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Translocation and Assembly of Mitochondrially Coded *Saccharomyces cerevisiae* Cytochrome *c* Oxidase Subunit Cox2 by Oxa1 and Yme1 in the Absence of Cox18

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ABSTRACT

Members of the Oxa1/YidC/Alb3 family of protein translocases are essential for assembly of energy-transducing membrane complexes. In *Saccharomyces cerevisiae*, Oxa1 and its paralog, Cox18, are required for assembly of Cox2, a mitochondrially encoded subunit of cytochrome *c* oxidase. Oxa1 is known to be required for cotranslational export of the Cox2 N-terminal domain across the inner mitochondrial membrane, while Cox18 is known to be required for post-translational export of the Cox2 C-tail domain. We find that overexpression of Oxa1 does not compensate for the absence of Cox18 at the level of respiratory growth. However, it does promote some translocation of the Cox2 C-tail domain across the inner membrane and causes increased accumulation of Cox2, which remains unassembled. This result suggests that Cox18 not only translocates the C-tail, but also must deliver it in a distinct state competent for cytochrome oxidase assembly. We identified respiring mutants from a *cox18*Δ strain overexpressing *OXA1*, whose respiratory growth requires overexpression of *OXA1*. The recessive nuclear mutations allow some assembly of Cox2 into cytochrome *c* oxidase. After failing to identify these mutations by methods based on transformation, we successfully located them to *MGR1* and *MGR3* by comparative hybridization to whole-genome tiling arrays and microarray-assisted bulk segregant analysis followed by linkage mapping. While Mgr1 and Mgr3 are known to associate with the Yme1 mitochondrial inner membrane *i*-AAA protease and to participate in membrane protein degradation, their absence does not appear to stabilize Cox2 under these conditions. Instead, Yme1 probably chaperones the folding and/or assembly of Oxa1-exported Cox2 in the absence of Mrg1 or Mgr3, since respiratory growth and cytochrome *c* oxidase assembly in a *cox18 mgr3* double-mutant strain overexpressing *OXA1* is *YME1* dependent.

CYTOCHROME *c* oxidase is the last enzyme in the pathway of aerobic respiration. Its catalytic core consists of the three largest subunits, Cox1, Cox2, and Cox3, which are encoded in mitochondrial DNA (mtDNA) in fungi and animals, and surrounded by nuclear gene products. The synthesis of these subunits and the assembly of active cytochrome oxidase is a highly complex process that requires the action of at least 30 nuclear genes in *Saccharomyces cerevisiae* (reviewed in Barrientos *et al.* 2002; Herrmann and Funes 2005; Cobine *et al.* 2006; Fontanesi *et al.* 2008). For example, functional expression of the mitochondrial *COX2* gene specifically requires, at least, Pet111 to activate mRNA translation; Oxa1 for translocation of the N-terminal domain through the inner membrane; Cox20 to chaperone the processing of the Cox2 leader peptide by the inner membrane protease (Imp1, Imp2, and Som1); Cox18, Mss2, and Pnt1 to translocate the Cox2 C-terminal domain; and Sco1 and Cox17 to insert copper into the CuA site in the C-terminal domain. These functions generate a mature protein with two transmembrane helices in the inner membrane and N- and C-tail domains in the intermembrane space (IMS) that is assembled into the complex in steps involving additional factors.

Oxa1 is the founding member of the Oxa1/YidC/Alb3 family of integral membrane proteins that facilitate the insertion of respiratory and energy-transducing complexes into bacterial, mitochondrial, and thylakoid membranes through protein translocase and membrane insertase activities (reviewed in Bonnefoy *et al.* 2009). Mitochondria of fungi, animals, and plants contain both Oxa1 proteins and paralogously related Cox18 (also known as Oxa2) proteins (Funes *et al.* 2004; Gaisne and Bonnefoy 2006). These proteins, and bacterial YidC

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TABLE 1

Strains and plasmids used in this study

Strain/plasmid	Genotype	Reference
CAB9	*MAT***a** *his3ΔHindIII leu2-3,112 lys2 ura3-52*	This study
CAB81	*MATα ade2 arg8Δ::hisG leu2-3,112 lys2 ura3-52 cox18Δ::KanMX4 yme1Δ::URA3*	This study
CAB115	*MATα ade2 arg8Δ::hisG leu2-3,112 lys2 ura3 cox18Δ::KanMX4* [YEpNB6]	This study
CAB115/1-2	*MATα ade2 arg8Δ::hisG leu2-3,112 lys2 ura3 cox18Δ::KanMX4 mgr1-1* [YEpNB6]	This study
CAB115/2-8	*MATα ade2 arg8Δ::hisG leu2-3,112 lys2 ura3 cox18Δ::KanMX4 mgr3-2* [YEpNB6]	This study
CUY564	*MATα ade2-101 ade3-24 leu2-3,112 ura3-52*	T. Huffaker
DFS188	*MAT***a** *arg8Δ::hisG leu2-3,112 lys2 ura3-52*	Saracco and Fox (2002)
JKR101	*MATα ade2 his4-519 leu2-3,112 lys2 ura3-Δ*	This study
HLF636	*MAT***a** *arg8Δ::hisG his3ΔHinDIII leu2-3,112 lys2 ura3-52 cox18Δ::URA3 mgr3Δ::KanMX4* [pNB189]	This study
HLF649	*MAT***a** *arg8Δ::hisG his3ΔHinDIII leu2-3,112 lys2 ura3-52 cox18Δ::URA3 mgr3Δ::KanMX4*	This study
HLF691	*MATα ade2 leu2-3,112 ura3 trp1-1 cox18Δ::KanMX4 mgr3Δ::kanMX4 yme1Δ::URA3*	This study
HFC1	*MAT***a** *arg8Δ::hisG his3ΔHinDIII leu2-3,112 lys2 ura3-52 cox18Δ::URA3 mgr1Δ::KanMX4* [pNB189]	This study
NB40-3C	*MAT***a** *arg8::hisG his3ΔHinDIII leu2-3,112 lys2 ura3-52 [rho$^+$ cox2-62]*	Bonnefoy and Fox (2000)
SCS59	*MAT***a** *arg8Δ::hisG his3ΔHinDIII leu2-3,112 lys2 ura3-52 cox18Δ::URA3*	Saracco and Fox (2002)
SCS207	*MAT***a** *arg8Δ::hisG his3ΔHinDIII leu2-3,112 lys2 ura3-52 cox18Δ::URA3 mgr3-1* [pNB189]	This study
pNB189	2μ *LEU2 OXA1*	Gift from N. Bonnefoy
YEpNB6	2μ *URA3 OXA1*	Bonnefoy *et al.* (1994a)
pRS314-*YME1*	*TRP1 CEN6/ARSH4 YME1*	Weber *et al.* (1996)
pRS314A6	*TRP1 CEN6/ARSH4 yme1(K327R)*	Weber *et al.* (1996)
pRS314Z3	*TRP1 CEN6/ARSH4 yme1(E541A)*	Weber *et al.* (1996)

