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Peripheral Mitochondrial Inner Membrane Protein, Mss2p, Required for Export of the Mitochondrially Coded Cox2p C Tail in *Saccharomyces cerevisiae*

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Cytochrome oxidase subunit 2 (Cox2p) is synthesized on the matrix side of the mitochondrial inner membrane, and its N- and C-terminal domains are exported across the inner membrane by distinct mechanisms. The *Saccharomyces cerevisiae* nuclear gene *MSS2* was previously shown to be necessary for Cox2p accumulation. We have used pulse-labeling studies and the expression of the *ARG8^m* reporter at the *COX2* locus in an *mss2* mutant to demonstrate that Mss2p is not required for Cox2p synthesis but rather for its accumulation. Mutational inactivation of the proteolytic function of the matrix-localized Yta10p (Afg3p) AAA-protease partially stabilizes Cox2p in an *mss2* mutant but does not restore assembly of cytochrome oxidase. In the absence of Mss2p, the Cox2p N terminus is exported, but Cox2p C-terminal export and assembly of Cox2p into cytochrome oxidase is blocked. Epitope-tagged Mss2p is tightly, but peripherally, associated with the inner membrane and protected by it from externally added proteases. Taken together, these data indicate that Mss2p plays a role in recognizing the Cox2p C tail in the matrix and promoting its export.

Expression of mitochondrial genes involves protein synthesis in the mitochondrial matrix, insertion of hydrophobic domains into the inner membrane, translocation of hydrophilic domains across the inner membrane, and assembly into functional respiratory complexes (18, 40). The processes by which mitochondrially encoded proteins translocate across the inner membrane have been difficult to study because there is no in vitro system for the expression of translation products encoded by mitochondrial DNA (mtDNA). We have therefore taken a genetic approach to studying export of protein domains encoded in mtDNA.

We have focused our attention on the translocation of the mitochondrially encoded Cox2p. The crystal structures of both bovine and *Paracoccus denitrificans* cytochrome oxidases have been determined (37, 61). Based on these structures and on other studies (42), the orientation of yeast Cox2p in the inner membrane has been firmly established. After or during synthesis, the amino- and carboxy-terminal tails of Cox2p are exported from the matrix into the intermembrane space (IMS), while its two transmembrane domains are embedded in the inner membrane.

In *Saccharomyces cerevisiae,* translation of the *COX2* mRNA is activated at the inner membrane by the protein Pet111p (17, 33, 41, 46). Cox2p is synthesized as a precursor protein whose N-terminal 15-amino-acid leader peptide is cleaved by the Imp peptidase complex in the IMS after translocation through the membrane (36, 43, 47, 50).

So far, two components of the Cox2p export machinery have been reported. Oxa1p (1, 4, 7) was shown to be a component of the export machinery (21, 23, 24, 25). In addition, *PNT1* was identified in a screen for export defective mutants and shown to encode a mitochondrial inner membrane protein (22).

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A previous report indicated that nuclearly encoded Mss2p is required for the expression of *COX2* (52). An *mss2* mutant was respiratory defective and failed to accumulate Cox2p, even though *COX2* mRNA was produced normally. In the present study, we demonstrate that Mss2p acts within mitochondria to posttranslationally stabilize Cox2p and is required to translocate the C-terminal domain of Cox2p through the inner membrane.

MATERIALS AND METHODS

Strains and plasmids. Standard yeast genetic methods were as previously described (14, 45). Strains used in this study are listed in Table 1. Strain SB44 is congenic to DBY947 (35). Strains J303-1A, SB48, SB49C, SB100, SB101, SB102, SB103, and YGS103 are congenic to W303 (59). All other strains listed in Table 1 are congenic to D273-10B (ATCC 25627). Fermentable medium was YPD (1% yeast extract, 2% Bacto Peptone, 100 mg of adenine/liter, and 2% glucose) or YPR (2% yeast extract, 2% Bacto Peptone, 100 mg of adenine/liter, and 2% raffinose) and nonfermentable medium was YPEG (1% yeast extract, 2% Bacto Peptone, 100 mg of adenine/liter, 3% ethanol, 3% glycerol). The minimal medium was SD (0.67% yeast nitrogen base without amino acids, 2% glucose), and it was supplemented with amino acids as needed. Transformations of plasmids and PCR products were accomplished by using the EZ-Transformation kit (Zymo Research).

Plasmids and DNA manipulation. To construct the *mss2*Δ*::LEU2.* deletion, a disruption cassette containing the *LEU2* gene flanked by 50 bp of sequence homologous to the *MSS2* coding region was PCR amplified, purified, and transformed into appropriate strains (HMD22, J303-1A, SH36, TF215, and YGS103). Deletion of *MSS2* was confirmed by PCR analysis. Strains containing the *yme1::URA3* deletion were constructed by using pPT45 (60) and verified by PCR. Tagging of *MSS2* was done by PCR amplifying a *HA-URA3-HA* cassette (48) with the primers TTCTTGAAAGTAGAAAAGATTCCATAAAGTTGCTGGACA AAGCACGGCTTAGGGAACAAAAGCTGG and GGTGGAGACATGTGT CCTTATATAAATCGCAAAAAGAATCGATCAGACATCTATAGGGCGA ATTGG. The resulting cassette, which targeted insertion of the hemagglutinin (HA) cassette directly before the *MSS2* stop codon, was transformed into TF215. Cells containing integration of the tagging cassette at the *MSS2* locus were identified by using PCR and plated on medium contain 5-fluoroorotic acid to pop out the *URA3* marker.

Mitochondrial purification, fractionation, and protein analysis. Mitochondrial purification and membrane fractionation, mitoplasting and protease protection, and alkaline extraction of mitochondrial proteins were performed as

