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Evidence for a Pro-oxidant Intermediate in the Assembly of Cytochrome Oxidase*[S]

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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 a reduction in either Cox1 expression or heme A formation but exacerbated by either enhanced Cox1 expression or heme A production arising from overexpression of *COX15*. Sco1 and Cox11 are implicated in the formation of the Cu_A and Cu_B sites of CcO, respectively. The respective wild-type genes suppress the peroxide sensitivities of *sco1*Δ and *cox11*Δ cells, but no cross-complementation is seen with noncognate genes. Copper-binding mutant alleles of Sco1 and Cox11 that are nonfunctional in promoting the assembly of CcO are functional in suppressing the peroxide sensitivity of their respective null mutants. Likewise, human Sco1 that is nonfunctional in yeast CcO assembly is able to suppress the peroxide sensitivity of yeast *sco1*Δ cells. Thus, a disconnect exists between the respiratory capacity of cells and hydrogen peroxide sensitivity. Hydrogen peroxide sensitivity of *sco1*Δ and *cox11*Δ 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),[2] 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 Cu_A and Cu_B 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 C*XXX*C 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 Cu_A and Cu_B 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 Cu_A 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, *sco1*Δ yeast cells were observed to be sensitive to hydrogen peroxide (7). In addition, *cox11*Δ cells, but not *cox17*Δ or *cox4*Δ cells, are also peroxide-sensitive (15, 16). The apparent selective peroxide sensitivity of *sco1*Δ and *cox11*Δ cells suggested that Sco1 and Cox11 have a mitochondrial function independent of copper metallation. To elucidate the mechanism of the peroxide sensitivity of *sco1*Δ and *cox11*Δ cells, we surveyed a wide array of yeast mutants and isolated genetic suppressors. We show that the hydrogen peroxide sensitivity of

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2 and Table 1.

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[2] The abbreviations used are: CcO, cytochrome *c* oxidase; IMS, intermembrane space; IM, inner membrane.

*sco1*Δ and *cox11*Δ 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*-C181A, *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

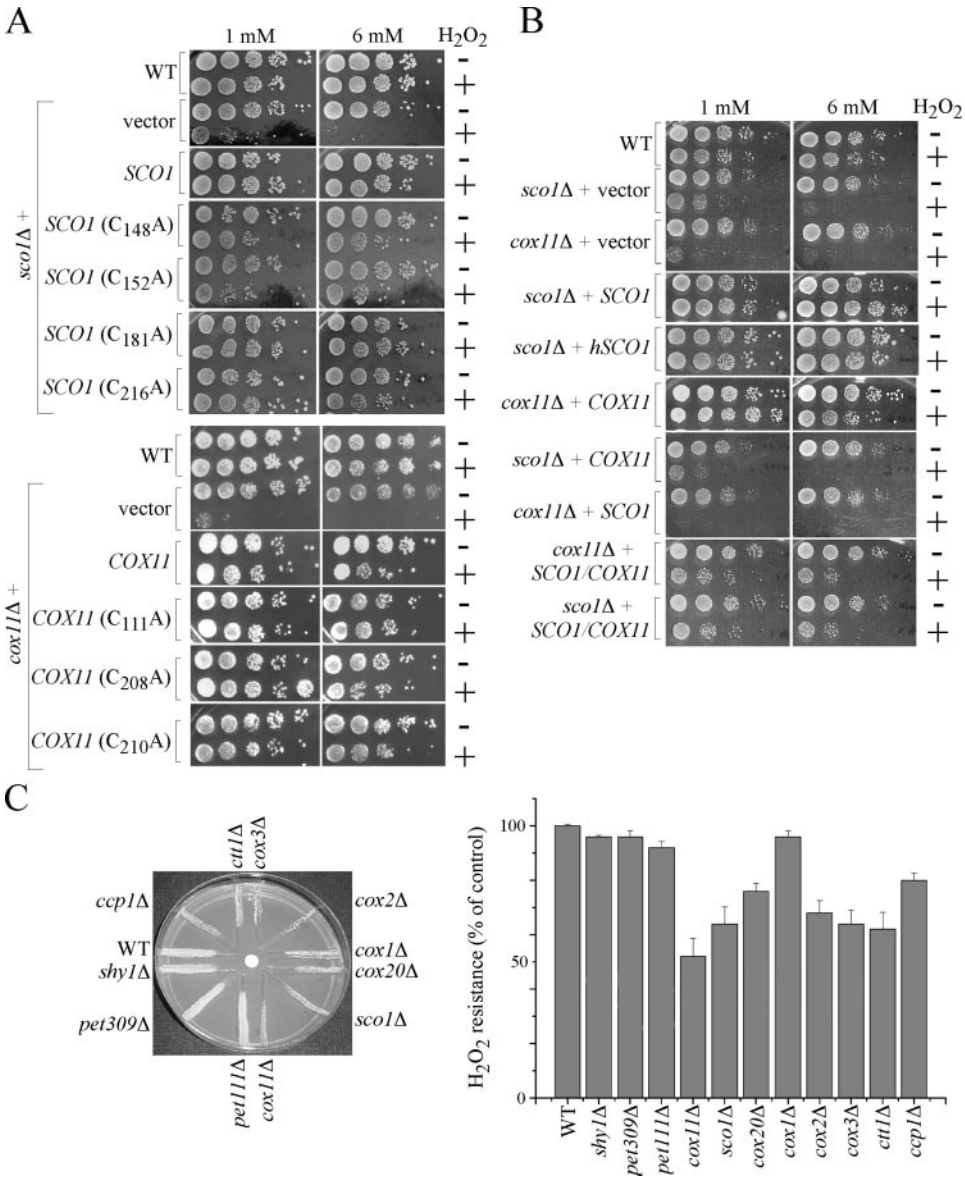


FIGURE 1. **Hydrogen peroxide sensitivity of *sco1*Δ and *cox11*Δ cells.** *A*, *sco1*Δ (*top*) or *cox11*Δ (*bottom*) cells containing an empty vector or plasmid expressing either wild-type (*WT*) *SCO1* or *COX11*, respectively, or mutant forms thereof, unable to bind Cu(I), were grown to midexponential phase and incubated with (+) or without (−) low (1 mM) or high (6 mM) H_2O_2 for 2 h at 30 °C. Respective serial dilutions were spotted onto plates containing 2% glucose and incubated for 36–48 h at 30 °C. *B*, *sco1*Δ or *cox11*Δ cells transformed with an empty vector or plasmid expressing either yeast or human *SCO1*, yeast *COX11*, or chimera consisting of the N-terminal portion of Sco1, including its transmembrane domain, and the C-terminal part of Cox11. Cells were treated and grown as described above. *C*, cells of various respiratory-deficient strains along with the wild-type control were grown on plates containing glucose. A sterile filter disc containing 50% H_2O_2 (3 μl) was placed in the middle of the plate. Lengths of the streaks were measured after 2 days of incubation at 30 °C and compared with the length of wild-type strain, which was set as 100%. The diagram represents the results of three independent experiments.