All strains are congenic to D273-10B except CUY564, which is congenic to S288C. All strains carry wild-type *rho*$^+$ mtDNA except NB40-3C, whose *rho*$^+$ mtDNA carries the *cox2-62* deletion.

proteins, share similar core topologies with five transmembrane domains. Oxa1 has a large C-terminal domain facing the matrix that interacts with mitochondrial ribosomes (Jia *et al.* 2003; Szyrach *et al.* 2003). Bacterial YidC and mitochondrial Cox18 proteins lack this domain.

In *S. cerevisiae*, Oxa1 and Cox18 have distinct functions in the biogenesis of Cox2. Oxa1 is required for translocation of the Cox2 N-tail domain (He and Fox 1997) via a cotranslational mechanism. It is also required for translocation of the C-tail domain, although it is unclear whether this requirement involves direct participation of Oxa1 or reflects a requirement of prior N-tail topogenesis for C-tail export (Bonnefoy *et al.* 2009). Yeast Oxa1 also participates in the assembly of the ATP synthase (Altamura *et al.* 1996; Hell *et al.* 2001; Jia *et al.* 2007). Yeast Cox18 is not required for N-tail export, but in conjunction with the peripheral inner membrane protein Mss2 and the integral membrane protein Pnt1, Cox18 is required for the export of the Cox2 C-tail post-translationally and has no other known substrate (He and Fox 1999; Broadley *et al.* 2001; Saracco and Fox 2002; Fiumera *et al.* 2007). These observations show that Oxa1 alone is not capable of translocating the Cox2 C-tail, whether or not it directly participates in that reaction.

S. cerevisiae OXA1 fails to complement *cox18* mutations when overexpressed in otherwise wild-type cells (Saracco and Fox 2002). Similarly, *COX18* fails to complement *oxa1* mutations (L. E. Elliott, H. L. Fiumera and T. D. Fox, unpublished results), confirming that these proteins have distinct functions. While the precise roles of human Oxa1 and Cox18 in human cells have not been established (Stiburek *et al.* 2007), expression in yeast of cDNAs encoding these human proteins does partially complement the corresponding yeast mutations (Bonnefoy *et al.* 1994b; Gaisne and Bonnefoy 2006). Furthermore, expression of mitochondrially targeted *Escherichia coli* YidC in yeast partially complements *cox18* mutations but not *oxa1* mutations (Preuss *et al.* 2005). Addition of the yeast Oxa1 C-terminal ribosome-binding domain to YidC allows it to partially complement *oxa1* mutations but not *cox18* mutations.

If Cox2 is correctly inserted into the inner membrane but prevented from assembling into cytochrome oxidase, it is degraded by a pathway largely dependent on Yme1 (Nakai *et al.* 1995; Pearce and Sherman 1995; Weber *et al.* 1996). Yme1 is a member of a conserved family of ATP-dependent AAA proteases (reviewed in Koppen and Langer 2007), whose human ortholog functions in yeast (Shah *et al.* 2000). Yme1 comprises the *i*-AAA protease, an integral inner membrane protein whose AAA and proteolytic domains are exposed in the IMS (Leonhard *et al.* 1996) where they interact with exported domains of Cox2 (Graef *et al.* 2007). When export of the Cox2 C-tail domain is prevented by an *mss2* deletion, Cox2 is instead largely degraded by the *m*-AAA protease (Broadley *et al.*

2001), an enzyme homologous to Yme1 with catalytic domains in the matrix (Leonhard *et al.* 1996). The AAA domain of Yme1 exhibits the chaperone-like property of binding to unfolded substrates in isolated mitochondria and *in vitro*, an interaction that precedes degradation (Leonhard *et al.* 1999; Graef *et al.* 2007). However, Yme1 has not previously been shown to participate as a chaperone in the productive folding of mitochondrial proteins *in vivo*.

In this study we have examined the phenotype of a nonrespiring *cox18*Δ deletion strain overproducing Oxa1 from multiple plasmid-borne copies of the wild-type *OXA1* gene. Surprisingly, we found that overproduced Oxa1 does support limited export of the Cox2 C-tail domain, but cytochrome oxidase is not assembled. Thus, in wild-type cells Cox18 appears not only to translocate the Cox2 C-tail, but also to do so in a fashion that promotes its proper folding and/or assembly. Respiring mutants selected from this strain inactivate either of two recently discovered adaptor subunits of the *i*-AAA protease. Respiratory growth of these strains remains dependent upon Yme1 activity, suggesting that under these conditions Yme1 can function as a chaperone in the assembly of Cox2 into cytochrome oxidase.