TABLE 1. Strains used in this study

Strain	Nuclear (mitochondrial) genotype	Source or reference
DBY947	*MAT***a** *ade2-101 ura3-52* [*rho*$^+$]	35
DFS188	*MAT***a** *arg8*::*hisG his3*Δ *leu2-3,112 lys2* [*rho*$^+$]	54
ECS108	*MAT*α *ade2 ino4-8 ino1-13 pet111-4* [*rho*$^+$]	This study
HMD22	*MAT***a** *arg8*::*hisG his3*Δ *leu2-3,112 lys2 ura3-52* [*rho*$^+$ *cox2*::*ARG8*^{m}]	13
J303-1A	*MAT***a** *ade2-1 his3-11,15 trp1-1 leu2,112 ura3-52* [*rho*$^+$]	2
NB39-9c	*MAT***a** *lys2 ura3-52 pet111-9* [*rho*$^+$]	This study
NSG192	*MAT***a** *ade2-101 arg8*::*hisG leu2-3,112 pet111-14 ura3-52* [*rho*$^+$ *cox2*::*ARG8*^{m}]	This study
PTH366	*MAT***a** *ade2-101 leu2*Δ *ura3-52* [*rho*$^+$]	This study
SB12	*MAT***a** *ade2-101 leu2*Δ *mss2*Δ::*LEU2 ura3-52* [*rho*$^+$]	This study
SB19A	*MAT*α *his4-519 leu2-3,112 MSS2-3xHA ura3*Δ*NS*	This study
SB20	*MAT***a** *arg8*::*hisG his3*Δ *leu2-3,112 lys2 mss2*Δ::*LEU2 ura3-52* [*rho*$^+$ *cox2*::*ARG8*^{m}]	This study
SB23B	*MAT*α *canR his3-11,15 leu2-112 pet111-1* [*rho*$^-$ *(COX3-COX2)*]	This study
SB26	*MAT***a** *canR his3-11,15 leu2-112 mss2*Δ::*LEU2 pet111-1* [*rho*$^-$ *(COX3-COX2)*]	This study
SB35	*MAT***a** *arg8*::*hisG his3*Δ *leu2-3,112 lys2 mss2*Δ::*LEU2 ura3-52* [*rho*$^+$ *COX2(1-67)*::*ARG8*^{m}]	This study
SB44	*MAT*α *ade2-101 arg8*Δ::*URA3 leu2*Δ *kar1-1 ura3-52 mss2*::Tn-*LEU2* [*rho*$^+$]	This study
SB48B	*MAT***a** *ade2-1 his3-11,15 mss2*Δ::*LEU2 trp1-1 leu2,112 ura3-52* [*rho*$^+$]	This study
SB49C	*MAT***a** *ade2-1 his3-11,15 leu2,112 mss2*Δ::*LEU2 trp1-1 ura3-52 yta10*::*HIS3MX6, YCplac22 (TRP1, CEN):ADH1-YTA10E559Q* [*rho*$^+$]	This study
SB100	*MAT***a** *ade2-1 his3-11,15 trp1-1 leu2,112 ura3-52 yme1*Δ::*URA3* [*rho*$^+$]	This study
SB101	*MAT***a** *ade2-1 his3-11,15 mss2*Δ::*LEU2 trp1-1 leu2,112 ura3-52 yme1*Δ::*URA3* [*rho*$^+$]	This study
SB103	*MAT***a** *ade2-1 his3-11,15 leu2,112 mss2*Δ::*LEU2 trp1-1 ura3-52 yta10*::*HIS3MX6, YCplac22 (TRP1, CEN):ADH1-YTA10E559Q, yme1*Δ::*URA3* [*rho*$^+$]	This study
SH36	*MAT***a** *arg8*::*hisG his3*Δ *leu2-3,112 lys2 ura3-52* [*rho*$^+$ *COX2(1-67)*::*ARG8*^{m}]	21
SH105	*MAT***a** *arg8*::*hisG his3*Δ *imp1-1 lys2 ura3-52* [*rho*$^+$]	This study
TF215	*MAT***a** *his4-519 leu2-3,112, ura3*Δ [*rho*$^+$]	This study
YGS103	*MAT***a** *ade2-1 his3-11,15 leu2,112 trp1-1 ura3-52 yta10*::*HIS3MX6, YCplac22 (TRP1, CEN):ADH1-YTA10E559Q* [*rho*$^+$]	2

previously described (15, 16, 21, 22). Pulse-labeling of mitochondrial translation products with [^{35}S]methionine Trans Label (ICN) was done essentially as described previously (6), except that cells were labeled with 0.2 mCi [^{35}S]methionine for 10 min in the presence of 0.2 mg of cycloheximide/ml. After pulse-labeling, cells were incubated with 2 mM cold methionine for 50 min or immediately chilled on ice. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12 or 15% gel, and a PhosphorImager was used to detect radioactivity within the dried gels. For steady-state analysis of proteins, total cellular protein was extracted from cells (65) in the presence of a protease inhibitor cocktail (Sigma) and analyzed, or purified mitochondria were analyzed. Protein was separated on a 10% polyacrylamide gel and probed with a monoclonal antibody to Cox2p (CCO6 [provided by T. L. Mason]) (39) that recognizes an epitope in the Cox2p C tail (21), a polyclonal antibody to cytochrome b_2, a polyclonal antibody to Arg8p, a polyclonal antibody to glucose-6-phosphate dehydrogenase (Sigma), a polyclonal antibody to citrate synthase (SS60 [provided by G. Schatz]), or a monoclonal antibody to the HA epitope (3F10 [Boehringer-Mannheim]). Secondary anti-mouse or anti-rabbit antibodies were detected by using the ECL kit (Amersham Pharmacia).

RESULTS

In the absence of Mss2p, Cox2p is synthesized normally but is destabilized. Our first goal was to understand the posttranscriptional role of Mss2p in *COX2* expression. We constructed a deletion mutant in which the *MSS2* coding region was largely replaced by the *LEU2* marker. In agreement with previous data (52), the *mss2*Δ mutant was respiratory defective, as judged by its inability to grow on the nonfermentable carbon source, YPEG (data not shown). We analyzed steady-state Cox2p levels in the *mss2*Δ mutant by using Western analysis of whole-cell extract (Fig. 1A) and confirmed that Cox2p accumulation was dramatically reduced. We next monitored Cox2p synthesis and accumulation by using cycloheximide pulse-labeling of mitochondrial translation products (Fig. 1B). Cells were subjected to cycloheximide poisoning to inhibit cytoplasmic translation, and mitochondrial translation products were pulse-labeled for 10 min with [^{35}S]methionine, followed with or without a 50-min chase with cold methionine, and analyzed by SDS-PAGE. After a short pulse, followed by a long chase, Cox2p was absent in the *mss2*Δ mutant, confirming that Mss2p is required for accumulation of Cox2p (Fig. 1B, lane 3). In addition, the absence of Mss2p caused a decrease in the accumulation of Cox1p, but no other mitochondrial translation products were affected. As expected, Cox2p was absent in the *pet111* mutant (Fig. 1B, lane 2). When mitochondrial translation products were radiolabeled for 10 min and immediately analyzed, Cox2p was labeled at nearly the wild-type rate in the *mss2*Δ mutant, showing that Mss2p is not required for Cox2p synthesis (Fig. 1B, lane 6). Thus, Mss2p is required for Cox2p stability but not for Cox2p synthesis. In the absence of either Mss2p or the *COX2* mRNA-specific translational activator Pet111p (Fig. 1B, lanes 5 and 6), Cox1p labeling was dramatically reduced. The decrease in Cox1p labeling caused by the absence of Cox2p is apparently an indirect effect (41). The data in Fig. 1 indicate that Mss2p has no role in synthesis or stability of the other mitochondrial translation products analyzed (Fig. 1B, lanes 3 and 6). Furthermore, spectroscopic analysis (51) of the *mss2*Δ mutant revealed the presence of cytochromes *c*, c_1, and *b*, while cytochrome aa_3 was absent (data not shown), indicating that the mutation affects cytochrome oxidase but not the bc_1 complex. Apparently the absence of Mss2p prevents accumulation of Cox2p, which in turn prevents the accumulation of the cytochrome oxidase complex.

