Absence of the Mitochondrial AAA Protease Yme1p Restores F_0-ATPase Subunit Accumulation in an *oxa*1 Deletion Mutant of *Saccharomyces cerevisiae*

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Absence of the Mitochondrial AAA Protease Yme1p Restores F_0-ATPase Subunit Accumulation in an *oxa1* Deletion Mutant of *Saccharomyces cerevisiae**

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The nuclear gene *OXA1* encodes a protein located within the mitochondrial inner membrane that is required for the biogenesis of both cytochrome *c* oxidase (Cox) and ATPase. In the absence of Oxa1p, the translocation of the mitochondrially encoded subunit Cox2p to the intermembrane space (also referred to as export) is prevented, and it has been proposed that Oxa1p could be a component of a general mitochondrial export machinery. We have examined the role of Oxa1p in light of its relationships with two mitochondrial proteases, the matrix protease Afg3p-Rca1p and the intermembrane space protease Yme1p, by analyzing the assembly and activity of the Cox and ATPase complexes in Δ*oxa1*, Δ*oxa1*Δ*afg3*, and Δ*oxa1*Δ*yme1* mutants. We show that membrane subunits of both complexes are specifically degraded in the absence of Oxa1p. Neither Afg3p nor Yme1p is responsible for the degradation of Cox subunits. However, the F_0 subunits Atp4p, Atp6p, and Atp17p are stabilized in the Δ*oxa1*Δ*yme1* double mutant, and oligomycin-sensitive ATPase activity is restored, showing that the increased stability of the ATPase subunits allows significant translocation and assembly to occur even in the absence of Oxa1p. These results suggest that Oxa1p is not essential for the export of ATPase subunits. In addition, although respiratory function is dispensable in *Saccharomyces cerevisiae*, we show that the simultaneous inactivation of *AFG3* and *YME1* is lethal and that the essential function does not reside in their protease activity.

In mitochondria, biogenesis of the respiratory complexes requires the expression of both the mitochondrial and nuclear genomes (1–3). The mitochondrial genome encodes only a few subunits of the respiratory complexes, whereas the other subunits and a number of proteins, which are not intrinsic components of the complexes but are required for their biogenesis, are nuclearly encoded.

The *Saccharomyces cerevisiae* nuclear gene *OXA1* encodes such an assembly-assisting factor that is required for the biogenesis of both cytochrome *c* oxidase (Cox)[1] and ATPase (4, 5). Oxa1p presents five hydrophobic segments and is located within the mitochondrial inner membrane (6–8). In the absence of Oxa1p, oligomycin-sensitive ATPase activity is significantly decreased, and Cox activity is totally abolished. On non-denaturing gels, the Cox complex of Δ*oxa1* strains displays a higher mobility probably due to the lack of accumulation of the mitochondrially encoded subunits (9, 10). In addition, *OXA1* inactivation prevents the N-terminal maturation of the precursor of Cox2p (pre-Cox2p) (11) and affects its translocation (export) from the matrix to the intermembrane space (12, 13). Defective insertion of several chimeric proteins in the inner membrane has also been observed in *oxa1* mutants, and it has been proposed that Oxa1p could be a component of a general mitochondrial export machinery (14). However, the fact that the absence of Oxa1p is compensated by point mutations in the cytochrome c_1 gene is difficult to conciliate with a unique role for Oxa1p as a mitochondrial channel (10).

Two mitochondrial proteases (Afg3p-Rca1p and Yme1p) are also involved in respiratory complex assembly in yeast (15–18). The hetero-oligomeric complex Afg3p-Rca1p acts on the matrix side of the inner membrane, whereas Yme1p is active in the intermembrane space (19, 20). Afg3p-Rca1p appears to be involved in the degradation of Cox and ATPase subunits (21), and Yme1p in the degradation of Cox2p (22–24). In addition to their proteolytic activity, these proteases seem to display a chaperone-like activity (25–28), and it has been shown that the overexpression of *OXA1* can partially compensate for the inactivation of *AFG3* (29).

Finally, Oxa1p as well as both protease complexes appear to be conserved through evolution, showing the importance of their function. The human, *Arabidopsis*, and *Schizosaccharomyces pombe OXA1* genes have been identified using functional complementation of a yeast *oxa1* mutation (30–32). Human cDNAs encoding proteins highly related to Afg3p-Rca1p have also been described, and one of them encodes paraplegin, which, when mutated, is responsible for a hereditary spastic paraplegia (33, 34).