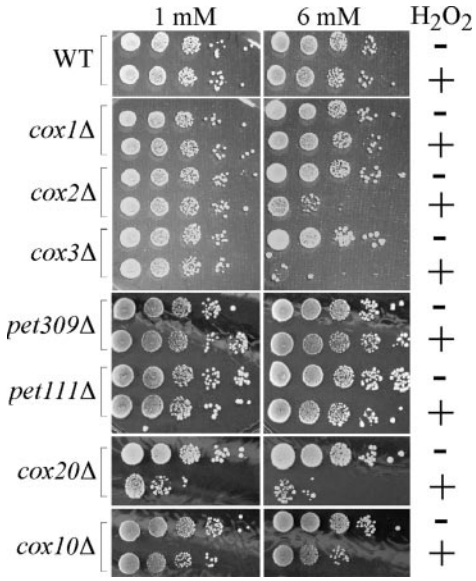


FIGURE 2. **Hydrogen peroxide sensitivity of cells devoid of mitochondrially encoded CcO subunits.** Cells of the strains depleted for mitochondrially coded *COX1*, *COX2*, or *COX3* genes as well as cells lacking translational activators Pet309 or Pet111 and CcO assembly proteins Cox20 or Cox10 were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

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). CcO activities were determined as described (23). Alkaline extraction of proteins was performed as described (24) using 0.1 M Na_2CO_3, 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 H_2O_2 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 [^{35}S]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/ijd-oshell.html).

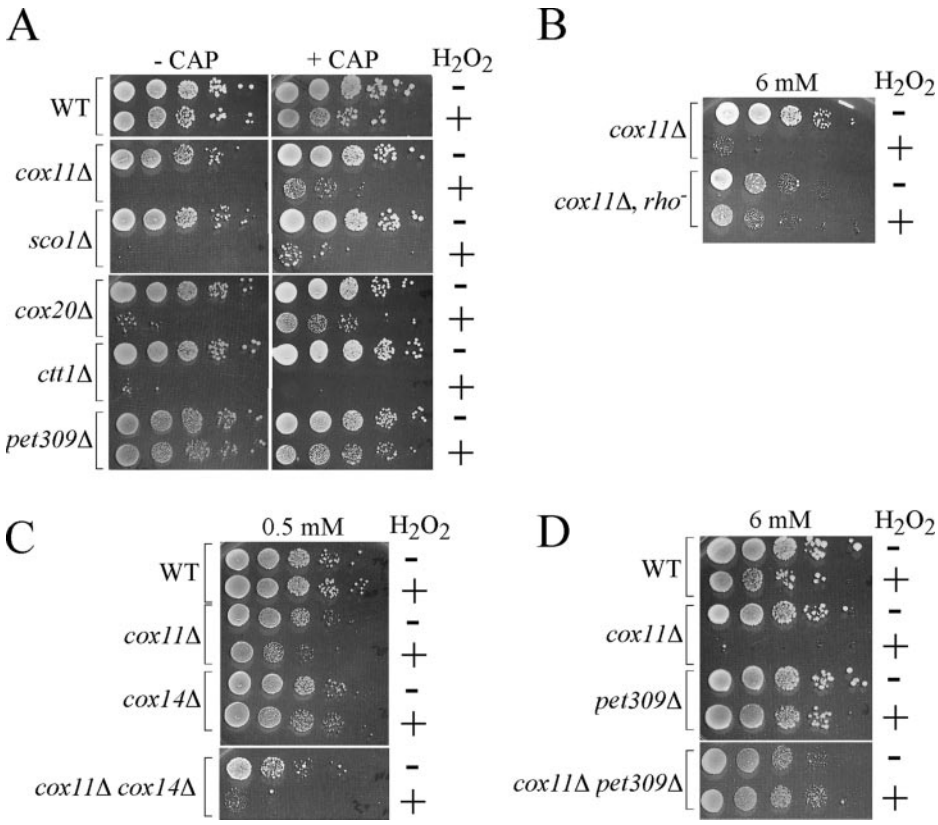


FIGURE 3. **Changes in mitochondrial translation affect H_2O_2 sensitivity of yeast cells.** *A*, *cox11*Δ, *sco1*Δ, and *cox20*Δ cells were grown to midexponential phase and incubated with (+*CAP*) or without (−*CAP*) chloramphenicol (8 mg/ml) prior to incubation with hydrogen peroxide. Then cells were treated and grown as described in the legend to Fig. 1. *B*, *cox11*Δ cells were turned into the *rho*$^-$ state by intensive treatment with ethidium bromide. The obtained cells along with the *cox11*Δ strain with intact mitochondrial genome were incubated with (+) or without (−) 6 mM H_2O_2 and grown as described in the legend to Fig. 1. *C*, *cox11*Δ, *cox14*Δ, and double *cox11*Δ *cox14*Δ mutant cells were incubated with (+) or without (−) 0.5 mM H_2O_2 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*, *cox11*Δ, *pet309*Δ, and double *cox11*Δ *pet309*Δ mutant cells were grown to midexponential phase and incubated with (+) or without (−) 1 or 6 mM H_2O_2 and grown as described in the legend to Fig. 1. *WT*, wild type.