To further monitor translation at the *COX2* locus, we took advantage of the synthetic, mitochondrially encoded *ARG8*^{m} gene. Arg8p is normally encoded in the nucleus and imported into the mitochondrial matrix where it participates in arginine biosynthesis. The synthetic *ARG8*^{m} produces the same biosynthetic enzyme from within the mitochondrial matrix and has been successfully used as a reporter for mitochondrial gene expression (8, 17, 21, 54). To address whether Mss2p has a role in the translation of *COX2*, the *COX2* coding region was replaced by the *ARG8*^{m} reporter gene in the *mss2*Δ background,

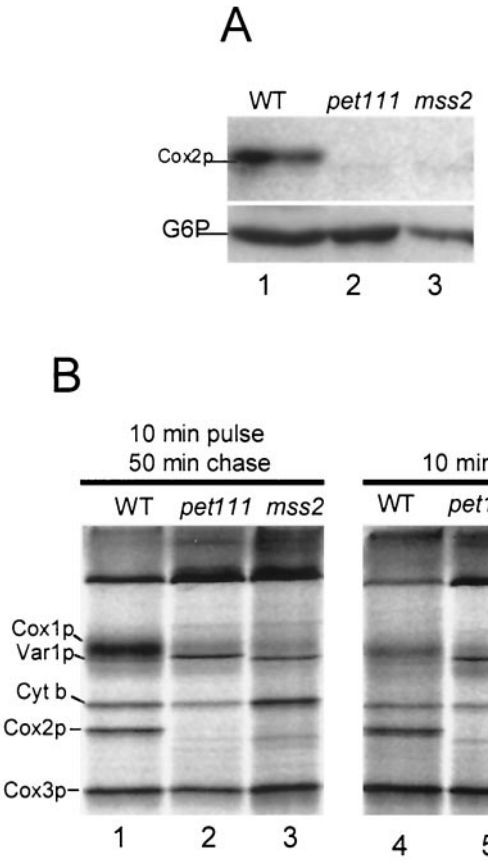

FIG. 1. Cox2p is unstable in the absence of Mss2p. (A) Cox2p steady-state levels are reduced in the *mss2*Δ mutant. Whole-cell extracts were prepared from cells grown overnight in YPR (see Materials and Methods). Extracts were analyzed by Western blotting with anti-Cox2p and anti-glucose-6-phosphate dehydrogenase (G6P) as a loading control. Lanes: 1, wild-type (PTH366); 2, *pet111* mutant (ECS108); 3, *mss2*Δ mutant (SB12). (B) Cox2p is synthesized but rapidly degraded in an *mss2*Δ mutant. Cells were incubated with cycloheximide and either pulsed with [^{35}S]methionine for 10 min and chased with cold methionine for 50 min or pulsed for 10 min with no chase, as indicated (see Materials and Methods). Mitochondria were isolated, and translation products were separated by SDS–15% PAGE and detected by autoradiography. Lanes: 1 and 4, wild type (WT; PTH366); 2 and 5, *pet111* (ECS108); 3 and 6, *mss2*Δ (SB12).

and the resulting cells were spotted onto medium lacking arginine (Fig. 2). The *mss2*Δ mutant was Arg$^+$, indicating efficient translation of the chimeric *cox2::ARG8^m* mRNA. In contrast, the *pet111* mutant was Arg$^-$, as expected for a mutant defective in *COX2* translation. Thus, the experiment whose results are shown in Fig. 2 verifies that Mss2p is not required for translation of mRNAs coded by the *COX2* locus.

PET111 interacts with the 5′ untranslated leader (5′-UTL) of *COX2* and is required for targeted translation of *COX2* (33, 46). If *PET111* is deleted, translation cannot occur, and the resulting cells are respiratory deficient (Pet$^-$). However, the requirement for *PET111* can be bypassed by replacing the 5′-UTL of *COX2* with the 5′-UTL of *COX3* (COX3-COX2) (32). In this case, pre-Cox2p can presumably be directed to the inner membrane by factors involved in targeted translation of the *COX3* mRNA. We sought to determine whether Mss2p was also interacting with the 5′-UTL of the *COX2* mRNA to

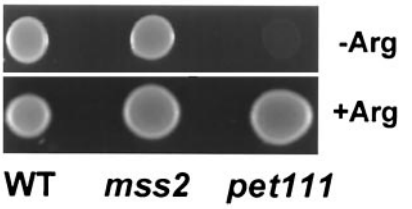

FIG. 2. Expression of a *cox2::ARG8^m* reporter gene in mtDNA is independent of Mss2p. Cells were spotted onto synthetic complete medium lacking arginine or synthetic complete medium containing arginine as indicated. Plates were incubated for 2 days at 30°C. Strains: WT, wild-type HMD22; *mss2*, SB20; *pet111*, NSG192.

tether translation of *COX2* to the inner membrane. To address this question, we tested whether the *PET111* suppressor, *COX3-COX2*, bypassed the requirement of Mss2p for respiration (Fig. 3). A homozygous *mss2*Δ diploid containing the *COX3-COX2* suppressor (ρ^-) in the presence of a functional mitochondrial genome (ρ^+) remained respiratory defective (Fig. 3, upper left quandrant), while a homozygous *pet111* diploid containing the ρ^- COX3-COX2/ρ^+ genome was respiratory competent (Fig. 3, lower right quandrant). These results suggest that *MSS2* is not acting through the *COX2* 5′-UTL to promote tethered translation of *COX2* in a *PET111*-like manner.

Inactivation of Yta10p (Afg3p) proteolytic activity increases Cox2p stability in the absence of Mss2p. There are two AAA proteases found in the mitochondrial inner membrane that expose catalytic domains to opposite surfaces of the membrane (30). The *i*-AAA protease complex contains Yme1p subunits and is active in the IMS, whereas the *m*-AAA protease comprises Yta10p (Afg3p) and Yta12p (Rca1p) subunits and is active in the mitochondrial matrix. Together, these proteases mediate degradation of several mitochondrial proteins.