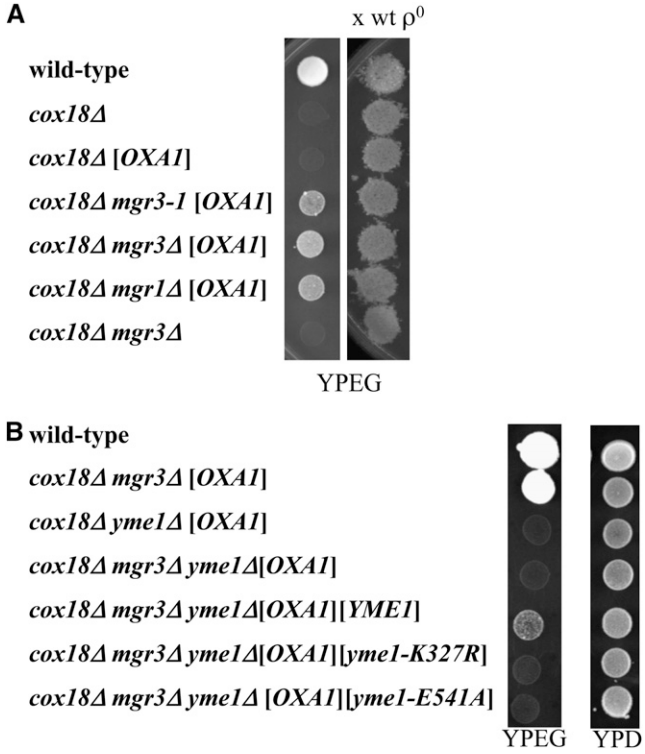

Figure 1.—Mutations in *mgr1* and *mgr3* allow respiratory growth of *cox18*Δ mutants overexpressing *OXA1*, which depends upon *YME1*. (A) Cells were grown in glucose CSM or glucose CSM lacking leucine (for maintenance of the *OXA1* plasmid) before spotting onto nonfermentable YPEG medium and then incubated at 30° for 6 days. To verify the presence of rho$^+$ mitochondria in the nonrespiring mutant strains, diluted cells were also spotted onto a lawn of a wild-type rho^0 tester strain on YPD media, allowed to mate for 2 days, and then replica plated to YPEG. Respiratory growth of the resulting diploids confirms the presence of rho$^+$ mtDNA. Strain names are given in brackets following the indicated relevant genotype: wild-type (DFS188), *cox18*Δ (SCS59), *cox18*Δ [OXA1] (SCS59 [pNB189]), *cox18*Δ *mgr3-1* [OXA1] (SCS207), *cox18*Δ *mgr3*Δ [*OXA1*] (HLF636), *cox18*Δ *mgr1*Δ [*OXA1*] (HFC1), and *cox18*Δ *mgr3*Δ (HLF649). HLF649 was derived by loss of the plasmid from HLF636. (B) Cells were grown in glucose CSM or glucose CSM lacking tryptophan and/or leucine (for plasmid maintenance) containing glucose before spotting onto YPEG medium and incubated at 30° for 10 days. Cells were also spotted onto YPD and incubated for 2 days. Strain names are given in brackets following relevant genotypes: wild-type (DFS188), *cox18*Δ *mgr3*Δ [*OXA1*] (HLF636), *cox18*Δ *yme1*Δ [*OXA1*] (CAB81 [pNB189]), and *cox18*Δ *mgr3*Δ *yme1*Δ [*OXA1*] (HLF691 [pNB189]). Plasmids carrying wild-type *YME1* [pRS314-*YME1*], *yme1-K327R* [pRS314A6], or *yme1-E541A* [pRS314Z3] were transformed into strain HLF691 as indicated.

MATERIALS AND METHODS

Yeast strains and genetic analysis of suppressor mutations: Standard genetic manipulations for nuclear genes of *S. cerevisiae* were performed as previously described (Guthrie and Fink 1991; Ausubel *et al.* 2007). Complete media containing dextrose, ethanol and glycerol, or raffinose were prepared as described. Minimal media (CSM based) were purchased from Bio101 Systems. Transformation of plasmids and PCR products was accomplished with the EZ-transformation kit (Zymo Research). *S. cerevisiae* strains used in this study are listed in Table 1. All strains are congenic to D273-10B (ATCC 24657), except CUY564, which is congenic to S288C.

Three spontaneously occurring, weakly respiring pseudorevertants were isolated from SCS59 [pNB189] (pseudorevertant strain SCS207) and CAB115 (pseudorevertants CAB115/1-2 and CAB115/2-8) on YPEG medium after incubation at 30° for 5–10 days. To determine the number of mutations in each pseudorevertant strain, pseudorevertants were mated with wild-type strains JKR101 (SCS207) or CAB9 (CAB115/1-2 and CAB115/2-8), diploids were sporulated, and *cox18*Δ meiotic progeny were analyzed for respiratory growth. In addition, *COX18* meiotic progeny carrying the nuclear recessive suppressor mutation from SCS207 were mated with pseudorevertant strain CAB115/1-2. The resulting diploids were sporulated and *cox18*Δ meiotic progeny were analyzed to determine that the complementing nuclear recessive loci in pseudorevertants SCS207 and CAB115/1-2 were unlinked. The *mgr1*Δ*::KanMX4* and *mgr3*Δ*::KanMX4* cassettes were amplified using PCR from Open Biosystems strains (Winzeler *et al.* 1999) and used to create strains HFC1 and HLF636, respectively, by transformation of a D273-10B congenic recipient.

Direct detection of mutations and genetic mapping by bulk segregant analysis using microarrays: To detect the suppressor mutations, genomic DNAs prepared from strains CAB115, CAB115/1-2, SCS59, and SCS207 were hybridized to Affymetrix GeneChip *S. cerevisiae* Tiling 1.0R arrays, and the data for each mutant were compared to those of the parent strain as previously described (Gresham *et al.* 2006). Bulk segregant analysis was performed as previously described (Brauer *et al.* 2006) from pools of genomic DNA with and without a suppressor mutation, prepared from *cox18*Δ meiotic progeny of a cross between SCS207 and CUY564.