In this work, we have studied the relationships between Oxa1p and the two inner membrane AAA (ATPases associated with diverse cellular activities) proteases by analyzing the assembly and activity of the Cox and ATPase complexes in Δ*oxa1*, Δ*oxa1*Δ*afg3*, and Δ*oxa1*Δ*yme1* mutants. We show that membrane subunits of both complexes, whether mitochondrially or nuclearly encoded, are rapidly degraded in the absence of Oxa1p. Interestingly, neither Afg3p nor Yme1p is responsible for the degradation of Cox subunits, whereas F_0-ATPase subunits are stabilized in the absence of Yme1p. The increased stability of the ATPase subunits in the Δ*oxa1*Δ*yme1* double mutant allows significant assembly to occur since oligomycin-

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[1] The abbreviation used is: Cox, cytochrome *c* oxidase.

sensitive ATPase activity is restored. Altogether, our results suggest that Oxa1p is not essential for the export of ATPase subunits. In addition, although respiratory function is dispensable in *S. cerevisiae*, we show that the simultaneous inactivation of the *AFG3* and *YME1* genes is lethal.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—Yeast genetic methods were previously described (35). All strains are isonuclear to the wild-type strain CW04 (α *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*), except for the mating type, *OXA1*, *AFG3*, and *YME1* loci. CW04 and NBT1 (α *oxa1::LEU2*) were previously described (4). AFG3-2 (a *afg3::TRP1*) exhibits the Yepafg3-2 plasmid carrying the *URA3* gene and the *afg3-E559Q* allele (21, 26). PHT7 (α *yme1::URA3*) and PHT9 (α *yme1::Kan^R*) were constructed by inactivating the *YME1* gene using a *URA3* cassette (15) or a *Kan^R* cassette (36). F01-6B (α *afg3::HIS3*) carries the *afg3::HIS3* inactivation (25). The four double mutants were constructed either by gene inactivation (PHT8, α *oxa1::LEU2 yme1::URA3*) or by crosses, sporulation, and microdissection (F01-6C, a *oxa1::LEU2 afg3::HIS3*; RF0-7C, α *oxa1::LEU2 afg3::HIS3*; and YA1-2C, α *afg3::TRP1 yme1:: Kan^R*). Strains RF0-7C and YA1-2C carry the Yepafg3-2 plasmid.

Purification and Extraction of Mitochondria—Purification of mitochondria and carbonate extraction were performed as described (6), except that the following protease inhibitors were added to each buffer: phenylmethylsulfonyl fluoride (1 mM), pepstatin (1 μg/ml), chymostatin (10 μg/ml), antipain (10 μg/ml), and leupeptin (10 μg/ml). The mitochondrial protein concentration was determined using the Bio-Rad assay.

In Vivo Labeling of the Mitochondrial Translation Products and Whole Cell Extracts—Yeast cells were grown on medium containing 1% yeast extract, 1% Bacto-peptone, 2% galactose, 0.1% glucose, and 20 μg/ml adenine and harvested at mid-exponential phase. *In vivo* labeling of the mitochondrial translation products was performed essentially as described (37). Cells (1.8×10^8) were harvested and resuspended in labeling medium (40 mM K_2HPO_4 (pH 7.4) and 2% galactose). They were labeled for 20 min at 30 °C with [^{35}S]methionine (130 μCi/ml) in the presence of cycloheximide (150 μg/ml) to inhibit cytosolic protein synthesis. The reaction was stopped by addition of methionine (10 mM final concentration). Total cell proteins were extracted by alkaline lysis (38).

Extraction of the F_1 Sector and F_0 Subunits—F_1 was released from mitochondrial membranes as described previously (39), except that protease inhibitors were added to the extraction buffer as in the carbonate extraction medium (see above). Mitochondria were suspended to a concentration of 5 mg/ml in extraction buffer (0.25 M sucrose, 10 mM Tris-HCl, and 1 mM EDTA (pH 7.5)), and 0.5 volume of chloroform was added. The two phases were vigorously mixed for 30 s and then separated by centrifugation at 400 × *g* for 5 min at 20 °C. The aqueous layer was centrifuged at 100,000 × *g* for 30 min at 20 °C to recover the supernatant, which contained the F_1 sector. F_1-ATPase activity was then measured as described (10). For gel electrophoresis analysis, F_1 was precipitated with $(NH_4)_2SO_4$ added to 60% saturation. Atp9p was extracted by organic solvents (40), except that mitochondrial protein (2 mg) was incubated for 2 h at room temperature with chloroform/methanol (1:1).

Cytochrome c Oxidase and ATPase Activity Measurements on Purified Mitochondria—Cox activity was estimated by measurement of cytochrome *c* oxidation performed spectrophotometrically at 550 nm (10). ATPase activity was measured by colorimetric determination of inorganic phosphate released from ATP (41).

