ctt1 catalase (Fig. 7C). Str2 may have a general role in protecting mitochondria from peroxide stress. Overexpression of Afg1 was also found to suppress the peroxide sensitivity of cox20Δ cells, suggesting that it has some specificity to the peroxide sensitivity of CoxO assembly mutants. Since Afg1 was selective in its suppression of mutants in the CoxO assembly pathway, we chose to restrict our focus to Afg1.

Cells lacking Afg1 showed a modest growth impairment on glycerol medium at 37 °C, and both CoxO and the bc1 complex activities are reduced (supplemental Fig. 1). A Myc-tagged Afg1 fusion was found to localize within the mitochondrial and be membrane-associated (Fig. 8). The fusion protein was insensitive to proteinase K in whole mitochondria and mitoplasts, suggesting that the protein faces the matrix side of the IM. Afg1 resembles the mitochondrial iAAA (Yme1) and mAAA (Yta10/Yta12) proteases but lacks the characteristic zinc protease domain (supplemental Fig. 2). The ATPase domain of Afg1 is important for its ability to suppress cox11Δ cells (data not shown).

To address whether Afg1 functions in degradation of Cox1, Cox2, and Cox3, cox11Δ cells in the presence or absence of AFG1 were pulse-labeled with [35S]methionine in the presence of cycloheximide, permitting only labeling of the mitochondrial translation products. The presence of AFG1 did not alter the incorporation into Cox1, Cox2, or Cox3 during the pulse phase of the reaction (Fig. 9A), but AFG1 did accentuate the diminution in labeled Cox1, Cox2, and Cox3 during the chase (Fig. 9, B–D). AFG1 had no effect on the turnover of Cob in the bc1 complex (Fig. 9E) or Var1 (data not shown). Thus, although Afg1 lacks a zinc protease domain, it facilitates the degradation of Cox1, Cox2, and Cox3 in cox11Δ cells. This observation was consistent with the peroxide sensitivity of cox11Δ cells arising from a Cox1 intermediate.

**DISCUSSION**

A subset of mutants that impair assembly of cytochrome c oxidase show a marked sensitivity to hydrogen peroxide in logarithmically growing cells (15, 16). The peroxide sensitivity is disconnected from the respiratory capacity of cells, since cells lacking the mitochondrial genome are resistant to hydrogen peroxide. The mutants exhibiting the peroxide sensitivity appear to generate a transient and low abundant pro-oxidant assembly intermediate consisting of Cox1. Cells lacking Cox1 or its translational activator Pet309 are peroxide-resistant, but cells stalled in CoxO assembly due to an inability to generate the Cu₄ site in Cox1 (cox11Δ mutant), an inability to fold or mature Cox2 (cox20Δ and sco1Δ mutants), or an absence of Cox2 or Cox3 result in varying levels of peroxide sensitivity. Since the peroxide sensitivity of cox11Δ cells is suppressed by the depletion of Cox1 (pet309Δ mutant) or inhibition of mitochondrial protein synthesis, nascent Cox1 chains appear to be deleterious with respect to hydrogen peroxide sensitivity. We postulate that the transient accumulation of unassembled Cox1 results in peroxide sensitivity. Importantly, these studies on hydrogen peroxide sensitivity in CoxO assembly shed insight into the assembly process.

Residual levels of unassembled Cox1 exist in CoxO mutant yeast strains (35), including cox11Δ cells (27). The pro-oxidant is probably a heme A-Cox1 complex. Cells unable to form heme A are peroxide-resistant (cox10Δ cells or cells treated with succinyl acetone), and cells overexpressing COX15 are hypersensitive to hydrogen peroxide. The lack of peroxide sensitivity in shy1Δ cells overexpressing Cox15 is consistent with a reported role of Shy1 in heme A insertion. *Rhodobacter* cells lacking Shy1 are compromised in heme A₁ insertion (31). Heme A₁ is the likely pro-oxidant in Cox1, since only heme A₁ has an open coordination site. Thus, shy1Δ cells would be peroxide-resistant if yeast Shy1 also functions in heme A₁ insertion, and that is what we observe. The candidate heme A₁:Cox1 unassembled pro-oxidant intermediate is probably so transient in nature that...
Heme-Cox1 Intermediate