The Yta10p/Yta12p (*m*-AAA) complex is capable of degrading a variety of mitochondrially synthesized subunits, including

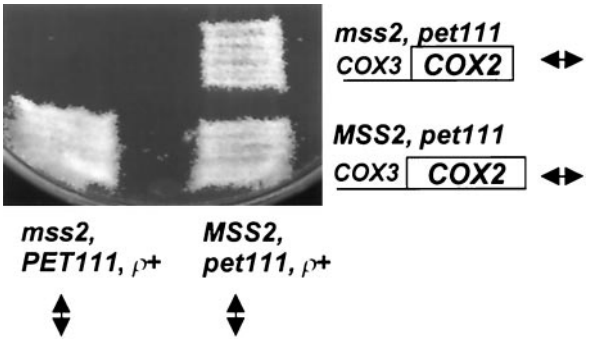

FIG. 3. *MSS2* function cannot be bypassed by placing the *COX3* mRNA 5′-UTL on the *COX2* mRNA. Haploid cells containing a synthetic ρ^- mtDNA bearing a chimeric gene specifying a *COX2* mRNA with the 5′-UTL of the *COX3* mRNA (33) were patched in horizontal stripes. Their relevant nuclear genotypes were *mss2 pet111* (SB26) and *MSS2 pet111* (SB23B). Cells containing wild-type ρ^+ mtDNA were patched in vertical stripes. Their relevant nuclear genotypes were *mss2 PET111* (SB12) and *MSS2 pet111* (NB39-9c). The stripes were cross-printed on complete medium, and diploids were selected. The diploids were printed to nonfermentable medium (YPEG) and incubated for 2 days at 30°C.

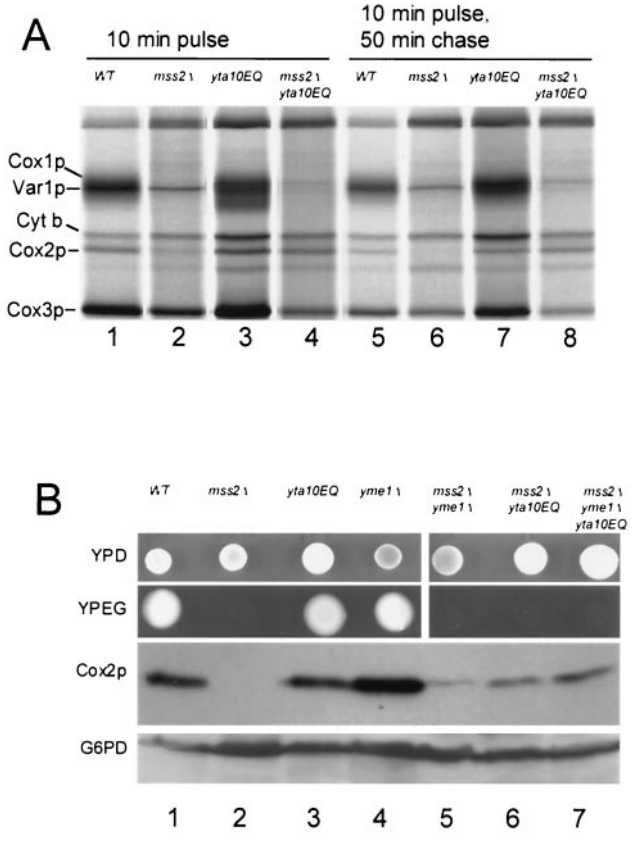

FIG. 4. Inactivation of Yta10p stabilizes Cox2p in an *mss2*Δ mutant but does not restore respiratory function. (A) Cells were incubated with cycloheximide, pulsed with [^{35}S]methionine for 10 min (lanes 1 to 4), and chased with cold methionine for 50 min (lanes 5 to 8). Crude mitochondria were analyzed on an SDS–15% polyacrylamide gel, followed by autoradiography. Lanes: 1 and 4, wild type (WT; J303-1a); 2 and 6, *mss2*Δ (SB12); 3 and 7, *yta10E559Q* (YGS103); 4 and 8, *mss2*Δ *yta10E559Q* double mutant (SB49C). (B) Cells were grown overnight in complete medium and spotted onto YPD and YPEG as indicated or used to prepare whole-cell extracts. Equal amounts of extracts were analyzed by Western blotting with anti-Cox2p and anti-glucose-6-phosphate dehydrogenase (G6PD). Lanes: 1, wild type (WT; J303-1a); 2, *mss2*Δ (SB48B); 3, *yta10E559Q* (YGS103); 4, *yme1*Δ (SB100); 5, *mss2*Δ *yme1*Δ (SB101); 6, *mss2*Δ *yta10EQ* (SB49C); 7, *mss2*Δ *yta10EQ yme1*Δ (SB103).

subunits I and III of cytochrome oxidase (3, 19). In addition to proteolytic activity, the *m*-AAA complex has chaperone activity required for assembly of respiratory complexes and respiration-dependent growth (2, 57, 62). Mutational inactivation of the proteolytic domain of Yta10p prevents proteolysis of unassembled respiratory subunits but does not affect assembly of respiratory subunits and respiratory competence (3). Although Cox2p has not previously been identified as a substrate of the *m*-AAA complex, we tested whether proteolytic inactivation of Yta10p by the *yta10E559Q* mutation stabilized Cox2p in the absence of Mss2p.

We first analyzed the accumulation of newly synthesized Cox2p in the *mss2*Δ *yta10E559Q* mutant by using cycloheximide pulse-labeling. After a 10-min pulse followed by a 50-min chase, we found that Cox2p was stabilized in the *mss2*Δ *yta10E559Q* double mutant (Fig. 4A, lane 8) relative to the *mss2*Δ single mutant (Fig. 4A, lane 6). In addition, Western analysis of whole-cell extracts revealed that the steady-state level of Cox2p was increased in the *mss2*Δ *yta10E559Q* double mutant relative to the *mss2*Δ single mutant (Fig. 4B, lane 2 and lane 6), although not to wild-type levels. These data indicate that the Yta10p protease participates in degradation of Cox2p in the absence of Mss2p. Nevertheless, although Cox2p was more stable in the double mutant, the *yta10E559Q* mutation did not restore any detectable respiratory growth in the absence of Mss2p (Fig. 4B).

Yme1p is required for the degradation of Cox2p that remains unassembled due to the absence of Cox4p (34, 64) or cytochrome *c* (38), and was thus a likely candidate for degrading Cox2p in the absence of Mss2p. We constructed an *mss2*Δ *yme1*Δ double mutant and examined Cox2p accumulation by using Western analysis of whole-cell extract (Fig. 4B). Cox2p was slightly stabilized in the *mss2*Δ *yme1*Δ double mutant relative to the *mss2*Δ single mutant (Fig. 4B, lanes 2 and 5). However, inactivation of both Yme1p and Yta10p proteases did not restore Cox2p levels to a much greater extent than inactivation of Yta10p protease alone (Fig. 4B, lanes 6 and 7). Thus, in contrast to its function in *MSS2* strains (34, 38, 64), Yme1p has only a minor role in degradation of Cox2p in the absence of Mss2p.