Analysis of mitochondrial proteins: Isolation and purification of mitochondria were carried out as previously described (Glick and Pon 1995). Mitochondrial protein concentrations were determined by the Lowry method using the DC protein assay kit (Bio-Rad). Protease treatments of solubilized mito-

chondrial and protease protections of mitoplasts were carried out as previously described (Broadley *et al.* 2001; Fiumera *et al.* 2007). Proteinase K was inactivated through incubation with 5% trichloroacetic acid and heat, as previously described (Glick 1995). For immunoblotting, proteins were separated on 12% SDS–PAGE, transferred to Immobilon-P polyvinylidene difluoride membrane (Millipore), and incubated with monoclonal anti-Cox2 CCO6, polyclonal anti-cytochrome *b2*, or polyclonal anti-citrate synthase antibodies. Secondary anti-mouse or anti-rabbit antibodies were detected using the ECL+ kit (Amersham Pharmacia).

RESULTS

In the absence of Cox18, overproduced Oxa1 increases Cox2 levels and promotes Cox2 C-tail export, but not cytochrome oxidase assembly: Mutants lacking Cox18 synthesize Cox2 in the matrix and export the Cox2 N-tail domain, but fail to export the C-tail domain (Souza *et al.* 2000; Saracco and Fox 2002; Fiumera *et al.* 2007). As a result, cytochrome oxidase is not assembled and the cells exhibit a Pet$^-$ nonrespiratory growth phenotype. Overexpression of *OXA1* from a multicopy plasmid does not compensate for the absence of Cox18 at the level of respiratory growth (Saracco and Fox 2002) (Figure 1A).

Since Cox2 is not assembled in *cox18* mutants, its steady-state level is greatly reduced relative to wild type. To determine whether overproduced Oxa1 affected Cox2 in the absence of Cox18, we first tested the effect of excess Oxa1 on the level of Cox2 in a *cox18*Δ mutant by immunoblotting whole-cell extracts with a Cox2-specific antibody. Interestingly, while overexpression of *OXA1* in a *cox18*Δ mutant does not support respiratory growth, it does lead to increased accumulation of Cox2 at steady state in these cells (Figure 2). Thus, overproduced Oxa1 interacts, directly or indirectly, with unassembled Cox2 to stabilize it.

We wanted to investigate whether overproduced Oxa1 could affect translocation of the Cox2 C-tail across the inner membrane in the absence of Cox18 by determining protease sensitivity in mitoplasts. For this purpose we previously used epitope tags placed at the C terminus of Cox2; these tags are accessible to proteases when exported, whether or not Cox2 is assembled into cytochrome oxidase (Saracco and Fox 2002; Fiumera *et al.* 2007). However, we found that, in the mutant strains described below, the addition of either HA or FLAG epitopes to Cox2 affected respiratory growth, rendering this approach unusable here (data not shown). Instead, we evaluated Cox2 C-tail translocation by examining protease sensitivity in mitoplasts using a monoclonal antibody (CCO6) that recognizes an endogenous epitope in the Cox2 C-tail domain (He and Fox 1997; H. L. Fiumera and T. D. Fox, unpublished results). In assembled cytochrome oxidase from wild-type cells, the Cox2 C-tail domain is protected from exogenous protease in mitoplasts, despite being exported to the IMS

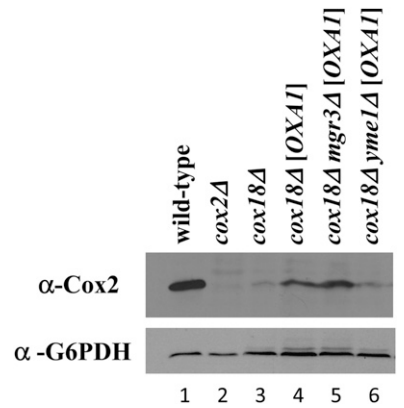

Figure 2.—Cox2 steady-state levels, reduced by *cox18*Δ, are increased by *OXA1* overexpression in a *YME1*-dependent manner. Whole-cell protein extracts were prepared from cells grown overnight in selective raffinose containing CSM, as appropriate for plasmid maintenance. Protein extracts (5 μg for wild type or 50 μg for all other strains) were analyzed by immunoblotting with anti-Cox2. As a loading control, equal amounts of extracts (10 μg) were analyzed by immunoblotting using an anti-glucose-6-phosphate dehydrogenase (α-G6DH) antibody. Lane 1, wild type (DFS188); lane 2, *cox2-62* (NB40-3C); lane 3, *cox18*Δ (SCS59); lane 4, *cox18*Δ [*OXA1*] (SCS59 [pNB189]); lane 5, *cox18*Δ *mgr3*Δ [*OXA1*] (HLF636); and lane 6, *cox18*Δ *yme1*Δ [*OXA1*] (CAB81 [pNB189]).

(Broadley *et al.* 2001) (Figure 3). In mitoplasts from *cox18* mutants, the unassembled Cox2 C-tail is resistant to protease since it remains in the matrix and is protected by the inner membrane (Saracco and Fox 2002). However, since the Cox2 N-tail is exported but not assembled in *cox18* mutants, the N-tail is digested by exogenous protease, resulting in a shorter protein fragment bearing the C-tail domain (Figure 3). In contrast, mitoplasts from a *cox18* mutant overexpressing Oxa1 failed to fully protect the Cox2 C-tail domain from protease, indicating that the C-tail was relatively efficiently exported to the IMS. Despite this C-tail export, Cox2 is apparently not assembled into active cytochrome oxidase since these cells fail to grow on nonfermentable carbon sources (Figure 1A).

After assembly into cytochrome *c* oxidase, Cox2 from wild-type mitochondria is highly resistant to degradation by proteases added to solubilized mitochondrial extracts (Eytan and Schatz 1975; Broadley *et al.* 2001). We used this test to confirm the presumed assembly states of Cox2 in these strains by evaluating protease sensitivity after solubilization of purified mitochondria with the nondenaturing detergent octylglucopyranoside (Figure 4). As expected, Cox2 from wild-type mitochondria was stable in the presence of 50 μg/ml proteinase K, whereas Cox2 from a *cox18*Δ mutant was sensitive to protease concentrations >1 μg/ml. Overexpression of Oxa1 in the *cox18* mutant had little, if any, effect on the protease resistance of Cox2, confirming that, despite the export of both N- and C-terminal domains under these conditions, Cox2 was not assembled into cytochrome *c* oxidase complexes.