Electrophoresis and Immunoblotting—Electrophoresis and electrophoretic transfers were performed as described previously (10), except for the analysis of the mitochondrial translation products (37). Immunodetection was carried out using the enhanced chemiluminescence method (Pierce). The anti-yeast Cox1p, Cox2p, Cox3p, and Cox4p monoclonal antibodies were purchased from Molecular Probes, Inc., whereas the anti-yeast Cox5p and Cox6ap monoclonal antibodies were a generous gift from R. A. Capaldi (University of Oregon) (42). The anti-yeast Cox6p polyclonal antibody was kindly provided by R. O. Poyton (University of Colorado). The anti-yeast ATPase subunit polyclonal antibodies were prepared against either the purified subunits (Atp1p, Atp2p, Atp3p, Atp4p, Atp5p, Atp16p, and Atp17p) or a synthetic peptide of the N-terminal part (Atp6p).

RESULTS

Oxa1p Is Required for the Accumulation of the Membrane Subunits of Cox and ATPase Complexes—To investigate the subunit composition of the Cox and ATPase complexes in the absence of Oxa1p, mitochondria of the wild-type and *oxa1*-deleted (Δ*oxa1*) strains were treated with carbonate, and the steady-state levels of the main subunits of these two complexes were systematically analyzed in the soluble and membrane fractions by immunoblotting. For the ATPase complex, the two sectors F_1 and F_0 were also separately extracted using different organic solvents, and the subunits were revealed by immunoblotting or silver staining.

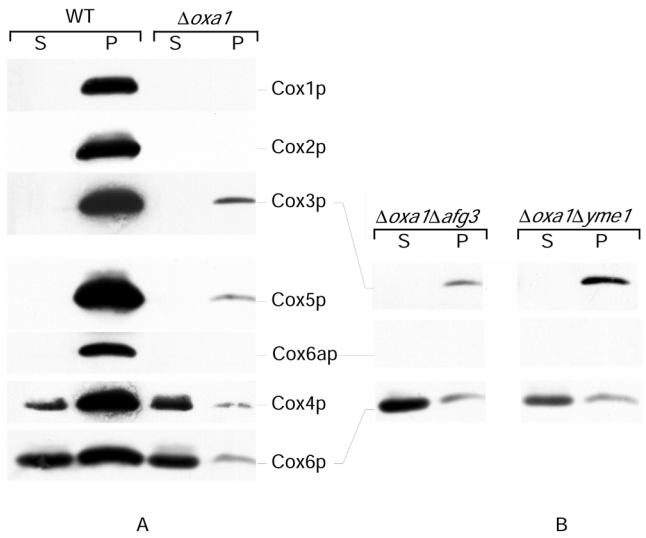

FIG. 1. **Accumulation of Cox subunits in mitochondrial membranes.** 300 μg of mitochondrial proteins were treated with sodium carbonate; the supernatants (*S*; soluble fraction) and pellets (*P*; membrane fraction) were separated on 12 or 15% SDS-polyacrylamide gels and then transferred to nitrocellulose. Western blots were probed with monoclonal antibodies raised against various Cox subunits. Cox1p, Cox2p, and Cox3p are mitochondrially encoded subunits, whereas the others are nuclearly encoded subunits. *A*, wild type (*WT*; CW04) and Δ*oxa1* (NBT1); *B*, Δ*oxa1*Δ*afg3* (F01--6C) and Δ*oxa1*Δ*yme1* (PHT8) strains. See "Experimental Procedures" for complete genotypes.

As previously shown (9, 10), the two membrane subunits Cox1p and Cox2p were not detectable in the absence of Oxa1p (Fig. 1*A*). The three other membrane Cox subunits were also either not detectable (Cox6ap) or poorly detectable (Cox3p and Cox5p) in the Δ*oxa1* mutant. The two subunits Cox4p and Cox6p, distributed between the membrane and the soluble fractions in the wild-type strain, were present mostly in the soluble fraction in the absence of Oxa1p, and their steady-state level was decreased.

The ATPase complex consists of a soluble sector (F_1) and a membrane-anchored sector (F_0) that assembles independently. The ATPase activity of the chloroform-extracted F_1 sector was lowered ~2-fold in the Δ*oxa1* strain compared with the wild-type strain (4815 ± 30 *versus* 9870 ± 450 nmol of ATP hydrolyzed per min/mg of protein). As shown by immunoblotting, the four F_1 subunits Atp1p, Atp2p, Atp3p, and Atp16p and the subunit Atp5p (oligomycin sensitivity-conferring protein subunit) were decreased by ~40% in the Δ*oxa1* extracts (Figs. 2*A* and 3*A*), which is consistent with the activity measurement. On the contrary, the three F_0 membrane subunits Atp6p, Atp17p (subunit f), and Atp4p exhibited dramatically reduced levels in the pellet fraction of the Δ*oxa1* strain (Fig. 2*A*). Organic solvent extraction of the F_0 subunits showed that Atp9p was decreased by ~40% in the Δ*oxa1* strain (Fig. 3*B*).