Mss2p is required for export of the Cox2p C-terminal domain. Normally, the nuclearly encoded Arg8p is synthesized in the cytoplasm and imported into the mitochondrial matrix where it participates in arginine biosynthesis. The synthetic mitochondrially encoded *ARG8^m* produces the same functional biosynthetic enzyme, which is able to complement a nuclear *arg8* mutation (54). When the Arg8p moiety is fused to the C terminus of Cox2p (Cox2-Arg8p), it is translocated as a passenger protein through the inner membrane to the IMS (21). In this case, the exported Arg8p is unable to participate in arginine synthesis, causing an Arg$^-$ phenotype in certain strain backgrounds (22). However, the Cox2p moiety of the fusion protein, which is largely detached from Arg8p by proteolysis in the IMS, assumes its correct membrane topology, and can assemble into active cytochrome oxidase. Thus, the resulting cells are respiratory competent (Pet$^+$). We have used the Cox2-Arg8p fusion to identify mutants which are export defective by selecting for Arg$^+$ Pet$^-$ phenotypes (22; S. A. Saracco and T. D. Fox, unpublished data).

We screened transposon mutagenized yeast cells (11) containing the *COX2::ARG8^m* fusion for the export-defective Pet$^-$ Arg$^+$ phenotype. Characterization of one such mutant revealed that the export defective phenotype was due to a transposon insertion at the *MSS2* locus. Sequence analysis indicated that this allele, *mss2::*Tn, would give rise to a truncated protein lacking the C-terminal third of Mss2p. The *mss2::*Tn allele appears to retain some function since a complete *mss2*Δ mutation, coupled with the *COX2::ARG8^m* fusion, caused an Arg$^-$ phenotype in this strain background. The basis for the difference in Arg growth phenotype between the *mss2::*Tn and the *mss2*Δ alleles remains unknown.

Previous studies suggested that the Cox2-Arg8p fusion is more difficult for mitochondria to export than wild-type Cox2p (22). We therefore first sought to determine whether the assembly of wild-type Cox2p was defective in the *mss2::*Tn mutant. Assembled Cox2p is resistant to proteolysis in solubilized mitochondria, whereas unassembled Cox2p is not (22). Mito-

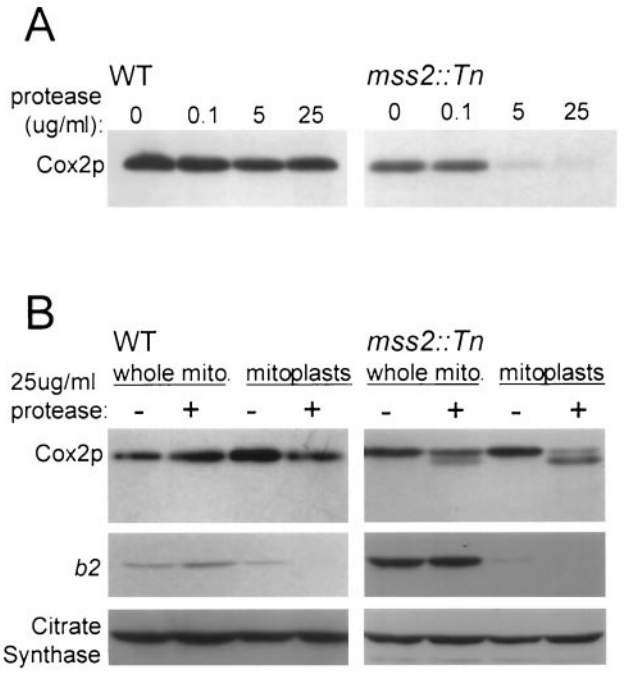

FIG. 5. Mss2p is required for export of the Cox2 C terminus. (A) *mss2* mutants are defective in assembly of Cox2p into the proteolytically resistant cytochrome oxidase complex. Purified mitochondria (180 μg) derived from wild type (WT; DBY947) or the *mss2::*Tn mutant (SB44) were solubilized in 1% octylglucopyranoside and incubated with proteinase K at the indicated concentrations. Samples were analyzed by Western blotting with a monoclonal antibody that recognizes an epitope in the Cox2p C terminus (CCO6) (21). Because steady-state levels of Cox2p are reduced in the *mss2::*Tn mutant, detection of Cox2p in *mss2::*Tn extract required a longer exposure time than detection of Cox2p in wild-type extract. (B) The Cox2p C-terminal domain is protected from protease by the inner membrane of mitoplasts. Mitochondria (180 μg) from wild type (WT; DBY947) or *mss2::*Tn (SB44) were incubated with or without 25 μg of proteinase K/ml or converted to mitoplasts and incubated with or without 25 μg of proteinase K/ml, as indicated. Samples were analyzed by Western blotting with the Cox2p antibody (CCO6), the cytochrome b_2 antibody, or the citrate synthase antibody. Cytochrome b_2 is a IMS marker which is used to assess mitoplasting efficiency. Citrate synthase is a matrix space marker used to demonstrate that the mitochondrial inner membrane is still intact.

chondria from wild-type or *mss2::*Tn cells were solubilized with detergent, subjected to increasing amounts of proteinase K, and analyzed by Western blotting with anti-Cox2p. Because steady-state levels of Cox2p are reduced in the *mss2::*Tn mutant, detection of Cox2p in *mss2::*Tn extract required a longer exposure time than detection of Cox2p in wild-type extract. As expected, assembled Cox2p from wild-type mitochondria was resistant to degradation (Fig. 5A). In contrast, Cox2p from *mss2::*Tn mitochondria was highly susceptible to degradation when exposed to as little as 5 μg of proteinase K/ml and was largely abolished when incubated with 25 μg of proteinase K/ml (Fig. 5A). Analysis of *mss2*Δ mitochondria yielded similar results (data not shown).

We next assessed whether the Cox2p C terminus is exported across the inner membrane in the absence of Mss2p function. If the unassembled Cox2p in the *mss2::*Tn mutant were exported across the inner membrane, then it would be sensitive to exogenously added protease in mitoplasts, lacking the outer membrane. If unassembled Cox2p were not exported in the mutant, it would remain in the matrix and be protected from protease degradation by the inner membrane. Purified mitochondria derived from the *mss2::*Tn mutant were converted to mitoplasts by osmotic shock. Mitochondria or mitoplasts were subjected to 25 μg of proteinase K/ml, sufficient to degrade unassembled Cox2p in solubilized mitochondria. Samples were analyzed by Western blotting with an antibody to the Cox2p C terminus. In mitoplasts derived from the *mss2::*Tn mutant, the Cox2p C terminus was protected from protease but shortened (Fig. 5B). Thus, the unassembled Cox2p was protected by the inner membrane. The shortening of Cox2p by added protease in this experiment was presumably due to successful export of the Cox2p N terminus in the absence of Mss2p (see below). Similar results were obtained in analysis of mitochondria from the *mss2*Δ mutant (data not shown). As expected, Cox2p derived from wild-type mitoplasts was resistant to degradation (Fig. 5B, left), even though it is properly exported, because it is assembled into a proteolytically resistant complex. Analogous experiments were performed on *mss2* mutants carrying the Cox2-Arg8 fusion protein. Consistent with the aforementioned results, the Cox2p-Arg8p fusion protein was protected from protease by the inner membranes of the *mss2* mutants, as previously observed for *pnt1* mutants (22) (data not shown). Taken together, these data indicate that the absence of Mss2p inhibits assembly of Cox2p into cytochrome oxidase, because the Cox2p C terminus is not exported across the inner membrane.

