**The EMBO Journal** Vol.17 No.16 pp.4837–4847, 1998

# The formation of respiratory chain complexes in mitochondria is under the proteolytic control of the *m*-AAA protease

**Heike Arlt, Gregor Steglich, Robert Perryman[1], Bernard Guiard[2], Walter Neupert and Thomas Langer[3]**

Institut für Physiologische Chemie der Universität München, Goethestrasse 33, 80336 München, Germany and [2]Centre de Genetique Moleculaire CNRS, Université Pierre et Marie Curie, 91190 Gif-sur-Yvette, France

[1]Present address: Department of Neuropathology, Beaumont Hospital, Dublin 9, Ireland

[3]Corresponding author
e-mail: Langer@bio.med.uni-muenchen.de

H.Arlt and G.Steglich contributed equally to this work

**Yta10p (Afg3p) and Yta12p (Rcal1p), members of the conserved AAA family of ATPases, are subunits of the mitochondrial *m*-AAA protease, an inner membrane ATP-dependent metallopeptidase. Deletion of *YTA10* or *YTA12* impairs degradation of non-assembled inner membrane proteins and assembly of respiratory chain complexes. Mutations of the proteolytic sites in either *YTA10* or *YTA12* have been shown to inhibit proteolysis of membrane-integrated polypeptides but not the respiratory competence of the cells, suggesting additional activities of Yta10p and Yta12p. Here we demonstrate essential proteolytic functions of the *m*-AAA protease in the biogenesis of the respiratory chain. Cells harbouring proteolytically inactive forms of both Yta10p and Yta12p are respiratory deficient and exhibit a pleiotropic phenotype similar to Δ*yta10* and Δ*yta12* cells. They show deficiencies in expression of the intron-containing mitochondrial genes *COX1* and *COB*. Splicing of *COX1* and *COB* transcripts is impaired in mitochondria lacking *m*-AAA protease, whilst transcription and translation can proceed in the absence of Yta10p or Yta12p. The function of the *m*-AAA protease appears to be confined to introns encoding mRNA maturases. Our results reveal an overlapping substrate specificity of the subunits of the *m*-AAA protease and explain the impaired assembly of respiratory chain complexes by defects in expression of intron-containing genes in mitochondria lacking *m*-AAA protease.**

*Keywords*: AAA-proteases/ATP-dependent proteolysis/mitochondria/mRNA splicing/Yta10p/Yta12p

## Introduction

Several proteases which serve a number of different purposes have been identified in mitochondria. Specific processing peptidases cleave off targeting sequences from nuclear-encoded mitochondrial preproteins by limited proteolysis (Pratje *et al.*, 1994; Brunner and Neupert, 1995; Isaya and Kalousek, 1995). Misfolded polypeptides possibly harmful to mitochondrial function are removed by ATP-dependent proteases which are present in various subcompartments of mitochondria (Langer and Neupert, 1996; Rep and Grivell, 1996; Suzuki *et al.*, 1997). Studies in yeast also indicate crucial regulatory functions of these proteases in mitochondrial biogenesis, although target proteins in mitochondria which are under proteolytic control remain to be identified.

The examination of the stability of mitochondrial translation products provided first evidence for the presence of ATP-dependent proteases in the inner membrane of mitochondria (Wheeldon *et al.*, 1974; Kalnov *et al.*, 1979; Yasuhara *et al.*, 1994). Energy-transducing complexes in the inner membrane are composed of nuclearly and mitochondrially encoded subunits. In the absence of a supply of cytoplasmically synthesized polypeptides, non-assembled mitochondrial translation products are subject to rapid proteolysis. Two homologous inner membrane proteins, Yta10p (Afg3p) and Yta12p (Rca1p), were shown to be essential for the proteolytic process (Pajic *et al.*, 1994; Arlt *et al.*, 1996; Guélin *et al.*, 1996). A mutation in the conserved consensus binding site for divalent metal ions in either protein abolished the degradation of non-assembled mitochondrial translation products, suggesting that Yta10p and Yta12p exert metal-dependent proteolytic activity (Arlt *et al.*, 1996; Guélin *et al.*, 1996). Both proteins form a high molecular weight complex in the inner membrane which presumably is composed of several copies of each protein (Arlt *et al.*, 1996). Substrate polypeptides were found exclusively in association with assembled Yta10p and Yta12p. Yta10p and Yta12p harbour a conserved ATPase domain of ~230 amino acids characteristic for the AAA family of P-loop ATPases (for ATPases associated with diverse cellular activities) (Beyer, 1997; Patel and Latterich, 1998). Therefore, the Yta10p–Yta12p complex was termed *m*-AAA protease (Arlt *et al.*, 1996; Leonhard *et al.*, 1996). The prefix denotes the presence of the catalytically active sites of Yta10p and Yta12p on the matrix side of the inner membrane. The *m*-AAA protease belongs to a novel conserved class of membrane-bound ATP-dependent proteases, members of which were identified in eubacteria, mitochondria and chloroplasts (Beyer, 1997; Patel and Latterich, 1998).