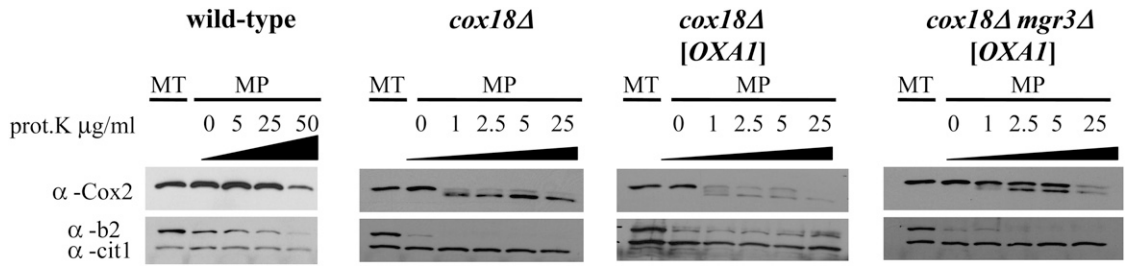

FIGURE 3.—Overproduced Oxa1 causes Cox2 C-tail translocation to the IMS in the absence of Cox18, but not cytochrome *c* oxidase assembly. Purified mitochondria (100 μg) were converted to mitoplasts through osmotic shock and incubated with the indicated amounts of proteinase K for 30 min on ice. Mitochondrial proteins were resolved by 12% SDS–PAGE and immunoblotted with anti-Cox2. The strength of the Cox2 bands were adjusted by loading less sample (10 μg equivalents for wild type) and by varying exposure times and do not reflect the relative amounts of protein in the different strains. The gel analyzing the *cox18*Δ [*OXA1*] mitoplasts was run longer than the others. As controls for mitoplasting efficiency, blots containing 5 μg of mitochondria were probed with antibodies against cytochrome b_2 (α-b2) and citrate synthase (α-Cit1). Strains were: wild type (DFS188), *cox18*Δ (SCS59), *cox18*Δ [*OXA1*] (SCS59 [pNB189]), and *cox18*Δ *mgr3*Δ [*OXA1*] (HLF636).

Mutations inactivating the nuclear genes *MGR1* and *MGR3* allow respiratory growth in a *cox18*Δ mutant overexpressing *OXA1*: While overexpression of *OXA1* does not affect respiratory growth of *cox18* mutants, we could select weakly respiring spontaneous pseudorevertants of *cox18*Δ mutants containing the *OXA1*, *LEU2*, 2μ plasmid pNB189 on YPEG medium after 5–10 days of incubation at 30°. Three such pseudorevertants were characterized in this study and shown to contain *mgr1* or *mgr3* mutations as described below. Respiratory growth of these pseudorevertants depended upon elevated levels of Oxa1p, since loss of the *OXA1* plasmid produced a tight Pet^- phenotype in both *mgr1* (not shown) and *mgr3* pseudorevertants (Figure 1A). Pseudorevertant strains whose *OXA1* plasmids had been lost were retransformed with the *OXA1* plasmid pNB189, resulting in restored respiratory growth (data not shown). Thus, the pseudorevertant phenotype did not depend upon plasmid-linked mutations.

The three pseudorevertants produced nonrespiring diploids when mated to *cox18*Δ rho^0 tester strains, indicating the presence of nuclear recessive mutations. The mutations segregated as single Mendelian loci in crosses of the pseudorevertants to wild type (MATERIALS AND METHODS), and 50% of the *cox18*Δ [*OXA1*] meiotic progeny of these crosses were weakly respiring ([*OXA1*] indicates the presence of a high-copy plasmid bearing *OXA1*). Two of the pseudorevertants, SCS207 and CAB115/2-8, formed weakly respiring diploids when mated to each other, indicating failure of the recessive mutations to complement. Tetrads from this respiring diploid all contained four Pet^+ spores, indicating that the noncomplementing suppressors were tightly linked and thus in the same gene. The nuclear recessive mutation in the third pseudorevertant strain, CAB115/1-2, complemented the other two and segregated independently, indicating an unlinked locus.

We first attempted to identify genes bearing these nuclear recessive mutations by transformation with a library of mini-transposon mutagenized genomic DNA fragments (BURNS *et al.* 1994). However, we were unable to recover pseudorevertants whose respiratory phenotype was linked to the transposon marker. We therefore employed two recently developed microarray-based mapping strategies (BRAUER *et al.* 2006; GRESHAM *et al.* 2006) to find the mutations.

To identify base-pair changes that could correspond to the mutations in the pseudorevertants, we employed comparative hybridization of genomic DNAs to DNA tiling microarrays (GRESHAM *et al.* 2006; MATERIALS AND METHODS). The highest-scoring predicted difference between CAB115/1-2 and its parent strain CAB115 was in a previously identified (DUNN *et al.* 2006) gene, *MGR1*. Sequence analysis of *MGR1* revealed a G-to-T transversion at nucleotide 778, resulting in a premature stop at codon 261 (of 417), termed *mgr1-1*. DNA sequence analysis of bulk meiotic segregants, pooled on the basis of their respiratory phenotype, confirmed that this substitution was genetically linked to the mutation. Furthermore, an *mgr1* deletion mutation constructed in our *cox18*Δ [*OXA1*] strain background caused a Pet^+ phenotype (Figure 1A) similar to that of *mgr1-1* (not shown), indicating that the stop codon causes loss of function.