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 *sco1*Δ and *cox11*Δ 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

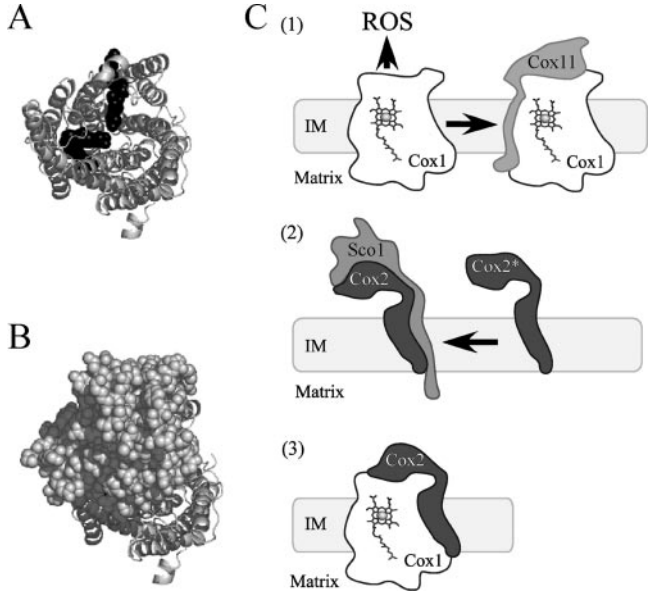


FIGURE 4. **Solvent-exposed channel in the heme A-Cox1 complex.** *A*, view of Cox1 (*light gray ribbon*) taken from the bovine CcO structure (47) and the Cox1 subunit with heme A/A_3 (*black space fill*). The heme moieties are packed in a channel-like cavity, partially exposed to the IMS, and accessible to H_2O_2. The structures were generated by Pymol software. *B*, Cox2 (*space-filled*) packs on Cox1 (*ribbon*) to mask the exposed hemes. The *black space-filled* hemes are no longer visible. *C*, schematic of the pro-oxidant heme A-Cox1 transient intermediate (*scheme 1*). We proposed that Cox11 transiently docks on Cox1 during CuB site formation, thereby sterically blocking the accessible channel in Cox1. *Scheme 2* depicts a model of Cox2 requiring Sco1 to induce a conformational state in Cox2 capable of docking properly on Cox1 shielding the heme A moieties as shown in *scheme 3*. *ROS*, reactive oxygen species.

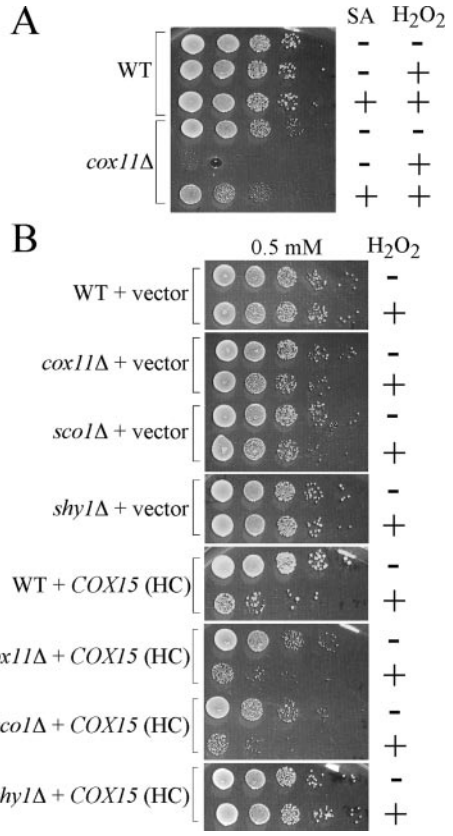


FIGURE 5. **Involvement of heme in the peroxide sensitivity.** *A*, inhibition of heme synthesis reverses the hydrogen peroxide sensitivity of *cox11*Δ cells. Cells were treated with 0.5 mM succinyl acetone (*SA*) in YPD for 3 h prior to incubation with 1 mM H_2O_2. *B*, effect of overexpression of *COX15* (designated *HC* for high copy) on peroxide sensitivity of assembly mutants. Cells were incubated with 0.5 mM H_2O_2 as described in *A*. *WT*, wild type.

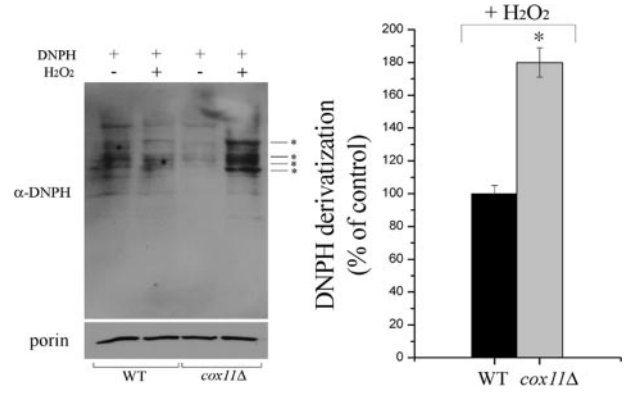


FIGURE 6. **Protein carbonylation levels are increased in Cox11-depleted mitochondria.** Wild-type (*WT*) or *cox11*Δ cells were grown to midexponential phase, and each culture was split into two halves, one left untreated (−) and the other incubated with 1 mM H_2O_2 for 2 h at 30 °C (+). Mitochondria were isolated and derivatized with 2,4-dinitrophenylhydrazine (*DNPH*), separated by SDS-PAGE (10% gel), and analyzed by immunoblotting using anti-2,4-dinitrophenylhydrazine antibodies. Anti-porin antibodies were used to ensure equal loading of samples. The four derivatized bands exhibiting an increase in intensity (marked by *asterisks* in the *left panel*) in *cox11*Δ cells were analyzed by densitometry to quantify the summation of intensities, and this summation was compared with the corresponding gel areas in wild-type cells (*right*).

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 *sco1*Δ cells and *COX11* transformants of *cox11*Δ cells were insensitive to hydrogen peroxide (Fig. 1, *A* and *B*). Surprisingly, Sco1 mutants with substitutions of the two Cu(I)-binding Cys residues, Cys^{148} and Cys^{152}, were also effective in suppressing the peroxide sensitivity of *sco1*Δ cells (Fig. 1*A*). Likewise, Cox11 mutants with substitutions of three Cu(I)-binding Cys residues also were effective in suppressing the peroxide sensitivity of *cox11*Δ cells (Fig. 1*A*). Whereas 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 *sco1*Δ and *cox11*Δ cells is protein-specific. High copy *SCO1* cannot suppress the peroxide sensitivity of *cox11*Δ cells, and overexpression of *COX11* fails to suppress the peroxide sensitivity of *sco1*Δ cells (Fig. 1*B*). 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 *sco1*Δ and *cox11*Δ cells (Fig. 1*B*).