In the absence of Mss2p, the Cox2 N terminus is exported normally. The experiment of Fig. 5 suggests that the N terminus of Cox2p may be successfully exported in the *mss2::*Tn mutant. To further examine Cox2p N-terminal export, we took advantage of a fusion protein in which the first 67 amino acids of Cox2p, containing the first transmembrane domain, are fused to mitochondrially coded Arg8p. In otherwise wild-type cells, the Cox2p N-terminal domain of Cox2 (amino acids 1 to 67)-Arg8p [Cox2(1-67)-Arg8p] is translocated through the inner membrane to the IMS, while the Arg8p moiety remains in the matrix (21). Mitochondria or mitoplasts from wild-type or *mss2*Δ cells containing the Cox2(1-67)-Arg8p fusion were incubated with or without proteinase K. In wild-type mitoplasts subjected to protease, Cox2-Arg8p was shortened by removal of the exposed Cox2p N-tail (Fig. 6A). The fusion protein behaved similarly in *mss2*Δ mitoplasts subjected to protease, indicating that its Cox2p N tail is exported in the absence of Mss2p function (Fig. 6A).

Cox2p is synthesized as a precursor protein (pre-Cox2p) (43, 50). After N-tail export, the first 15 amino acids of pre-Cox2p are cleaved by the Imp protease complex, producing mature Cox2p (36, 43, 47). In an *imp1* mutant, the pre-Cox2p leader peptide is not processed, and the precursor can be detected as a slower-migrating protein after pulse-labeling in the presence of cycloheximide. We therefore used pulse-labeling to study the relative size of Cox2p in the *mss2*Δ mutant. Wild-type, *pet111*, *imp1*, or *mss2*Δ cells were pulse-labeled for 10 min, and mitochondrial translation products were analyzed on a 12% gel (Fig. 6B). As expected, no Cox2p accumulated in the *pet111* mutant that is unable to synthesize Cox2p. However, Cox2p derived from the *mss2*Δ mutant was similar in size to wild-type

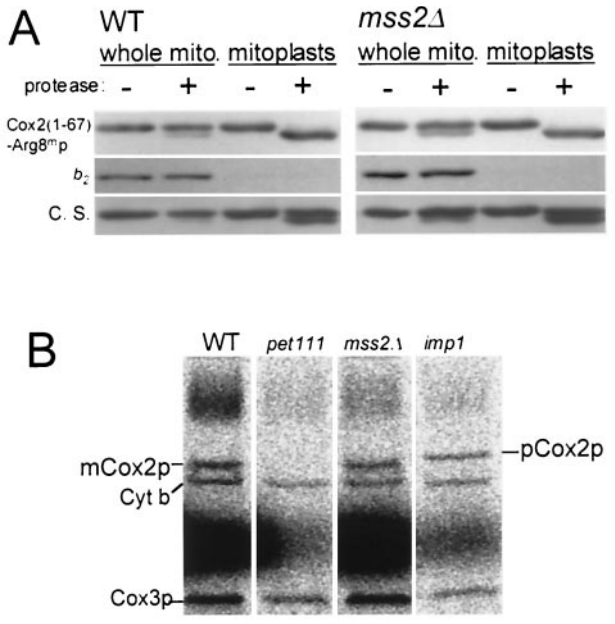

FIG. 6. Mss2p is not required for export of the Cox2 N terminus. (A) The N tail of Cox2 (1-67)-Arg8^{m}p is exported and susceptible to exogenously added protease. Purified mitochondria and mitoplasts derived from wild type (WT; SH36) or *mss2*Δ (SB35) cells were incubated with or without 75 μg of proteinase K/ml, and samples were analyzed by Western blotting with anti-Arg8p, anti-b_2, and anti-citrate synthase (C.S.). (B) The leader peptide of pre-Cox2p is processed normally in the absence of Mss2p. Cells were incubated in the presence of cycloheximide and pulsed for 10 min with [^{35}S]methionine. Mitochondrial translation products were separated on an SDS–12% polyacrylamide gel and analyzed by autoradiography. (The relative mobilities of Cox2p and cytochrome *b* are reversed on 12% gels relative to the 15% gels used in other figures.) Strains: wild type (WT; PTH366); *pet111*, ECS108; *mss2*, SB12; *imp1*, SH105. The mature cleaved form of Cox2p is indicated as mCox2p, while uncleaved pre-Cox2p is indicated as pCox2p.

Cox2p and shorter than Cox2p from the *imp1* mutant. These data confirm that N-tail export and subsequent processing occur normally in the absence of Mss2p.

Mss2p is a mitochondrial matrix protein that is peripherally associated with the inner membrane. To determine the cellular localization of Mss2p, we tagged the protein by attaching codons for a triple HA epitope to the chromosomal *MSS2* gene (see Materials and Methods). Cells containing the tagged protein were respiratory competent, indicating that Mss2p-HA was functional. Whole-cell extracts derived from cells containing *MSS2-HA* or *MSS2* were analyzed by Western blotting with the anti-HA antibody (Fig. 7A). Anti-HA specifically reacted with a doublet band of the expected size for Mss2p-HA in the extract derived from the tagged strain (Fig. 7A). Purified mitochondria and cytosolic fractions derived from the *MSS2-HA* strain were analyzed by Western blotting with the anti-HA antibody (Fig. 7B). Mss2p-HA was found predominantly in the mitochondrial fraction (Fig. 7B, lane 1). Thus, Mss2p-HA is localized to the mitochondria.

We next determined the submitochondrial location of Mss2p-HA. Mitochondria derived from *MSS2-HA* cells were sonicated to disrupt mitochondrial membranes and centrifuged to separate insoluble membrane proteins from soluble proteins. Western analysis revealed that Mss2p-HA was found in the insoluble membrane fraction (Fig. 7C, lane 1) and not in the soluble supernatant (Fig. 7C, lane 2), indicating that Mss2p is associated with mitochondrial membranes. Consistent with membrane association, Mss2p-HA was solubilized by the addition of 1% Triton X-100 (not shown). To determine whether Mss2p is peripherally or integrally associated with mitochondrial membranes, alkaline carbonate extraction was performed on the insoluble membrane fraction. Peripheral membrane proteins are extracted from membranes by using alkaline carbonate, while integral membrane protein are not (15). Mss2p-HA was largely extracted from the membrane pellet (Fig. 7C, lane 4), indicating that Mss2p is a peripheral membrane protein. Consistent with this observation, the hydrophobicity profile (29) of Mss2p does not indicate the presence of any membrane spanning domains (data not shown). Finally, we analyzed the protease sensitivity of Mss2p-HA in mitoplasts (Fig. 7D). Mss2p-HA was protected from degradation by the inner membrane of mitoplasts. Taken together, these data indicate that Mss2p is a mitochondrial matrix protein that is peripherally associated with the inner surface of the inner membrane.