We were unable to identify a clear candidate mutation by comparative hybridization of DNA from pseudorevertant SCS207 and its parent, SCS59. Instead, we employed microarray-assisted bulk segregant analysis (BRAUER *et al.* 2006) to detect genetic linkage between the mutation and polymorphic differences between SCS207 (D273-10B genetic background) and a wild-type strain related to S288c (MATERIALS AND METHODS). This analysis indicated linkage to polymorphisms on chromosome XIII in the region around base pair 560,000 (not shown). We confirmed that the mutation was in this region by meiotic mapping, which located the mutation to a position 9.7 cM from *YMR102C* and 21.4 cM from *IMP1* (not shown). This location corresponded closely to that of ORF *YMR115W*, centered around base pair 498,500 of chromosome XIII. This ORF was first

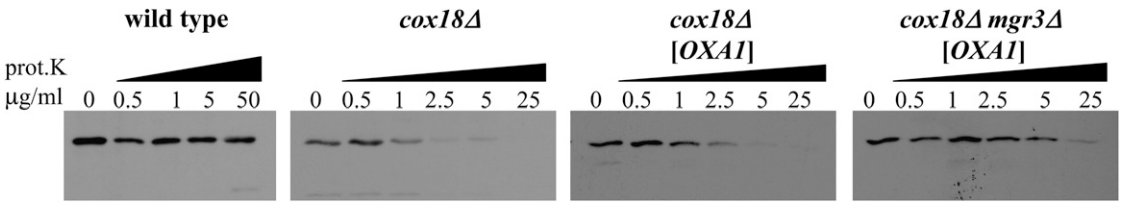

FIGURE 4.—Cox2 is assembled into protease-resistant cytochrome *c* oxidase in a *cox18*Δ *mgr3*Δ [*OXA1*] strain. Purified mitochondria (10 μg for wild type, 100 μg for all other strains) were solubilized in 1% octylglucopyranoside and incubated with increasing amounts of proteinase K for 30 min on ice. Proteinase K was inactivated through acid and heat treatment after which samples were analyzed by immunoblotting using an anti-Cox2 antibody. The strength of the Cox2 bands were adjusted by varying exposure times and do not reflect the relative amounts of protein in the different strains. See legend for Figure 3 for strains corresponding to the indicated relevant genotypes.

characterized as encoding a mitochondrial protein (SICKMANN *et al.* 2003) and was later named *MGR3* (DUNN *et al.* 2008). DNA sequence analysis of *MGR3* amplified from pseudorevertant SCS207 revealed a deletion of a single A within a monotonous run of 11 A's (nucleotides 217–227), leading to a frameshift early in the 501-codon *MGR3* ORF, termed *mgr3-1*. The nature of this mutation probably accounts for the failure of direct detection by hybridization to tiling microarrays. We cannot account for the small but significant discrepancy between the actual position and that predicted by microarray-assisted bulk segregant analysis. As expected, an *mgr3* deletion mutation also caused a Pet^+ phenotype in the *cox18*Δ [*OXA1*] background (Figure 1A). Furthermore, the mutation in pseudorevertant CAB115/2-8, which failed to complement *mgr3-1* and was genetically linked to it, also affected the *MGR3* ORF: insertion of a single T into a monotonous run of six T's (nucleotides 913–918), causing a frameshift. This pseudorevertant strain exhibited a weaker respiratory phenotype (not shown), suggesting an incomplete loss of function possibly due to ribosomal frameshifting.

The Mgr1 and Mgr3 proteins have recently been shown to associate with each other and with Yme1 (DUNN *et al.* 2006, 2008), the inner mitochondrial membrane AAA ATP-dependent zinc protease (NAKAI *et al.* 1995; PEARCE and SHERMAN 1995; WEBER *et al.* 1996) facing the intermembrane space (LEONHARD *et al.* 1996). These newly discovered subunits of the *i*-AAA complex bind to known Yme1 substrates and are required for normal degradation rates *in vivo*, suggesting that they recruit substrate proteins to Yme1 for degradation (DUNN *et al.* 2008).

Respiratory growth of a *cox18*Δ *mgr3*Δ [*OXA1*] strain is Yme1 dependent: Mutant strains lacking either Mgr1 or Mgr3, or both, exhibit normal respiratory growth (DUNN *et al.* 2008). Similarly, *yme1* mutants exhibit robust respiratory growth at 30°, although they are Pet^- at 37° (THORSNESS and FOX 1993). The genetic analysis described above demonstrated that the absence of Mgr1 and/or Mgr3 proteins allows overproduced Oxa1 to partially compensate for the lack of Cox18 in the assembly of cytochrome *c* oxidase. Since *mgr1* and *mgr3* mutations appear to decrease proteolysis by Yme1 (DUNN *et al.* 2006, 2008), we asked whether a *yme1* deletion might also allow respiratory growth in the *cox18*Δ [*OXA1*] background. Interestingly, the *yme1*Δ *cox18*Δ [*OXA1*] strain failed to grow on nonfermentable carbon sources (Figure 1B). Furthermore, an *mgr3*Δ *yme1*Δ *cox18*Δ [*OXA1*] strain was also nonrespiratory. Thus, the respiratory growth of *mgr3*Δ *cox18*Δ [*OXA1*] strains is not simply due to loss of Yme1 activity, and indeed depends upon functional Yme1.

Yme1 contains a highly conserved Walker A motif within the AAA ATPase domain and a conserved metallopeptidase catalytic site. Missense mutations that catalytically inactivate either of these active sites have been previously observed to cause the same phenotypes as the *yme1* deletion (LEONHARD *et al.* 1996, 1999; WEBER *et al.* 1996). We nevertheless tested whether both of these Yme1 catalytic domains were required for the respiratory growth of a *yme1*Δ *cox18*Δ *mgr3*Δ strain containing extra copies of *OXA1* and transformed with additional plasmids bearing defined *yme1* missense alleles. Neither *yme1-K327R* nor *yme1-E541A* (WEBER *et al.* 1996) supported respiratory growth, regardless of the incubation time, indicating that both the ATP-binding and zinc-dependent protease domains, respectively, are required for this activity (Figure 1B). Transformation with a corresponding plasmid carrying wild-type *YME1* was able to restore respiration, albeit not to the level supported by the chromosomal *YME1* in the control strain, perhaps because competition between the two related plasmids in these transformants reduced the Oxa1 levels.