Human *SCO1* fails to complement the respiratory defect of yeast *sco1*Δ cells, yet human *SCO1* does reverse the peroxide sensitivity of *sco1*Δ cells (Fig. 1*B*). 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. 1*C* and 2). Cells lacking Cox2 or Cox3 are resistant to 1 mM peroxide but show sensitivity at 6 mM (Figs. 1*C* and 2). Cells lacking the

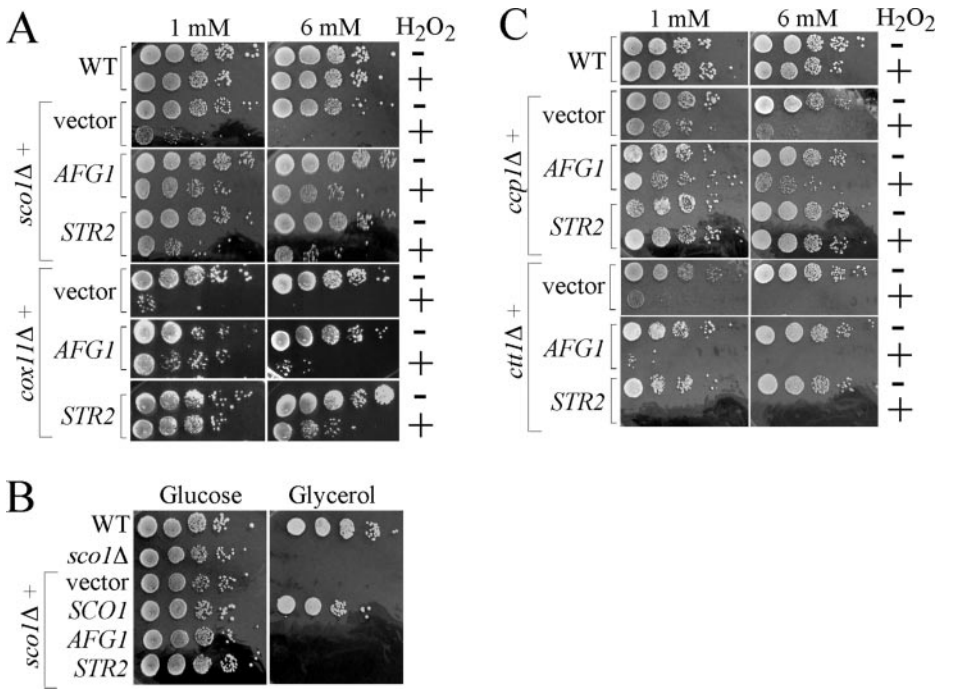


FIGURE 7. **AFG1 and *STR2* are specific high copy suppressors of the H_2O_2 sensitivity of *sco1*Δ and *cox11*Δ mutants.** *A*, *sco1*Δ or *cox11*Δ cells containing empty vector or plasmids expressing high copy suppressor *AFG1* or *STR2* were treated with H_2O_2, and serial dilutions were spotted as described in the legend to Fig. 1. *B*, serial dilutions of *sco1*Δ 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 (*ccp1*Δ) and cytosolic catalase (*ctt1*Δ) transformed with an empty vector or plasmids expressing either *AFG1* or *STR2* were treated with hydrogen peroxide and grown as described above. *WT*, wild type.

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. 1*C* 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 *sco1*Δ, *cox11*Δ, and *cox20*Δ cells is partially reversed by preincubation of those cultures with chloramphenicol for 10 min prior to exposure to peroxide (Fig. 3*A*). The presence of chloramphenicol inhibits mitochondrial protein synthesis. Chloramphenicol failed to suppress the peroxide sensitivity of *ctt1*Δ cells lacking the cytosolic Ctt1 catalase. In addition, the peroxide sensitivity of *cox11*Δ cells is abrogated by conversion of *cox11*Δ cells to a *rho*$^-$ state (Fig. 3*B*).

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. 1*C* and 2). To substantiate the role of Cox1 in the peroxide sensitivity, *COX14* was deleted, creating the *cox11*Δ*cox14*Δ 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 H_2O_2 (Fig. 3*C*). The role of Cox1 was substantiated by the demonstration that the peroxide sensitivity of *cox11*Δ cells is attenuated by disruption of the Cox1 translational activator Pet309. The *cox11*Δ*pet309*Δ double mutant was peroxide-resistant, consistent with Cox1 being a component of the pro-oxidant (Fig. 3*D*).

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 A_3 moiety in Cox1. Heme A_3 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. 4*A*). The peroxide sensitivity of *cox11*Δ cells may arise from the transient accumulation of a heme A-Cox1 intermediate (Fig. 4*C*, *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 *cox11*Δ cells should be reversed if heme A formation is blocked and exacerbated if heme A production is increased. The inhibition of heme synthesis in *cox11*Δ cells by the addition of 0.5 mM succinyl acetone abrogated the sensitivity to 1 mM hydrogen peroxide (Fig. 5*A*). 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 *cox11*Δ and *sco1*Δ cells (Fig. 5*B*). A growth defect was observed with 0.5 mM H_2O_2 in the overexpressor cells. Overexpression of *COX15* also induced mild sensitivity in wild-type cells to 0.5 mM H_2O_2 but curiously no sensitivity in *shy1*Δ cells. The lack of peroxide sensitivity in *shy1*Δ cells is consistent with a defect in heme A_3 insertion in these cells. *Rhodobacter* cells lacking ShyI are compromised in heme A_3 insertion (31).

The accumulation of a heme A-Cox1 pro-oxidant intermediate in *cox11*Δ cells may result in generalized reactive oxygen damage. Wild-type and *cox11*Δ cells were cultured in the presence of 1 mM H_2O_2 for 2 h at 30 °C for isolation of mitochondria. The accumulation of carbonylated proteins was assessed by immunoblotting after dinitrophenyl hydrazone derivatization. *cox11*Δ cells showed nearly 2-fold enhancement in carbonylation, consistent with enhanced reactive oxygen sensitivity (Fig. 6).

Multicopy suppressors of the peroxide sensitivity of *cox11*Δ 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