Insertion of Cox1, Cox2, and Cox3 in the IM requires the translocation of polypeptide segments across the IM by the translocases, OxaI and Cox18 (39–41). The lack of sensitivity to hydrogen peroxide observed in *oxa1Δ* and *cox18Δ* strains suggests that both translocases are important for Cox1 insertion. Both translocases are well established to function on Cox2, but if Cox2 were their only substrate, the clear prediction is that the null mutants would accumulate the pro-oxidant Cox1 intermediate and become peroxide-sensitive.

Recently, a truncated Cox1 polypeptide was reported to form in cells lacking Mss51 (42). This novel 15-kDa polypeptide *mp15* is not the pro-oxidant Cox1 intermediate discussed presently, since the *mp15* polypeptide also forms in *shy1Δ* and *cox17Δ* cells that are resistant to hydrogen peroxide (data not shown).

The peroxide sensitivity of *sco1Δ* cells is suppressed by either wild-type Sco1, mutant Sco1 lacking the ability to bind Cu(I), or human Sco1. Neither the copper-binding mutants nor human Sco1 is competent to restore respiration to *sco1Δ* cells, but they are able to suppress the peroxide sensitivity, again highlighting the disconnection between respiration and peroxide sensitivity. The transmembrane domain of Sco1 is important for peroxide resistance. Like Cox11, Sco1 may have a protein chaperone role in the assembly of CcO. The suppression of peroxide sensitivity of *sco1Δ* cells by mutant Sco1 may arise from a stabilizing effect of Sco1 on Cox2 in occluding the putative solvent channel in Cox1.

No redox function has been confirmed in Sco proteins. Sco proteins in prokaryotes can have a function independent of CuA site formation, since a number of bacterial species lacking CuA, CcO proteins have Sco homologs. *Neisseria* contain a Sco protein that is not essential for maturation of the *cbb3* oxidase (43). *Neisseria* cells lacking this Sco protein are sensitive to visualizing it biochemically would be nearly impossible. Its existence is largely corroborated by genetic evidence.

Heme A insertion occurs in Cox1 prior to the addition of Cox2. An open channel exists in Cox1 through which heme A is probably inserted from the IMS side of the IM (Fig. 4A). The channel is sterically blocked upon the insertion of Cox2 (Fig. 4B). The globular domain of Cox2 packs onto Cox1, occluding the accessible channel. CuB site formation in Cox1 occurs either in synchrony or sequentially with heme A insertion. Assembly of the CuB site is precluded in *cox11Δ* cells, and these cells are peroxide-sensitive. We propose that the globular domain of Cox11 projecting into the IMS is able to transiently occlude the Cox1 channel, thereby yielding peroxide resistance (Fig. 4C). This putative Cox11-Cox1 complex is expected to mediate Cu(I) transfer to the Cox1 CuB site through ligand exchange reactions. However, the ability of Cox11 to mediate peroxide resistance is unrelated to the ability of Cox11 to bind Cu(I). Cox11 may bind and stabilize Cox1 through transient interactions involving both the C-terminal globular domain and its transmembrane domain. We show presently that the Cox11 transmembrane domain is important for the ability of Cox11 to efficiently confer peroxide resistance. A transient interaction of Cox11 with Cox1 may stabilize a conformer of Cox1 that has less solvent accessibility to the heme A3.