Altogether, these data clearly establish that there is a selective decrease in the accumulation of the membrane subunits of Cox and F_0-ATPase in the absence of Oxa1p. It was previously shown that the mitochondrially encoded subunits are still synthesized in the absence of Oxa1p (4, 11). Using a PhosphorIm-

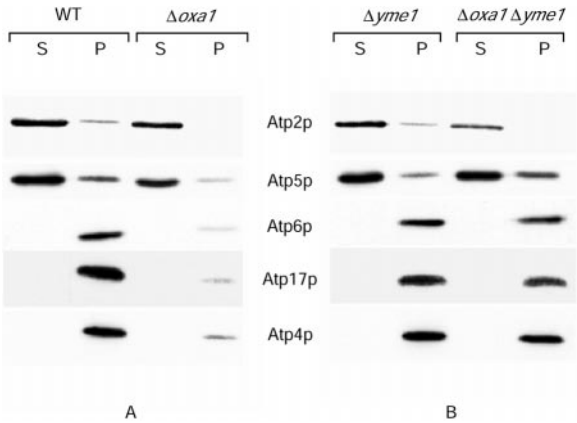

FIG. 2. **Accumulation of ATPase subunits in mitochondrial membranes.** Western blot analysis of mitochondrial proteins was performed as described in the legend to Fig. 1. *S*, supernatant; *P*, pellet. The blots were probed with polyclonal antibodies raised against the F_1 β-subunit (Atp2p), the oligomycin sensitivity-conferring protein subunit (Atp5p), and F_0 subunits (Atp6p, Atp17p, and Atp4p). Atp6p is a mitochondrially encoded subunit, whereas the others are nuclearly encoded. *A*, wild type (*WT*; CW04) and Δ*oxa1* (NBT1); *B*, Δ*yme1* (PHT7) and Δ*oxa1*Δ*yme1* (PHT8).

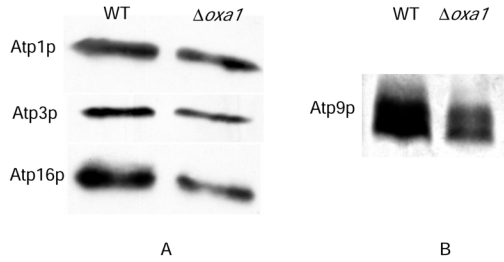

FIG. 3. **Organic solvent extraction of the F_1-ATPase sector and of the F_0 subunit Atp9p.** The F_1 sector and Atp9p were extracted by chloroform and chloroform/methanol, respectively (see "Experimental Procedures"). Proteins were fractionated on 15% SDS-polyacrylamide gels. *A*, F_1 subunits were transferred to nitrocellulose, and the blots were probed with antibodies against Atp1p, Atp3p, and Atp16p. *B*, the Atp9p subunit of F_0 was revealed by silver staining (48). The wild-type (*WT*; CW04) and Δ*oxa1* (NBT1) strains were used.

ager, the synthesis level of the Cox and ATPase mitochondrial subunits was quantified after ^{35}S labeling using the mitoribosomal protein Var1p as an internal control. The synthesis of Cox1p, Cox2p, and Atp6p was diminished by ~50%, whereas the synthesis of Cox3p and Atp9p was not affected in the absence of Oxa1p (data not shown). Thus, the fact that these subunits do not accumulate in the mitochondrial membranes or at a reduced level despite significant synthesis indicates that they are prone to degradation.

The Proteases Afg3p-Rca1p and Yme1p Are Not Responsible for the Degradation of Cox Subunits in the Δoxa1 Strain—To assess the possible roles of the two inner membrane AAA proteases Afg3p-Rca1p and Yme1p in the membrane subunit degradation occurring in the absence of Oxa1p, we inactivated the genes encoding these proteases in a Δ*oxa1* background. The single mutants Δ*afg3*, Δ*rca1*, and Δ*oxa1* are respiratory-deficient, whereas Δ*yme1* exhibits only a thermosensitive respiratory growth. The double mutants Δ*oxa1*Δ*afg3*, Δ*oxa1*Δ*rca1*, and Δ*oxa1*Δ*yme1* were all respiratory-deficient, showing that the lack of protease does not fully compensate for the absence of Oxa1p. The Δ*oxa1* mutant does not accumulate *rho*$^-$ mitochondrial mutants. In the Δ*afg3* and Δ*oxa1*Δ*afg3* strains, the percentage of *rho*$^-$ mutants was ~15% under standard conditions of culture.

As shown in Table I, cytochrome *c* oxidase activity was still defective in the double mutants, and the membrane subunits

TABLE I
Activities of Cox and ATPase complexes

Measurements of cytochrome *c* oxidase and ATPase activities were made on purified mitochondria. The values are an average of measurements obtained from two separate mitochondrial preparations. They are expressed as a percentage of the wild-type (WT) strain with an S.D. of ~10%. The cytochrome *c* oxidase activity of the wild-type strain is 770 nmol of oxidized cytochrome *c*/min/mg of protein. The specificity of cytochrome *c* oxidase activity was tested by addition of 4 μM KCN. The total ATPase activity of the wild-type strain is 1.57 μmol of P_i/min/mg of protein, and the oligomycin-sensitive activity is 1.43 μmol of P_i/min/mg of protein.