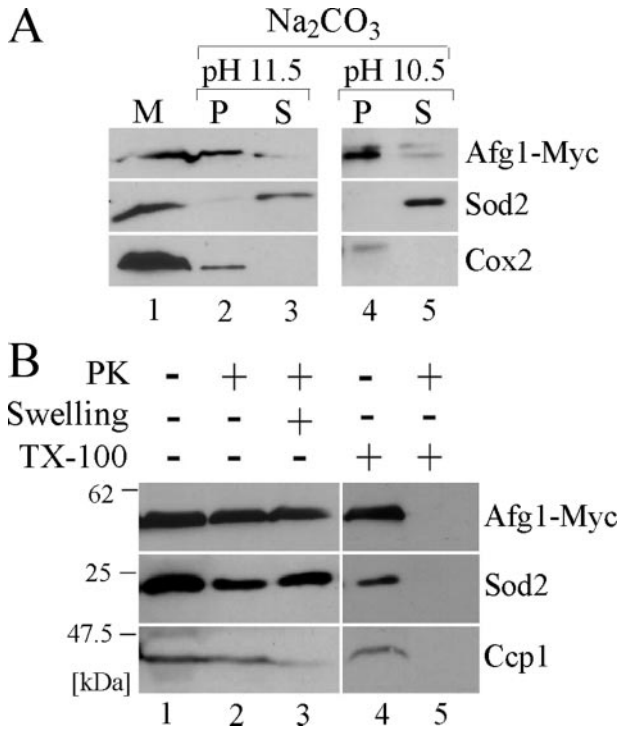


FIGURE 8. **Inner mitochondrial membrane localization of Afg1.** *A*, isolated mitochondria of a strain expressing C-terminally Myc-tagged Afg1 (*M*, *lane 1*) were treated with 0.1 M Na_2CO_3 and fractionated by high speed centrifugation. Obtained pellet (*P*, *lanes 2* and *4*) and supernatant (*S*, *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. 7*A*). Overexpression of *STR2* or *AFG1* was found to suppress the peroxide sensitivity but not the respiratory deficiency of both *sco1*Δ and *cox11*Δ cells (Fig. 7*B*). 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 *ccp1*Δ cells lacking cytochrome *c* peroxidase localized to the IMS. Neither gene suppressed the peroxide sensitivity of cells lacking the cytosolic Ctt1 catalase (Fig. 7*C*). Str2 may have a general role in protecting mitochondria from peroxide stress. Overexpression of Afg1 was also found to suppress the peroxide sensitivity of *cox20*Δ cells, suggesting that it has some specificity to the peroxide sensitivity of CcO assembly mutants. Since Afg1 was selective in its suppression of mutants in the CcO 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 CcO and the bc_1 complex activities are reduced (supplemental Fig. 1). A Myc-tagged Afg1 fusion was found to localize within the mitochondrion 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 *cox11*Δ cells (data not shown).

To address whether Afg1 functions in degradation of Cox1, Cox2, and Cox3, *cox11*Δ cells in the presence or absence of *AFG1* were pulse-labeled with [^{35}S]methionine in the presence of cycloheximide, permitting only labeling of the mitochondrial translation products. The presence of *AFG1* did not alter the ^{35}S incorporation into Cox1, Cox2, or Cox3 during the pulse phase of the reaction (Fig. 9*A*), 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 bc_1 complex (Fig. 9*E*) or Var1 (data not shown). Thus, although Afg1 lacks a zinc protease domain, it facilitates the degradation of Cox1, Cox2, and Cox3 in *cox11*Δ cells. This observation was consistent with the peroxide sensitivity of *cox11*Δ 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 CcO assembly due to an inability to generate the Cu_B site in Cox1 (*cox11*Δ mutant), an inability to fold or mature Cox2 (*cox20*Δ and *sco1*Δ mutants), or an absence of Cox2 or Cox3 result in varying levels of peroxide sensitivity. Since the peroxide sensitivity of *cox11*Δ cells is suppressed by the depletion of Cox1 (*pet309*Δ 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 CcO assembly shed insight into the assembly process.

Residual levels of unassembled Cox1 exist in CcO mutant yeast strains (35), including *cox11*Δ cells (27). The pro-oxidant is probably a heme A-Cox1 complex. Cells unable to form heme A are peroxide-resistant (*cox10*Δ cells or cells treated with succinyl acetone), and cells overexpressing *COX15* are hypersensitive to hydrogen peroxide. The lack of peroxide sensitivity in *shy1*Δ cells overexpressing Cox15 is consistent with a reported role of ShyI in heme A insertion. *Rhodobacter* cells lacking ShyI are compromised in heme A_3 insertion (31). Heme A_3 is the likely pro-oxidant in Cox1, since only heme A_3 has an open coordination site. Thus, *shy1*Δ cells would be peroxide-resistant if yeast ShyI also functions in heme A_3 insertion, and that is what we observe. The candidate heme A_3-Cox1 unassembled pro-oxidant intermediate is probably so transient in nature that

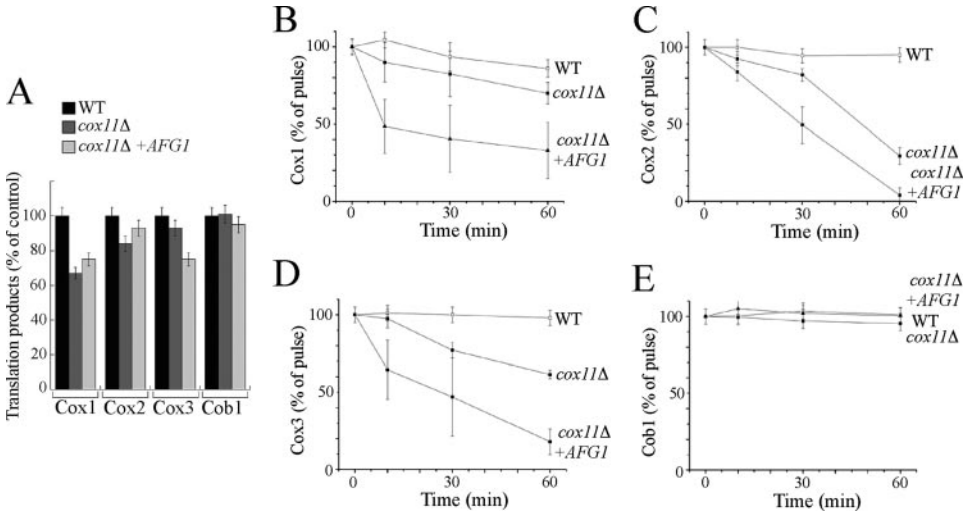


FIGURE 9. **Overexpression of *AFG1* in *cox11*Δ cells increases degradation of newly synthesized CcO subunits.** Wild-type (*WT*), *cox11*Δ, or *cox11*Δ cells overexpressing *AFG1* were radiolabeled with [^{35}S]methionine at 30 °C for 30 min in the presence of cycloheximide. Reaction has been stopped by the addition of an excess of unlabeled methionine. *A*, labeling of Cox1, Cox2, and Cox3 and Cob1 in wild-type, *cox11*Δ, or *cox11*Δ cells overexpressing *AFG1* is shown during the pulse phase. The actual labeling of Cox1 in *cox11*Δ 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 ^{35}S radioactivity in each band during the chase to the pulse time.

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. 4*A*). The channel is sterically blocked upon the insertion of Cox2 (Fig. 4*B*). The globular domain of Cox2 packs onto Cox1, occluding the accessible channel. Cu_B site formation in Cox1 occurs either in synchrony or sequentially with heme A insertion. Assembly of the Cu_B site is precluded in *cox11*Δ 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. 4*C*). This putative Cox11-Cox1 complex is expected to mediate Cu(I) transfer to the Cox1 Cu_B 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 A_3.