DISCUSSION

The nuclear gene *MSS2* was originally identified by mutations that prevented accumulation of mitochondrially coded Cox2p by blocking an undefined posttranscriptional step (52), and we have set out to further elucidate its function. We found that the gene product, Mss2p, is present in the mitochondrial matrix as a peripherally bound inner membrane protein and thus presumably has a direct role in mitochondrial gene expression.

Mss2p is not required for translation of the *COX2* mRNA, since Cox2p was pulse-labeled normally in an *mss2*Δ mutant. In addition, the *mss2*Δ had no effect on expression from the *COX2* locus of the mitochondrial reporter gene *ARG8^{m}*. Despite normal synthesis in the absence of Mss2p, pulse-labeled Cox2p was largely degraded during a chase period, and steady-state accumulation was dramatically decreased. Therefore, Mss2p functions to stabilize Cox2p. Proper localization of Cox2p synthesis depends upon targeting information in the untranslated portions of its mRNA, and incorrect targeting can lead to degradation of the protein (46). However, the Mss2p function could not be bypassed by synthesis of Cox2p from a chimeric mRNA with the 5′-untranslated leader of the *COX3* mRNA, indicating that Mss2 is not involved in mRNA localization.

A key clue to the function of Mss2p was our isolation of an *mss2* mutant in a genetic screen (22) for strains with defects in the ability to export the C-terminal domain of a Cox2p-Arg8p fusion protein from the matrix. By examining the protease sensitivity of Cox2p in mitochondria and mitoplasts from the *mss2* mutant, we found that it is not assembled into the cytochrome oxidase complex and that the Cox2p C-terminal domain remained inside the inner membrane. However, the Cox2p N-terminal domain was exported efficiently. A previous study demonstrated that export of the Cox2p C-terminal domain depends upon a potential across the inner membrane, while export of the N-terminal domain does not (21). Our observation that the *mss2*Δ blocks C-tail export, but not N-tail

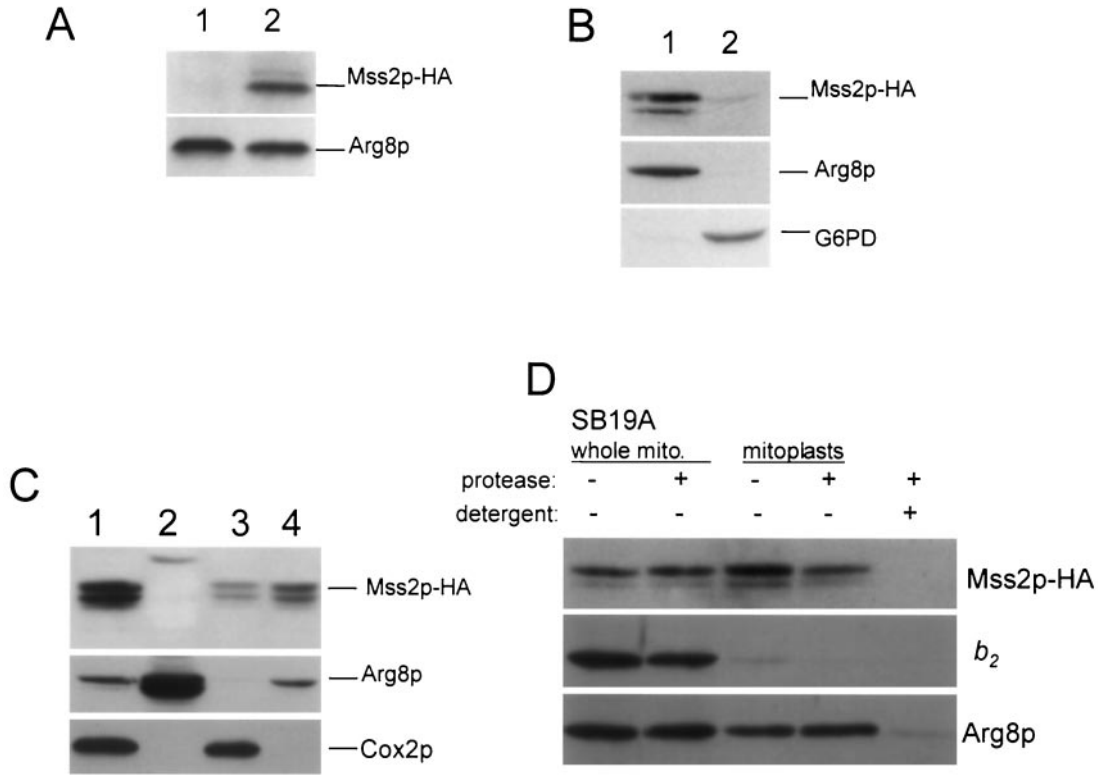

FIG. 7. Mss2p is a mitochondrial matrix protein that is peripherally associated with the inner membrane. (A) Whole-cell extracts derived from cells containing *MSS2-HA* or *MSS2* were analyzed by Western blotting with the antibody against the HA epitope (3F10) and anti-Arg8p. Lanes: 1, *MSS2* (TF215); 2, *MSS2-HA* (SB19A). (B) Mss2p-HA copurifies with mitochondria. Purified mitochondria (see Materials and Methods) (lane 1) or cytosol (lane 2) derived from the *MSS2-HA* strain (SB19A) were analyzed by Western blotting with anti-HA, anti-Arg8p, and anti-glucose-6-phosphate (G6PD). (C) Mss2p-HA is a peripheral membrane protein. Purified mitochondria from the *MSS2-HA* strain were sonicated and centrifuged to separate the insoluble membrane fraction (lane 1) and the soluble fraction (lane 2). The membrane pellet was extracted with sodium carbonate and centrifuged to separate insoluble integral membrane proteins (lane 3) from solubilized peripheral membrane proteins (lane 4). Samples were analyzed by using Western blotting with anti-HA, anti-Arg8p, and anti-Cox2p. (D) Mss2p-HA is largely protected from protease by the inner membrane. Purified mitochondria or mitoplasts containing Mss2p-HA were subjected to digestion with proteinase K. Samples were analyzed by using Western blotting with anti-HA, anti-cytochrome b_2, and anti-Arg8p.

export, confirms that these two processes are mechanistically distinct.