Peroxide sensitivity arising from a transient accumulation of heme A3-Cox1 is consistent with the observed peroxide sensitivity of *sco1Δ*, *cox20Δ*, and *cox23Δ* cells. The lack of Sco1 or Cox2 may destabilize Cox2 sufficiently that it fails to stably interact with Cox1, leading to peroxide sensitivity (Fig. 4C). Whereas the lack of Cox2 may be expected to result in accumulation of the pro-oxidant Cox1 intermediate, only modest peroxide sensitivity is observed in *cox23Δ* cells, and *pet111Δ* cells are peroxide-resistant. Cells containing mutant alleles of COX2 with mutations in the 5′-untranslated region showed the expected diminution in Cox2 nascent chains but also reduced Cox1 nascent chains (36). Likewise, cells lacking Pet111 show the expected loss of Cox2 translation and also diminution in Cox1 translation (37). We confirmed that *pet111Δ* cells have attenuated Cox1 translation (data not shown). Furthermore, overexpression of Pet111 results in a diminution in translation of Cox1 (38), suggesting that translational activators function in a balanced manner. Thus, the limited peroxide sensitivity observed in *cox2Δ* and *pet111Δ* cells probably arises from reduced levels of the Cox1 intermediate.

Growth susceptibility to hydrogen peroxide may be a useful assay to assess Cox1 translation under certain conditions when CcO assembly is stalled from defects arising at the IM/IMS interface.

**FIGURE 9.** Overexpression of AFG1 in *cox11Δ* cells increases degradation of newly synthesized CcO subunits. Wild-type (WT), *cox11Δ*, or *cox11Δ*+AFG1 cells were radiolabeled with [35S]methionine at 30 °C for 30 min in the presence of cycloheximide. Reaction has been stopped by the addition of an excess of unlabeled methionine. A, labeling of Cox1, Cox2, and Cox3 and Cob1 in wild-type, *cox11Δ*, or *cox11Δ*+AFG1 cells overexpressing AFG1 is shown during the pulse phase. The actual labeling of Cox1 in *cox11Δ* cells was only 67% of that of wild-type cells. B–E, each reaction mixture was split into four parts that were incubated at 37 °C for 0, 10, 30, and 60 min, respectively. Cells were trichloroacetic acid-precipitated and subjected to SDS-PAGE and autoradiography. Degradation kinetics of newly synthesized Cox1 (B), Cox2 (C), Cox3 (D), and Cob1 (E) proteins was evaluated by densitometry. Degradation is assessed as a percentage of [35S] radioactivity in each band during the chase to the pulse time.
Heme-Cox1 Intermediate

paraoquat (43). If the deletion strain accumulates a heme B intermediate of the cbb₃ oxidase, a similar mechanism may confer oxidative stress.

The mitochondrial Afg1 ATPase was found to be an efficient suppressor of the peroxide sensitivity of CcO assembly mutants. Afg1 was an inefficient suppressor of the peroxide sensitivity of ccp1Δ and unable to suppress ctt1Δ cells, suggesting some specificity. Although Afg1 lacks the zinc protease domain of the AAA proteases of the mitochondria (Yme1 and Yta10/Yta12), it does mediate the degradation of unassembled Cox1, Cox2, and Cox3 polypeptides in Cox1ΔΔ cells. Afg1 localizes to the IM and may collaborate with a protease(s) for the extrusion and degradation of unassembled subunits. The mp15 Cox1 truncate is degraded by the Yta10/Yta12 protease (42), suggesting that Yta10/Yta12 may mediate the degradation stimulated by Afg1.

Afg1 is a highly conserved eukaryotic protein with a robust human homolog (supplemental Fig. 2). The similarity of Afg1 with the Cdc48 (p97) suggests that Afg1 forms a homohexameric ring (44, 45). Yeast Cdc48 functions as an unfolding chaperone for the degradation of endoplasmic reticulum membrane proteins by the proteasome. The mammalian p97 stimulates the degradation of polytopic membrane proteins through extraction of the protein from the endoplasmic reticulum membrane (46). Likewise, Afg1 may function in the extraction of mitochondrial IM proteins, including Cox1, and subsequent presentation to a protease(s). Cdc48 interacts with a number of adapter proteins that modulate its function. Afg1 may also interact with adapter proteins as well as the actual protease. Future studies will attempt to identify interacting partners of Afg1.