Peroxide sensitivity arising from a transient accumulation of heme A_3-Cox1 is consistent with the observed peroxide sensitivity of *sco1*Δ, *cox20*Δ, and *cox2*Δ cells. The lack of Sco1 or Cox20 may destabilize Cox2 sufficiently that it fails to stably interact with Cox1, leading to peroxide sensitivity (Fig. 4*C*). 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 *cox2*Δ cells, and *pet111*Δ 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 *pet111*Δ 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 *cox2*Δ and *pet111*Δ 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.

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 *oxa1*Δ and *cox18*Δ 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 *shy1*Δ and *cox17*Δ cells that are resistant to hydrogen peroxide (data not shown).

The peroxide sensitivity of *sco1*Δ 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 *sco1*Δ 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 *sco1*Δ 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 Cu_A CcO proteins have Sco homologs. *Neisseria* contain a Sco protein that is not essential for maturation of the cbb_3 oxidase (43). *Neisseria* cells lacking this Sco protein are sensitive to

paraquat (43). If the deletion strain accumulates a heme B intermediate of the cbb_3 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 *ccp1*Δ and unable to suppress *ctt1*Δ 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 *cox11*Δ 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

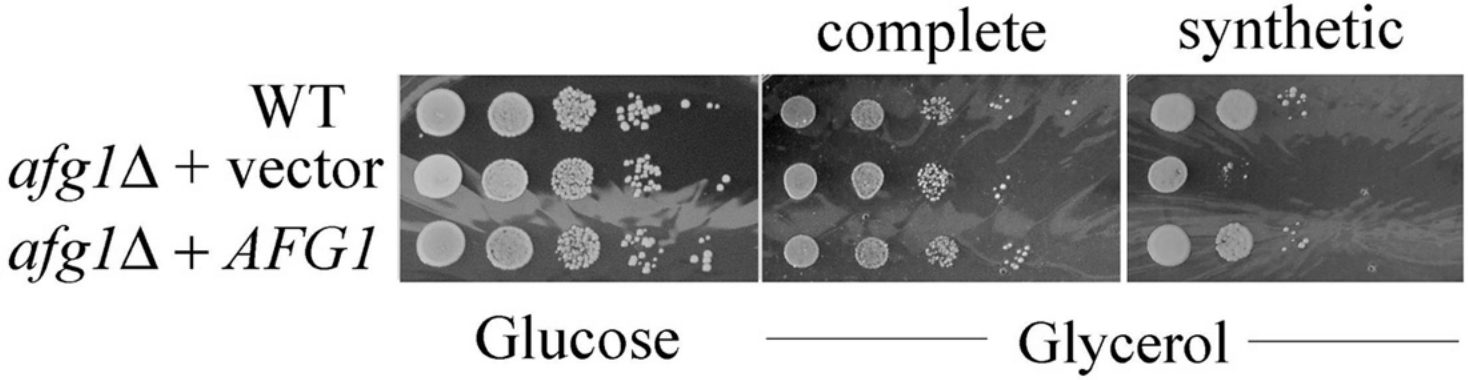
1. Khalimonchuk, O., and Rodel, G. (2005) *Mitochondrion* **5,** 363–388
2. Cobine, P. A., Pierrel, F., and Winge, D. R. (2006) *Biochim. Biophys. Acta* **1763,** 759–772
3. Fontanesi, F., Soto, I. C., Horn, D., and Barrientos, A. (2006) *Am. J. Physiol.* **291,** C1129–C1147
4. Horng, Y. C., Cobine, P. A., Maxfield, A. B., Carr, H. S., and Winge, D. R. (2004) *J. Biol. Chem.* **279,** 35334–35340
5. Khalimonchuk, O., Ostermann, K., and Rodel, G. (2005) *Curr. Genet.* **47,** 223–233
6. Balatri, E., Banci, L., Bertini, I., Cantini, F., and Cioffi-Baffoni, S. (2003) *Structure* **11,** 1431–1443
7. Williams, J. C., Sue, C., Banting, G. S., Yang, H., Glerum, D. M., Hendrickson, W. A., and Schon, E. A. (2005) *J. Biol. Chem.* **280,** 15202–15211
8. Abajian, C., and Rosenzweig, A. C. (2006) *J. Biol. Inorg. Chem.* **11,** 459–466
9. Nittis, T., George, G. N., and Winge, D. R. (2001) *J. Biol. Chem.* **276,** 42520–42526
10. Carr, H. S., George, G. N., and Winge, D. R. (2002) *J. Biol. Chem.* **277,** 31237–31242
11. Carr, H. S., and Winge, D. R. (2003) *Acc. Chem. Res.* **36,** 309–316
12. Ye, Q., Imriskova-Sosova, I., Hill, B. C., and Zia, Z. (2005) *Biochemistry* **44,** 2934–2942
13. Banci, L., Bertini, I., Calderone, V., Ciofi-Baffoni, S., Mangani, S., Marinelli, M., Palumma, P., and Wang, S. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103,** 8595–8600
14. Chinenov, Y. V. (2000) *J. Mol. Med.* **78,** 239–242
15. Pungartnik, C., Kern, M. F., Brendel, M., and Henriques, J. A. P. (1999) *Curr. Genet.* **36,** 124–129
16. Banting, G. S., and Glerum, D. M. (2006) *Eukaryot. Cell* **5,** 568–578
17. Sambrook, J., Fritsch, E. F., and Mantiatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
18. Schiestl, R. H., and Gietz, R. D. (1989) *Curr. Genet.* **16,** 339–346
19. Carr, H. S., Maxfield, A. B., Horng, Y.-C., and Winge, D. R. (2005) *J. Biol. Chem.* **280,** 22664–22669
20. 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
21. Mumberg, D., Muller, R., and Funk, M. (1994) *Nucleic Acids Res.* **22,** 5767–5768
22. Daum, G., Bohni, P. C., and Schatz, G. (1982) *J. Biol. Chem.* **257,** 13028–13033
23. Capaldi, R. A., Marusich, M. F., and Taanman, J. W. (1995) *Methods Enzymol.* **260,** 117–132
24. Fijiki, Y., Hubbard, A. L., Fowler, S., and Lazarow, P. B. (1982) *J. Cell Biol.* **93,** 97–102
25. Khalimonchuk, O., Ott, M., Funes, S., Ostermann, K., Rodel, G., and Herrmann, J. M. (2006) *Eukaryot. Cell* **5,** 997–1006
26. Barrientos, A., Korr, D., and Tzagoloff, A. (2002) *EMBO J.* **21,** 43–52
27. Barrientos, A., Zambrano, A., and Tzagoloff, A. (2004) *EMBO J.* **23,** 3472–3482
28. Williams, S. L., Valnot, I., Rustin, P., and Taanman, J.-W. (2004) *J. Biol. Chem.* **279,** 7462–7469
29. Barros, M. H., and Tzagoloff, A. (2002) *FEBS Lett.* **516,** 119–123
30. Barros, M. H., Nobrega, F. G., and Tzagoloff, A. (2002) *J. Biol. Chem.* **277,** 9997–10002
31. Smith, D., Gray, J., Mitchell, L., Antholine, W. E., and Hosler, J. P. (2005) *J. Biol. Chem.* **280,** 17652–17656
32. Hansen, J., and Johannesen, P. F. (2000) *Mol. Gen. Genet.* **263,** 535–542
33. 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
34. Lee, Y.-J., and Wickner, R. B. (1992) *Yeast* **8,** 787–790
35. Horan, S., Bourges, I., Taanman, J.-W., and Meunier, B. (2005) *Biochem. J.* **390,** 703–708
36. Mulero, J. J., and Fox, T. D. (1993) *Mol. Biol. Cell* **4,** 1327–1335
37. Poutre, C. G., and Fox, T. D. (1987) *Genetics* **115,** 637–647
38. Fiori, A., Perez-Martinez, X., and Fox, T. D. (2005) *Mol. Microbiol.* **56,** 1689–1704
39. Souza, R. L., Green-Willms, N. S., Fox, T. D., Tzagoloff, A., and Nobrega, F. G. (2000) *J. Biol. Chem.* **275,** 14898–14902
40. Hell, K., Neupert, W., and Stuart, R. A. (2001) *EMBO J.* **20,** 1281–1288
41. Saracco, S. A., and Fox, T. D. (2002) *Mol. Biol. Cell* **113,** 1122–1131
42. Zambrano, A., Fontanesi, F., Solans, A., Leite de Oliveira, R., Fox, T. D., Tzagoloff, A., and Barrientos, A. (2007) *Mol. Biol. Cell* **18,** 523–535
43. Seib, K. L., Jennings, M. P., and McEwan, A. G. (2003) *FEBS Lett.* **546,** 411–415
44. Elsasser, S., and Finley, D. (2005) *Nat. Cell Biol.* **7,** 742–749
45. Halawani, D., and Latterich, M. (2006) *Mol. Cell* **22,** 713–717
46. Carlson, E. J., Pitonzo, D., and Skach, W. R. (2006) *EMBO J.* **25,** 4557–4566
47. Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguichi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., and Yoshikawa, S. (1996) *Science* **272,** 1136–1144