To better understand the basis for the Yme1-dependent respiratory growth of the *cox18*Δ *mgr3*Δ [*OXA1*] pseudorevertant, we examined the status of Cox2 in cells of that genotype. Interestingly, the steady-state level of Cox2 was only modestly elevated in these cells relative to the nonrespiring *cox18*Δ [*OXA1*] parent strain (Figure 2). Thus, the absence of Mgr3 did not appear to greatly reduce degradation of Cox2 under these conditions, despite the fact that Mgr3 appears to function in delivering substrates to the Yme1 protease (DUNN *et al.* 2008). Furthermore, deletion of *YME1* actually reduced the steady-state level of Cox2 relative to the *cox18*Δ [*OXA1*] parent strain to roughly the level seen in the

simple *cox18*Δ mutant (Figure 2). Thus, unassembled Cox2 molecules whose C-tails are exported by excess Oxa1 appear to be stabilized by Yme1, suggesting that, under these conditions, Yme1 provides a chaperone-like function that, in the absence of Mgr3, can promote limited assembly of active cytochrome oxidase. In this connection, it is interesting to note that plasmid-borne extra copies of *YME1* did not promote respiratory growth of a *cox18*Δ [*OXA1*] strains (not shown).

To confirm that limited amounts of Cox2 were assembled in the *cox18*Δ *mgr3*Δ [*OXA1*] pseudorevertant, we examined its sensitivity to protease digestion in mitoplasts (Figure 3) and in detergent solubilized mitochondria (Figure 4). In both cases, full-length Cox2 from the pseudorevertant exhibited increased protease stability relative to the *cox18*Δ [*OXA1*] parent, which is consistent with its assembly into functional cytochrome *c* oxidase complexes.

DISCUSSION

Functional expression of protein-coding genes in yeast mtDNA requires a surprisingly complex suite of accessory functions, many of which are specific for individual mitochondrially encoded proteins. In this study, we have explored the functions of two paralogous, nuclearly encoded mitochondrial inner membrane translocases, Oxa1 and Cox18, in the topogenesis and subsequent assembly of the mitochondrially encoded Cox2 subunit of cytochrome *c* oxidase. Our results have revealed a new role for Cox18 and latent activities of Oxa1 and the *i*-AAA ATP-dependent protease Yme1. Orthologs of all three of these proteins are encoded in the human genome.

Previous studies have shown that Cox18 is specifically required in otherwise wild-type cells for translocation of the mitochondrially synthesized Cox2 C-tail through the inner membrane to the IMS (Saracco and Fox 2002). This fact alone would account for the cytochrome *c* oxidase deficiency of *cox18* mutants and the low steady-state level of Cox2 (Souza *et al.* 2000). However, we now find that elevated levels of Oxa1 in cells lacking Cox18 lead to increased accumulation of Cox2, although not to wild-type levels, and significant translocation of the Cox2 C-tail through the inner membrane to the IMS. Despite this overproduced-Oxa1-dependent C-tail export, there is no assembly of active cytochrome *c* oxidase. Thus, the Oxa1-translocated Cox2 C-tail is not available for assembly in otherwise wild-type cells despite the fact that it exhibits an N-out, C-out topology. We conclude that, under normal conditions, Cox18 not only translocates the Cox2 C-tail to the IMS, but also is required to deliver in a state, as yet unknown, that is competent for downstream steps leading to active cytochrome *c* oxidase.

Cox18 interacts functionally and physically with Mss2 (Saracco and Fox 2002), a peripheral inner membrane protein on the matrix side that is also required for C-tail export (Broadley *et al.* 2001). Both Cox18 and Mss2 interact physically with full-length amino terminally processed, newly synthesized Cox2, and C-tail export depends upon the amino acid sequence and/or structural features of the 40 C-terminal Cox2 residues (Fiumera *et al.* 2007). These facts indicate that Cox2 C-tail translocation is post-translational (Fiumera *et al.* 2007), in contrast to cotranslational Oxa1-dependent N-tail export (Szyrach *et al.* 2003). One may conjecture that the normal C-tail export apparatus delivers this mitochondrially synthesized domain to the IMS in a specific, partially folded conformation, while, in the absence of Cox18, overproduced Oxa1 does not.

Why does excess Oxa1 promote limited Cox2 C-tail export in the absence of Cox18, while normal levels of Oxa1 do not? We do not have a clear answer to this question. Oxa1 has a large C-terminal matrix localized domain that interacts with mitochondrial ribosomes to promote cotranslational protein insertion (Jia *et al.* 2003; Szyrach *et al.* 2003). Perhaps overproduction increases the number of Oxa1 molecules to the point that many are unable to bind to ribosomes and are thus available to act post-translationally on Cox2 C-tail domains left on the inner surface of the membrane in the absence of Cox18. Our data do not shed light on the question of whether Oxa1 participates directly in Cox2 C-tail export in wild-type cells or indirectly through a requirement for prior N-tail export. It is noteworthy, however, that Oxa1 can be crosslinked to newly synthesized full-length mitochondrial translation products, including Cox2, in wild-type mitochondria (Hell *et al.* 1998).

The presence of the C-terminal ribosome-binding domain on Oxa1, and its absence from Cox18, appears to play a role in the functional distinction between these paralogous proteins. The homologous YidC translocase of *E. coli* resembles Cox18 in lacking this domain (reviewed in Bonnefoy *et al.* 2009), and expression in yeast of mitochondrially targeted YidC partially complements a *cox18* deletion, but not an *oxa1* deletion (Preuss *et al.* 2005). Addition of the yeast Oxa1 C-terminal ribosome-binding domain to mitochondrially targeted YidC allows it to partially complement *oxa1*, but not *cox18*, deletions at the level of respiratory growth (Preuss *et al.* 2005). Whether this modified YidC-Oxa1 chimeric protein causes translocation of the Cox2 C-tail domain in the *cox18*Δ mutant, but fails to allow cytochrome oxidase assembly, was not determined. In any event, it is also clear that the presence or absence of the Oxa1 C-terminal domain is not the only feature underlying the distinct functions of Oxa1 and Cox18 since expression of truncated forms of Oxa1 lacking its C-terminal ribosome-binding domain (Lemaire *et al.* 2004), at either normal or high levels, does not suppress the respiratory defect caused by a *cox18* deletion (H. L. Fiumera, L. E. Elliott and T. D. Fox, unpublished results).