Strain	Cytochrome *c* oxidase activity	ATPase activities	
		Total	Oligomycin-sensitive
	%		%
WT (CW04)	100	100	100
Δ*oxa1* (NBT1)	0	88	20
Δ*afg3* (F01–6B)	60	70	3
Δ*oxa1*Δ*afg3* (F01–6C)	0	73	5
Δ*yme1* (PHT7)	62	95	89
Δ*oxa1*Δ*yme1* (PHT8)	0	104	80

Cox1p, Cox2p, Cox3p, Cox5p, and Cox6ap were still degraded (Fig. 1*B* and data not shown). In addition, the extramembrane subunits, Cox4p and Cox6p, also displayed the same pattern as in the Δ*oxa1* single mutant (Fig. 1*A* and data not shown). Similarly, Cox2p remained undetectable in whole cell extracts from Δ*oxa1*Δ*rca1* and Δ*oxa1*Δ*rca1*Δ*afg3* strains (data not shown), suggesting that the accumulation of Cox1p, Cox3p, and Cox4p is also affected since the accumulation of these four subunits is interdependent (43). Thus, the absence of Afg3p-Rca1p or Yme1p does not seem to restore the stability of the Cox subunits.

Since it has been proposed that Afg3p-Rca1p could have a dual protease and chaperone activity (26, 27), the subunit degradation occurring in the Δ*oxa1*Δ*afg3* double mutant could be due to the lack of the chaperone function of Afg3p-Rca1p. The absence of Afg3p-Rca1p leads to defects in respiratory complex assembly (17, 18, 25) that can be complemented by a proteolytically inactive variant of Afg3p in which glutamic acid 559 of the proteolytic site is changed to a glutamine (21, 27). Thus, we have constructed a strain (RF0-7C) with the double inactivation Δ*oxa1*Δ*afg3* and a plasmid carrying the *afg3-E559Q* allele (21). In this strain, the chaperone activity of Afg3p should still be functional, whereas the protease activity of the Afg3-Rca1p complex is abolished. We found that Cox2p remained undetectable in whole cell extracts of RF0-7C, showing that the Cox2p degradation in the Δ*oxa1*Δ*afg3* strain is not due to the absence of the chaperone activity of Afg3p-Rca1p. In conclusion, Afg3p-Rca1p is not responsible for the degradation of membrane subunits of Cox that occurs in the absence of Oxa1p.

F_0-ATPase Membrane Subunits Defective in the Δoxa1 Strain Are Stabilized in the Double Mutant Δoxa1Δyme1—The ATPase activities of the double mutants Δ*oxa1*Δ*afg3* and Δ*oxa1*Δ*yme1* were compared with those of the corresponding wild-type and single mutant strains (Table I). Although the total ATPase activity was not significantly diminished in the various strains, the oligomycin-sensitive ATPase activity was strongly decreased in the Δ*oxa1*, Δ*afg3*, and Δ*oxa1*Δ*afg3* strains. Surprisingly, whereas Yme1p has not to date been reported to have a role in the degradation of ATPase subunits, we found that the inactivation of the *YME1* gene in the Δ*oxa1* strain restored an oligomycin-sensitive ATPase activity, *i.e.* Δ*oxa1*Δ*yme1* reached 80% of the wild-type activity (Table I). Thus, the defect in the formation of the F_1F_0 complex occurring in the Δ*oxa1* mutant was restored in the Δ*oxa1*Δ*yme1* strain since the oligomycin-sensitive ATPase activity reflects a well

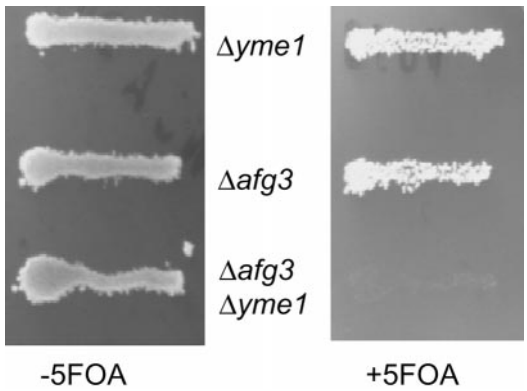

FIG. 4. **Simultaneous inactivation of *AFG3 and YME1* is lethal.** The two single mutants and the double mutant carrying the YepAfg3-2 plasmid were patched on uracil-containing media without (*−5FOA*) or with (*+5FOA*) 1 mg/ml 5-fluoroorotic acid. Cells were incubated for 3 days at 28 °C. The Δ*yme1* (PHT9 + YepAfg3-2), Δ*afg3* (AFG3-2), and Δ*afg3*Δ*yme1* (YA1-2C) strains were used.