Degradation of Cox2p in the *mss2*Δ mutant is presumably triggered by failure to export the C tail and thus likely to initiate on the matrix side of the inner membrane. Consistent with this idea, we found that the IMS-localized ATP-dependent *i*-AAA protease Yme1p plays only a minor role in degrading Cox2p in an *mss2* mutant, in contrast to its role in *MSS2* strains (34, 38, 64). We found instead that in vivo degradation of newly synthesized pulse-labeled Cox2p was almost entirely blocked by inactivation of the matrix localized *m*-AAA protease subunit, Yta10p(Afg3p). However, when we examined the steady-state level of Cox2p in an *mss2*Δ mutant lacking this proteolytic activity, it was increased substantially relative to the *mss2*Δ containing the protease but was still far lower than that of the wild type. Thus, it appears that rapid degradation of Cox2p in the absence of Mss2p is carried out largely by Yta10p, but it is not the only protease to participate in Cox2p degradation over longer time periods. Since proteolytic inactivation of both Yta10p and Yme1p did not completely restore Cox2p levels, it is clear that other mitochondrial proteases must be involved in the degradation of Cox2p in the absence of Mss2p. Candidates include the Yta12p(Rca1p) component of the *m*-AAA protease and the *lon* homolog Pim1p (56, 63).

It is important to note that although Cox2p accumulation is increased by inactivation of Yta10p, there is no detectable suppression of the *mss2*Δ respiratory growth defect. Thus, it appears that the primary effect of *mss2*Δ is to prevent export of the Cox2p C tail and that the stability defect is secondary. In addition to Mss2p, three other proteins are known to have roles in exporting domains of Cox2p through the inner membrane. N-tail export requires the activity of Oxa1p (21, 23), a conserved integral inner membrane protein (4, 7, 9, 25, 27, 49), which interacts directly with mitochondrially synthesized polypeptides (24). Cox2p C-tail export is also dependent upon Oxa1p (21, 23), but this could be an indirect effect if C-tail export is dependent for other reasons upon prior translocation of the N-tail. Pnt1p is an integral inner membrane protein required for export of the Cox2p-Arg8p fusion protein (22). Pnt1p is not essential for C-tail export in *S. cerevisiae* but is essential in *Kluyveromyces lactis* (22). Finally, Cox18p, an integral inner membrane protein required for cytochrome oxidase assembly (53), has been found to be necessary for C-tail but not for N-tail export (Saracco and Fox, unpublished).

The precise role of Mss2p in Cox2 C-tail export is unclear.

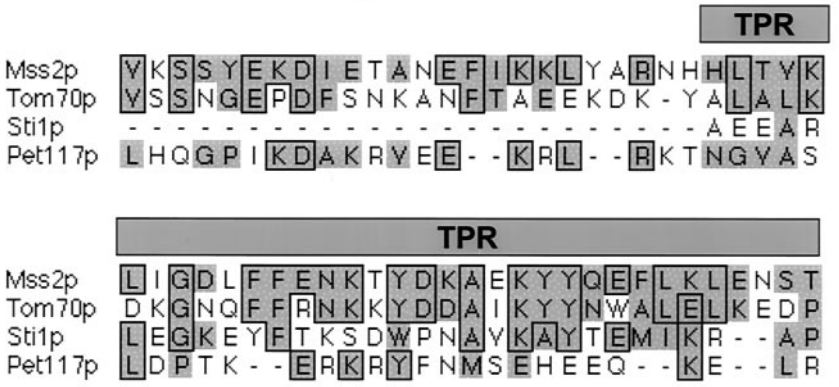

FIG. 8. Mss2p has at least one TPR-like motif. Analysis of the Mss2p sequence by using the ProDom protein domain database (http://www.toulouse.inra.fr/prodom.html) indicated the presence of a TPR-like motif (12). This region of Mss2p is aligned with TPR motifs of Tom70p, Sti1p, and Pet117 (see Discussion). Boxed letters represent amino acid identity with Mss2p sequence. Shaded letters represent amino acid similarity with the Mss2p sequence. The gray bar above the sequences delimits the TPR-like motif in Mss2p.

Mss2p is unlikely to function in establishing the inner membrane potential necessary for Cox2 C-tail export, because inner membrane potential is required for the essential process of protein import (40), while *MSS2* is not an essential gene. Mss2p is unlikely to function as a translocase since it is not embedded in the inner membrane. However, Mss2p could function as a receptor and/or chaperone to deliver the Cox2p C-terminal domain to its translocation system. In this scenario, Mss2p could either interact directly with Cox2p or indirectly through other components. For example, it could organize other mitochondrial chaperones (mtHsp70) or translocation machinery components (Pnt1p, Oxa1p, and Cox18p). Implicit in this proposal is the idea that Cox2p C-tail export may be a posttranslational process (see below), in contrast to N-tail export, which is likely to occur during synthesis (21, 24, 42).

We selected the *mss2::*Tn allele owing to the fact that it prevents export of the Cox2p-Arg8p fusion protein and thereby causes an Arg^+ growth phenotype. Surprisingly, however, the *mss2*Δ allele in the same strain background does not cause an Arg^+ phenotype as a result of blocking export. This is intriguing since both strains contain similar steady-state levels of the Cox2p-Arg8p fusion protein in the mitochondrial matrix (data not shown). Clearly the *mss2::*Tn allele has a partial function lacking in the deletion. Perhaps this partial function assists folding of the Arg8p enzymatic domain fused to Cox2p. However, Mss2p is not absolutely required for folding Arg8p, since in another strain background with more robust mitochondrial gene expression, the *mss2*Δ does not cause an Arg^- phenotype in strains expressing the Cox2-Arg8p fusion. The Pet^- phenotype of *mss2* mutations is not affected by strain background.

Analysis of the Mss2p sequence has not revealed any close homologs. However, Mss2p contains at least one motif resembling a TPR sequence (Fig. 8). The TPR motif comprises a highly degenerate 34-amino-acid sequence which has been implicated in a variety of protein-protein interactions (5). Interestingly, the Mss2p TPR domain is very similar to one of the TPR domains present in Tom70p, a receptor on the surface of the mitochondrial outer membrane for certain proteins imported from the cytoplasm (10, 26, 28, 55). The TPR motifs of Tom70p are thought to facilitate interactions with other proteins involved in the import process (20, 28). The Mss2p TPR-like motif is also similar to a domain of Sti1p, a cytosolic cochaperonin whose TPR motifs mediate interaction with Hsp90p (44). Interestingly, the uncharacterized product of the yeast gene *PET117*, which is required for cytochrome oxidase assembly (31), also contains a TPR motif similar to that of Mss2p. We suggest that, based on these observations, the folding of the Cox2 C tail may be obligatory for subsequent export to the IMS and that Mss2p may function to organize this folding process on the matrix side of the inner membrane. The export pathway for the Cox2p C tail may be similar to several other pathways known to translocate folded proteins across membranes (58).

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