Supplement

Fig. s1 Panel A. Serial dilutions of *afg1_* cells harboring an empty vector or plasmid expressing *AFG1* were spotted onto plates with either complete or synthetic media containing 2% glucose or 2% lactate/glycerol as a carbon source. Panel B. Cytochrome *c* oxidase and SDH/bc_1 activities. Activities of the wild-type mitochondria were set as 100%.

Fig. s2 Alignment of Afg1 proteins from different organisms. Highly conserved Walker A motif is underlined and marked by an asterisk.

A


complete
synthetic
WT
afg1Δ + vector
afg1Δ + AFG1
Glucose
Glycerol

B

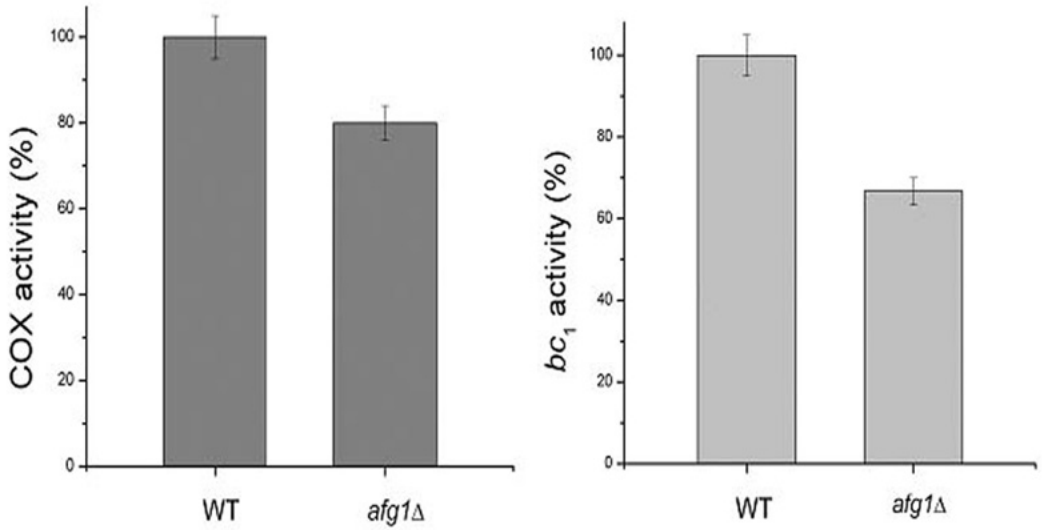

COX activity (%)
100
80
60
40
20
0
WT
afg1Δ
bc1 activity (%)
100
80
60
40
20
0
WT
afg1Δ