assembled F_1F_0 complex. In addition, Western blot analysis showed that the accumulation of the F_0 membrane subunits Atp6p, Atp17p, and Atp4p, which was strongly diminished in the Δ*oxa1* mutant and not affected in the Δ*yme1* mutant, was restored in the double mutant Δ*oxa1*Δ*yme1* (Fig. 2*B*). On the contrary, the accumulation of these subunits was not restored in the Δ*oxa1*Δ*afg3* strain carrying the *afg3-E559Q* plasmid (RF0-7C) (data not shown). Thus, the absence of Yme1p restores the F_0-ATPase subunit accumulation, which is defective in the absence of Oxa1p.

Simultaneous Inactivation of AFG3 and YME1 Is Lethal—Since the Cox subunit stability was restored neither by *YME1* nor by *AFG3-RCA1* single inactivations, it was tempting to test the effect of the double inactivation. To construct the triple mutant Δ*oxa1*Δ*afg3*Δ*yme1*, we crossed the Δ*oxa1*Δ*afg3* double mutant (F01-6C) with the Δ*yme1* strain (PHT7) and dissected the asci. However, no spore carrying the inactivation of both *AFG3* and *YME1* germinated. Similar results were obtained by crossing the Δ*afg3* strain (or Δ*rca1*) with the Δ*yme1* strain, suggesting that the simultaneous absence of Afg3p-Rca1p and Yme1p is lethal whether *OXA1* is deleted or not.

To test if this lethality was specifically due to the absence of the protease function of Afg3p-Rca1p, we crossed the Δ*afg3* strain, which expresses the proteolytically inactive variant *afg3-E559Q* (AFG3-2) on a *URA3* plasmid, with the Δ*yme1* strain (PHT9). After dissection, all the $URA3^+$ spores carrying the plasmid were viable. We then forced the loss of the plasmid by growing the cells on uracil-containing medium supplemented with 5-fluoroorotic acid, which is toxic for uracil prototrophs; thus, cells that lose the plasmid and become uracil auxotrophs are able to grow on 5-fluoroorotic acid. The single mutant strains were able to grow on 5-fluoroorotic acid, but the double mutant Δ*afg3*Δ*yme1* (YA1-2C) was not (Fig. 4), showing that it cannot lose the plasmid. Since the plasmid expresses a proteolytically inactive variant of Afg3p, this shows that the protease activity is not the essential function.

DISCUSSION

In this study, we show that Oxa1p is specifically required for the stability of the membrane subunits of the Cox and ATPase complexes. Such specificity is consistent with the inner membrane location of Oxa1p and with a role in export and/or assembly of the membrane subunits of these two complexes. We then tested whether the unexported and/or unassembled membrane subunits could be the target of the known inner membrane protease complexes Yme1p and Afg3p-Rca1p.

Concerning the Cox subunits, Cox2p is a known substrate of Yme1p (20, 23, 24), and the complex Afg3p-Rca1p has been shown to participate in the degradation of Cox1p and Cox3p (21, 27). Since pre-Cox2p export is blocked in the Δ*oxa1* mutant (11–13) and Yme1p is active in the intermembrane space, it is not surprising that Yme1p does not degrade pre-Cox2p in the Δ*oxa1* mutant. However, the fact that the inactivation of neither *YME1* nor *AFG3* could restore pre-Cox2p or the other Cox subunit stability suggests that either another protease is responsible for the degradation of these subunits or that they can be degraded by Yme1p and Afg3p-Rca1p. We rather favor the first hypothesis since the Cox subunits are still unstable when both Yme1p and Afg3p protease activities are blocked.

It has been shown that the assembly of Cox1p, Cox2p, Cox3p, and Cox4p is interdependent (43). For example, in a *cox2* mutant, the accumulation of the three other subunits is reduced. A specific pre-Cox2p export defect could then explain the general instability of most of the other Cox subunits found in Δ*oxa1* strains. However, the fact that the suppressor mutations that compensate for the absence of Oxa1p fully compensate for the maturation defect of Cox2p but only partially restore Cox activity (10) suggests that Oxa1p is not required only for Cox2p export. Whether the primary function of Oxa1p in Cox biogenesis is linked to export of membrane subunits and/or their assembly still remains an open question, both processes being tightly linked.