REFERENCES

1. Khalimonchuk, O., and Rodel, G. (2005) *Mitochondrion* 5, 363–388
2. Cobine, P. A., Pierrel, F., and Winge, D. R. (2006) *Biochim. Biophys. Acta* 1763, 759–772
3. Fontanesi, F., Soto, I. C., Horn, D., and Barrientos, A. (2006) *Am. J. Physiol. Cell Physiol.* 291, C1129–C1147
4. Horng, Y. C., Cobine, P. A., Maxfield, A. B., Horng, Y.-C., and Winge, D. R. (2005) *J. Biol. Chem.* 280, 22664–22669
5. Horng, Y.-C., Leary, S. C., Cobine, P. A., Young, F. B. J., George, G. N., Shoubridge, E. A., and Winge, D. R. (2005) *J. Biol. Chem.* 280, 34113–34122
6. Balatri, E., Banci, L., Bertini, I., Cantini, F., and Cioffi-Baffoni, S. (2003) *J. Biol. Chem.* 278, 223–233
7. Williams, J. C., George, G. N., and Winge, D. R. (2005) *Curr. Genet.* 47, 223–233
8. Balatinecz, J., and Pumplin, T. (2005) *Biochim. Biophys. Acta* 1763, 759–772
9. Fontanesi, F., Soto, I. C., Horn, D., and Barrientos, A. (2006) *Am. J. Physiol. Cell Physiol.* 291, C1129–C1147
10. Horng, Y. C., Cobine, P. A., Maxfield, A. B., Carr, H. S., and Winge, D. R. (2004) *J. Biol. Chem.* 279, 35334–35340
11. Khalimonchuk, O., Ott, M., Funes, S., Ostermann, K., Rodel, G., and Herrmann, J. M. (2006) *Eukaryot. Cell* 5, 997–1006
12. Barrientos, A., Zambroano, A., and Tzagoloff, A. (2004) *EMBO J.* 23, 3472–3482
13. Williams, S. L., Valnot, I., Rustin, P., and Taanman, J.-W. (2004) *J. Biol. Chem.* 279, 7462–7469
14. Barros, M. H., and Tzagoloff, A. (2002) *FEBS Lett.* 516, 119–123
15. Barros, M. H., Nobrega, F. G., and Tzagoloff, A. (2002) *J. Biol. Chem.* 277, 9997–10002
16. Smith, D., Gray, J., Mitchell, L., Antholine, W. E., and Hosler, J. P. (2005) *J. Biol. Chem.* 280, 17652–17656
17. Hansen, J., and Johansen, P. F. (2000) *Mol. Gen. Genet.* 263, 535–542
18. Huh, W. K., Falvo, J. V., Gerke, L. C., Carroll, A. S., Howson, R. W., Weissman, J. S., and O’Shea, E. K. (2003) *Nat. Cell Biol.* 5, 742–749
19. Carr, H. S., Maxfield, A. B., Horng, Y.-C., and Winge, D. R. (2005) *Nat. Cell Biol.* 11, 713–717
20. Saracco, S. A., and Fox, T. D. (2002) *Mol. Cell. Biol.* 22, 1122–1131
21. Hell, K., Neupert, W., and Stuart, R. A. (2001) *EMBO J.* 20, 1281–1288
22. Hell, K., Neupert, W., and Stuart, R. A. (2001) *EMBO J.* 20, 1281–1288
23. Barrientos, A., Zambroano, A., and Tzagoloff, A. (2002) *J. Biol. Chem.* 277, 1122–1131
24. Zambroano, A., Fontanesi, F., Solans, A., Leite de Oliveira, R., Fox, T. D., Tzagoloff, A., and Barrientos, A. (2007) *Mol. Biol. Cell* 18, 523–535
25. Seib, K. L., Jennings, M. P., and McEwan, A. G. (2003) *FEBS Lett.* 546, 411–415
26. Elssasser, S., and Finley, D. (2005) *Nat. Cell Biol.* 7, 742–749
27. Halawani, D., and Latterich, M. (2006) *Mol. Cell* 22, 713–717
28. Carlson, E. J., Pitonzo, D., and Skach, W. R. (2006) *EMBO J.* 25, 4557–4566
29. Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., and Yoshikawa, S. (1996) *Science* 272, 1136–1144