Fig. S1

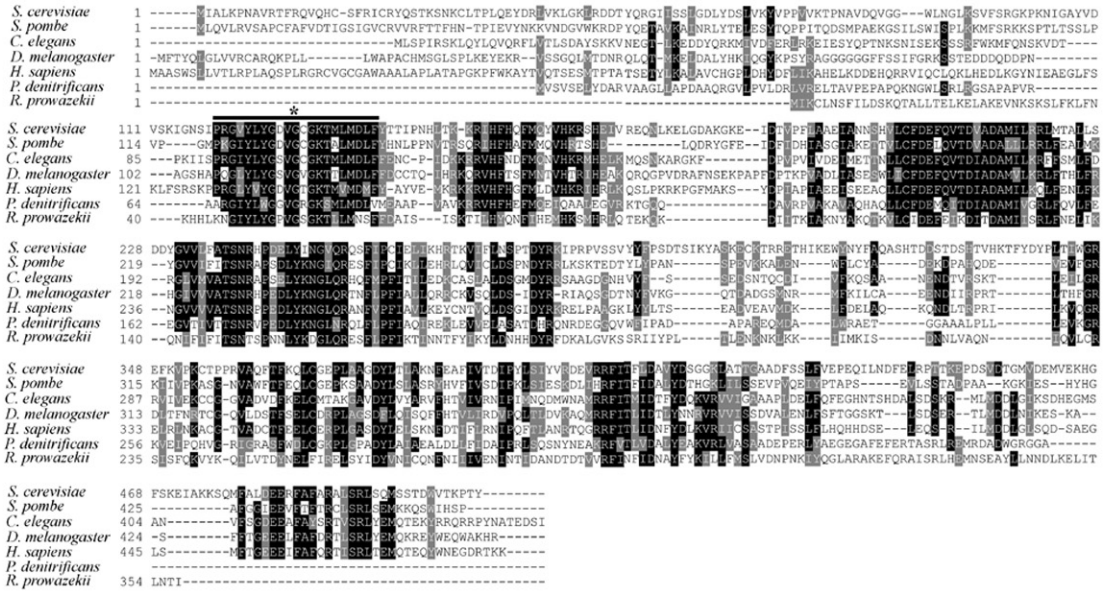

S. cerevisiae
S. pombe
C. elegans
D. melanogaster
H. sapiens
P. denitrificans
R. prowazekii

Fig. s2

Table 1
List of strains used in this study. Resgen signifies strains purchased from Research Genetics.

Strain	Allele	Source
WT	BY4741 *MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rho*$^+$	Resgen
WT	W303 *MAT*a *ura3-1 ade2-1 trp1-1 his3-11,15 leu2-3,112 rho*$^+$	
WT	NB80 *MAT*a *lys2 arg8::hisG ura3-52 leu2-3,112 his3-ΔHindIII rho*$^+$	(1)
*cox11*Δ	W303 *MAT*a *ura3-1 ade2-1 trp1-1 his3-11,15 leu2-3,112 cox11::HIS3 rho*$^+$	(2)
*cox11*Δ	BY4741 *MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 cox11::kanMX rho*$^+$	Resgen
*sco1*Δ	W303 *MAT*a *ura3-1 ade2-1 trp1-1 his3-11,15 leu2-3,112 sco1::URA3 rho*$^+$	(3)
*sco1*Δ	BY4741 *MAT*a his3*Δ*1 leu2*Δ0* met15*Δ*0 ura3*Δ*0 *sco1*:: *kanMX4 rho*$^+$	Resgen
*cox1*Δ	NB80 *MAT*a *lys2 arg8::hisG ura3-52 leu2-3,112 his3-ΔHindIII cox1::ARG8m rho*$^+$	(4)
*cox2*Δ	NB80 *MAT*a *lys2 arg8::hisG ura3-52 leu2-3,112 his3-ΔHindIII cox2-62 rho*$^+$	(1)
*cox3*Δ	NB40 *MAT*a *arg8::URA3 ura3Δ0 leu2-3,112 his4-519 cox3::ARG8m rho*$^+$	(5)
*pet309*Δ	BY4741 *MAT*a his3*Δ*1 leu2*Δ0* met15*Δ*0 ura3*Δ*0 *pet309*:: *kanMX4 rho*$^+$	Resgen
*pet111*Δ	BY4741 *MAT*a his3*Δ*1 leu2*Δ0* met15*Δ*0 ura3*Δ*0 *pet111*:: *kanMX4 rho*$^+$	Resgen
*cox10*Δ	W303 *MAT*a *ura3-1 ade2-1 trp1-1 his3-11,15 leu2-3,112 cox10::HIS3 rho*$^+$	(6)
*cox10*Δ	BY4741 *MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 cox10::kanMX rho*$^+$	Resgen
*cox20*Δ	BY4741 *MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 cox20::kanMX rho*$^+$	Resgen
*ccp1*Δ	BY4741 *MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 ccp1::kanMX rho*$^+$	Resgen
*ctt1*Δ	BY4741 *MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 ctt1::kanMX rho*$^+$	Resgen
*afg1*Δ	BY4741 *MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 afg1::kanMX rho*$^+$	Resgen
*cox14*Δ	BY4741 *MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 cox14::kanMX rho*$^+$	Resgen
*cox11*Δ *cox14*Δ	BY4741 *MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 cox11::kanMX cox14::CaURA3 rho*$^+$	This study
*cox11*Δ *pet309*Δ	BY4741 *MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 cox11::kanMX pet309::CaURA3 rho*$^+$	This study
*shy1*Δ	W303 *MAT*a *ura3-1 ade2-1 trp1-1 his3-11,15 leu2-3,112 shy1::URA3 rho*$^+$	(7)
*shy1*Δ	BY4741 *MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 shy1::kanMX rho*$^+$	Resgen

*cox18*Δ	BY4741 *MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 cox18::kanMX rho*$^+$	Resgen
*oxa1*Δ	BY4741 *MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 oxa1::kanMX rho*$^+$	Resgen

1. Bonnefoy, N. and Fox, T.D. (2000) In vivo analysis of mutated initiation codons in the mitochondrial COX2 gene of *Saccharomyces cerevisiae* fused to the reporter gene ARG8m reveals lack of downstream initiation. *Mol. Gen. Genet.*, **262**, 1036-1046.
2. Tzagoloff, A., Capitanio, N., Nobrega, M.P. and Gatti, D. (1990) Cytochrome oxidase assembly in yeast requires the product of COX11, a homolog of the *P. denitrificans* protein encoded by ORF3. *EMBO J.*, **9**, 2759-2764.
3. Glerum, D.M., Shtanko, A. and Tzagoloff, A. (1996) *SCO1* and *SCO2* Act as High Copy Suppressors of a Mitochondrial Copper Recruitment Defect in *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **271**, 20531-20535.
4. Perez-Martinez, X., Broadley, S.A. and Fox, T.D. (2003) Mss51p promotes mitochondrial Cox1p synthesis and interacts with newly synthesized Cox1p. *EMBO J.*, **22**, 5951-5961.
5. Steele, D.R., Butler, C.A. and Fox, T.D. (1996) Expression of a recoded nuclear gene inserted into yeast mitochondrial DNA is limited by mRNA-specific translational activation. *Proc. Natl. Acad. Sci. USA*, **93**, 5253-5257.
6. Nobrega, M.P., Nobrega, F.G. and Tzagoloff, A. (1990) COX10 codes for a protein homologous to the ORF1 product of *Paracoccus denitrificans* and is required for the synthesis of yeast cytochrome oxidase. *J. Biol. Chem.*, **265**, 14220-14226.
7. Barrientos, A., Korr, D. and Tzagoloff, A. (2002) Shy1p is necessary for full expression of mitochondrial COX1 in the yeast model of Leigh's syndrome. *EMBO J.*, **21**, 43-52.