As far as the ATPase is concerned, we found that the F_0 membrane subunits are stabilized in the absence of Yme1p. Cross-linking experiments have shown that the subunits Atp4p, Atp6p, and Atp17p display accessible targets in the intermembrane space (41). However, it is difficult to determine whether they all represent true targets of Yme1p or whether only one subunit is degraded by this protease and exerts a protective effect upon the others. Nevertheless, the restoration of oligomycin-sensitive ATPase activity in the Δ*oxa1*Δ*yme1* double mutant shows that the increased stability of the ATPase membrane subunits allows significant translocation and assembly to occur. This result suggests that Oxa1p is not essential for the export of the ATPase subunits.

It has been proposed that the assembly of the three mitochondrially encoded subunits of the F_0 sector (Atp6p, Atp8p, and Atp9p) occurs first and is required for the subsequent assembly of Atp4p and then Atp5p (44–46). We found that Atp6p and Atp4p levels are dramatically reduced in the Δ*oxa1* strain, whereas Atp9p and Atp5p levels are not. For Atp9p, our results can be related to the data showing that Atp9p is associated with the F_1 sector independently of the other F_0 subunits (44). For Atp5p, our data suggest that its accumulation is independent of the formation of the F_0 complex. In accordance with these results, we found that Atp5p was also stable in rho^0 cells (data not shown), which are devoid of mitochondrial DNA and therefore of mitochondrially encoded subunits of F_0.

Finally, we have shown that the simultaneous absence of Afg3p (or Rca1p) and Yme1p is lethal and that viability is restored by the introduction of the proteolytically inactive variant of Afg3p. This suggests that Afg3p-Rca1p and Yme1p have an overlapping essential function that is probably due to the chaperone-like function. Alternatively, the cell viability might require both protease and chaperone activities to be functional. Although Oxa1p partially compensates for the *AFG3* inactivation and could also display a "chaperone-like" function, the lethality of the double inactivation of *AFG3* and *YME1* cannot be cured by overexpressing the *OXA1* gene (data not shown). It has been shown that the growth of Δ*yme1* rho^- cells is severely affected (47). However, the lethality of the double mutant Δ*afg3*Δ*yme1* is probably not simply due to the accumulation of rho^- mutants since the percentage of rho^- mutants is ~15% in

the Δ*afg3* mutant. In *S. cerevisiae*, respiratory function is dispensable, but the integrity of the mitochondrial compartment is essential. Thus, it is tempting to propose that Afg3p-Rca1p and Yme1p would be involved in the biogenesis of both respiratory complexes and other protein complexes controlling mitochondrial compartment integrity, whereas Oxa1p would play a role only in export and assembly of the membrane subunits of respiratory complexes.