Spontaneous recessive nuclear mutations in two genes allowed *cox18*Δ strains overexpressing Oxa1 to assemble low levels of active cytochrome *c* oxidase and exhibit weak respiratory growth on nonfermentable carbon sources. We mapped these mutations to *MGR1* and *MGR3* by comparative hybridization of genomic DNAs to tiling microarrays (Gresham *et al.* 2006) and by microarray-assisted bulk segregant analysis (Brauer *et al.* 2006) after failing to identify them by transposon-tagging mutagenesis.

Mgr1 and Mgr3 have recently been identified as physically and functionally associated with the *i*-AAA protease Yme1, which is bound to the inner membrane with catalytic domains facing the IMS (Dunn *et al.* 2006, 2008). Mgr1 and Mgr3 interact with each other, with substrates of the Yme1 protease, and together bind to Yme1 itself. Both Mgr1 and Mgr3 are required for normal binding of proteolytic substrates to Yme1 and for normal rates of substrate degradation. Thus, Mgr1 and Mgr3 appear to play the role of adaptor subunits in the recognition and delivery of substrates to be degraded by Yme1 (Dunn *et al.* 2008). For reasons that are not clear (see below), *yme1* mutations are synthetically lethal in combination with the loss of mtDNA (*rho*$^-$ or *rho*^{0}) (Thorsness *et al.* 1993), as are *mgr1* and *mgr3* mutations in certain strain backgrounds (Dunn *et al.* 2006, 2008). However, respiratory growth of *mgr1* and *mgr3* mutants is normal, while that of *yme1* mutants is defective only at elevated temperature, and steady-state levels of Cox2 are normal in all three mutants (Dunn *et al.* 2008). Thus, these functions are not needed for assembly of cytochrome *c* oxidase or other respiratory complexes in otherwise wild-type strains.

The fact that Mgr1 and Mgr3 are necessary for normal proteolysis by Yme1 initially suggested that the respiratory growth of *cox18*Δ *mgr1* and *cox18*Δ *mgr3* strains overproducing Oxa1 could be due to decreased degradation of Cox2 by Yme1. An effect like this has been previously reported, in which a *yme1*Δ mutant can reverse the *oxa1*Δ-induced defect in ATP synthase activity by stabilizing the integral membrane components of ATP synthase, although *yme1*Δ has no effect on the cytochrome *c* oxidase deficiency (Lemaire *et al.* 2000).

However, our data clearly indicate that decreased proteolysis by Yme1 is not the mechanism operating here. First, the steady-state level of Cox2 in these strains was not greatly increased by the *mgr3* deletion. Second, a *yme1* deletion itself did not allow respiratory growth of a *cox18*Δ strain overexpressing Oxa1 and actually decreased the steady-state level of Cox2 relative to the corresponding *YME1* control. Thus, in this situation Yme1 partially protects Cox2 from degradation by other, unknown, proteases. Finally, both *yme1* deletion and catalytically inactivating *yme1* missense mutations prevented the respiratory growth of a *cox18*Δ *mgr3*Δ strain overexpressing Oxa1. Thus, cytochrome *c* oxidase assembly and respiratory growth are dependent on active Yme1 under these conditions.

For reasons that are not clear, *yme1* mutations are synthetically lethal in combination with the loss of mtDNA (*rho*$^-$ or *rho*^{0}), as are *mgr1* and *mgr3* mutations in certain strain backgrounds. *rho*^{0} cells lacking Yme1 are virtually inviable (Thorsness *et al.* 1993), and a similar phenotype is observed in some yeast strain backgrounds for *rho*^{0} cells lacking Mgr1 and Mgr3 (Dunn *et al.* 2006, 2008). This inviability is apparently due to the inability of these nonrespiratory mitochondria to maintain an inner-membrane potential by F_1-ATPase-dependent hydrolysis of fermentatively derived ATP in the matrix, although the mechanism underlying this defect remains unclear (Kominsky *et al.* 2002). Since both *yme1*Δ and *mgr3*Δ cause similar membrane potential related growth phenotypes in *rho*^{0} cells, it is unlikely that the distinct respiratory growth phenotypes caused by these deletions in the *cox18* [*OXA1*] strains reported here are due to differential effects on membrane potential.

We propose that, in the absence of Cox18 and Mgr1 or Mgr3, Yme1 acts as a chaperone to promote the assembly of Cox2 C-tail domains that were aberrantly exported by excess Oxa1. In this view, the absence of the substrate adaptor subunits could allow latent chaperone activity of Yme1 to affect the structure of the Cox2 C-tail domain neighboring it on the outer surface of the inner membrane. While evidence indicating chaperone activity of Yme1 *in vivo* has not been previously reported, the purified AAA domain of Yme1 does exhibit chaperone-like function *in vitro*, preferentially binding to denatured dihydrofolate reductase and promoting its renaturation (Leonhard *et al.* 1999).

The homologous, matrix-facing, *m*-AAA protease complex was previously thought to have chaperone activity in the assembly of respiratory complexes (Paul and Tzagoloff 1995; Arlt *et al.* 1996). However, the phenotypes caused by mutations affecting the *m*-AAA protease are now known to be due to a defect in the processing of an imported mitochondrial ribosomal protein (Nolden *et al.* 2005). Thus, the apparent *in vivo* chaperone activity of Yme1 in the absence of Mgr1 or Mgr3 is a novel observation for AAA proteases.

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