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REFERENCES

1. Costanzo, M. C., and Fox, T. D. (1990) *Annu. Rev. Genet.* **24,** 91–113
2. Tzagoloff, A., and Dieckmann, C. L. (1990) *Microbiol. Rev.* **54,** 211–225
3. Grivell, L. A. (1995) *Crit. Rev. Biochem. Mol. Biol.* **30,** 121–164
4. Bonnefoy, N., Chalvet, F., Hamel, P., Slonimski, P. P., and Dujardin, G. (1994) *J. Mol. Biol.* **239,** 201–212
5. Altamura, N., Capitanio, N., Bonnefoy, N., Papa, S., and Dujardin, G. (1996) *FEBS Lett.* **382,** 111–115
6. Kermorgant, M., Bonnefoy, N., and Dujardin, G. (1997) *Curr. Genet.* **31,** 302–307
7. Meyer, W., Bauer, M., and Pratje, E. (1997) *Curr. Genet.* **31,** 401–407
8. Herrmann, J. M., Neupert, W., and Stuart, R. A. (1997) *EMBO J.* **16,** 2217–2226
9. Meyer, W., Bömer, U., and Pratje, E. (1997) *Biol. Chem. Hoppe-Seyler* **378,** 1373–1379
10. Hamel, P., Lemaire, C., Bonnefoy, N., Brivet-Chevillotte, P., and Dujardin, G. (1998) *Genetics* **150,** 601–611
11. Bauer, M., Behrens, M., Esser, K., Michaelis, G., and Pratje, E. (1994) *Mol. Gen. Genet.* **245,** 272–278
12. He, S., and Fox, T. D. (1997) *Mol. Biol. Cell* **8,** 1449–1460
13. Hell, K., Herrmann, J., Pratje, E., Neupert, W., and Stuart, R. A. (1997) *FEBS Lett.* **418,** 367–370
14. Hell, K., Herrmann, J., Pratje, E., Neupert, W., and Stuart, R. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 2250–2255
15. Thorsness, P. E., White, K. H., and Fox, T. D. (1993) *Mol. Cell. Biol.* **13,** 5418–5426
16. Guélin, E., Rep, M., and Grivell, L. A. (1994) *Yeast* **10,** 1389–1394
17. Tauer, R., Mannhaupt, G., Schnall, R., Pajic, A., Langer, T., and Feldmann, H. (1994) *FEBS Lett.* **353,** 197–200
18. Tzagoloff, A., Yue, J., Jang, J., and Paul, M.-F. (1994) *J. Biol. Chem.* **269,** 26144–26151
19. Pajic, A., Tauer, R., Feldmann, H., Neupert, W., and Langer, T. (1994) *FEBS Lett.* **353,** 201–206
20. Leonhard, K., Herrmann, J. M., Stuart, R. A., Mannhaupt, G., Neupert, W., and Langer, T. (1996) *EMBO J.* **15,** 4218–4229
21. Guélin, E., Rep, M., and Grivell, L. A. (1996) *FEBS Lett.* **381,** 42–46
22. Pearce, D. A., and Sherman, F. (1995) *J. Biol. Chem.* **270,** 20879–20882
23. Nakai, T., Yasuhara, T., Fujiki, Y., and Ohashi, A. (1995) *Mol. Cell. Biol.* **15,** 4441–4452
24. Weber, E. R., Hanekamp, T., and Thorsness, P. E. (1996) *Mol. Biol. Cell* **7,** 307–317
25. Paul, M.-F., and Tzagoloff, A. (1995) *FEBS Lett.* **373,** 66–70
26. Rep, M., van Dijl, J. M., Suda, K., Schatz, G., Grivell, L. A., and Suzuki, C. K. (1996) *Science* **274,** 103–106
27. Artl, H., Tauer, R., Feldmann, H., Neupert, W., and Langer, T. (1996) *Cell* **85,** 875–885
28. Leonhard, K., Stiegler, A., Neupert, W., and Langer, T. (1999) *Nature* **398,** 348–351
29. Rep, M., Nooy, J., Guélin, E., and Grivell, L. A. (1996) *Curr. Genet.* **30,** 206–211
30. Bonnefoy, N., Kermorgant, M., Groudinsky, O., Minet, M., Slonimski, P. P., and Dujardin, G. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 11978–11982
31. Hamel, P., Sakamoto, W., Wintz, H., and Dujardin, G. (1997) *Plant J.* **12,** 101–109
32. Bonnefoy, N., Kermorgant, M., Groudinsky, O., and Dujardin, G. (2000) *Mol. Microbiol.* **35,** 1135–1145
33. Casari, G., De Fusco, M., Ciarmatori, S., Zeviani, M., Mora, M., Fernandez, P., De Michele, G., Filla, A., Cocozza, S., Marconi, R., Dürr, A., Fontaine, B., and Ballabio, A. (1998) *Cell* **93,** 973–983
34. Banfi, S., Bassi, M. T., Andolfi, G., Marchitiello, A., Zanotta, S., Ballabio, A., Casari, G., and Franco, B. (1999) *Genomics* **59,** 51–58
35. Dujardin, G., Pajot, P., Groudinsky, O., and Slonimski, P. P. (1980) *Mol. Gen. Genet.* **179,** 469–482
36. Wach, A., Brachat, A., Pohlmann, R., and Philippsen, P. (1994) *Yeast* **10,** 1793–1808
37. van Dyck, L., Neupert, W., and Langer, T. (1998) *Genes Dev.* **12,** 1515–1524
38. Yaffe, M. P. (1991) *Methods Enzymol.* **194,** 627–643
39. Beechey, R. B., Hubbard, S. A., Linnett, P. E., Mitchell, A. D., and Munn, E. A. (1975) *Biochem. J.* **148,** 533–537
40. Paul, M.-F., Velours, J., Arselin de Chateaubodeau, G., Aigle, M., and Guérin, B. (1989) *Eur. J. Biochem.* **185,** 163–171
41. Spannagel, C., Vaillier, J., Chaignepain, S., and Velours, J. (1998) *Biochemistry* **37,** 615–621
42. Taanman, J. W., and Capaldi, R. A. (1993) *J. Biol. Chem.* **268,** 18754–18761
43. Lemaire, C., Robineau, S., and Netter, P. (1998) *Curr. Genet.* **34,** 138–145
44. Hadikusumo, R. G., Meltzer, S., Choo, W. M., Jean-François, M. J. B., Linnane, A. W., and Marzuki, S. (1988) *Biochim. Biophys. Acta* **933,** 212–222
45. Straffon, A. F. L., Prescott, M., Nagley, P., and Devenish, R. J. (1998) *Biochim. Biophys. Acta* **1371,** 157–162
46. Mukhopadhyay, A., Zhou, X., Uh, M., and Mueller, D. M. (1992) *J. Biol. Chem.* **267,** 25690–25696
47. Weber, R. E., Rooks, R. S., Shafer, K. S., Chase, J. W., and Thorsness, P. E. (1995) *Genetics* **140,** 435–442
48. Görg, A., Postel, W., Weser, J., Schiwara, H. W., and Boesken, W. H. (1985) *Sci. Tools* **32,** 5–9