The *m*-AAA protease is required for the maintenance of mitochondrial functions. Cells lacking either Yta10p or Yta12p lose respiratory competence and exhibit deficiencies in the assembly of the ATP synthase and respiratory chain complexes in the inner membrane (Guélin *et al.*, 1994; Tauer *et al.*, 1994; Tzagoloff *et al.*, 1994; Paul and Tzagoloff, 1995). A mutation in the consensus metal-binding site of Yta10p or Yta12p abolished the proteolytic breakdown of non-assembled inner membrane proteins, but neither the assembly of energy-transducing

complexes nor the respiratory competence of the cells (Arlt *et al.*, 1996; Guélin *et al.*, 1996). This observation was taken as evidence for additional functions of Yta10p or Yta12p which may be independent of their proteolytic activity (Arlt *et al.*, 1996; Guélin *et al.*, 1996). In support of this assumption, multicopy suppressors lacking proteolytic activity have been identified which maintain respiration in the absence of Yta10p or Yta12p (Rep *et al.*, 1996a). As a stable association of non-native polypeptides with the *m*-AAA protease has been observed, this complex may exert chaperone-like functions during the assembly of membrane protein complexes (Arlt *et al.*, 1996).

In an attempt to characterize the role of the *m*-AAA protease in mitochondrial biogenesis, we constructed a Δ*yta10*Δ*yta12* strain expressing proteolytically inactive forms of both Yta10p and Yta12p. The mutant proteins formed the same high molecular weight complex in the inner membrane as the wild-type proteins. Respiration and the assembly of respiratory chain complexes, however, were impaired in these cells, as they were in the absence of Yta10p or Yta12p. Further analysis of cells containing proteolytically inactive variants of both Yta10p and Yta12p revealed deficiencies in the synthesis of subunit 1 of the cytochrome *c* oxidase (Cox1) and of cytochrome *b* (Cob) which both are encoded by intron-containing genes. These results establish essential proteolytic functions of the ATP-dependent *m*-AAA protease in mitochondrial biogenesis.

## Results

### *Cells harbouring proteolytically inactive Yta10p and Yta12p exhibit a pleiotropic phenotype similar to yta10- and yta12-null mutants*

Yta10p and Yta12p contain a consensus binding site for divalent metal ions, HEXXH, which is characteristic of the proteolytic centre of metal-dependent peptidases (Jiang and Bond, 1992; Hooper, 1994; Barrett, 1997). The two histidine residues in this motif together with a variable third ligand and a water molecule mediate the binding of metal ions. The glutamate residue is dispensable for binding and serves exclusively a catalytic function during proteolysis (Vazeux *et al.*, 1996). To examine the importance of the proteolytic activity of the *m*-AAA protease for mitochondrial function, glutamate residues in the metal-binding sites of Yta10p and Yta12p were replaced by glutamine. The mutant variants were constitutively expressed from a centromere-based vector in Δ*yta10* (*yta10*$^{E559Q}$), Δ*yta12* (*yta12*$^{E614Q}$) and Δ*yta10*Δ*yta12* cells (*yta10*$^{E559Q}$*yta12*$^{E614Q}$). Respiration of Δ*yta10* and Δ*yta12* cells was restored upon expression of Yta10$^{E559Q}$p and Yta12$^{E614Q}$p, respectively (Arlt *et al.*, 1996; Guélin *et al.*, 1996). On the other hand, *yta10*$^{E559Q}$*yta12*$^{E614Q}$ mutant cells were respiration-deficient (Figure 1A). Cytochrome *b* and cytochromes *aa*$_3$ were absent in spectra of *yta10*$^{E559Q}$*yta12*$^{E614Q}$ cells, as they were in cells lacking Yta10p, Yta12p or both (data not shown). In contrast, mitochondrial cytochromes were detectable in Δ*yta10* and Δ*yta12* cells expressing Yta10$^{E559Q}$p and Yta12$^{E614Q}$p, respectively (data not shown).

To assess the assembly of the cytochrome *c* oxidase, we took advantage of the observation that only assembled subunits of the cytochrome *c* oxidase are resistant to added protease (Mason *et al.*, 1973; Rep *et al.*, 1996b). Mitochondria were isolated from the various mutant strains and, after solubilization with detergent, the trypsin resistance of subunit 4 of the cytochrome *c* oxidase (Cox4) was analysed (Figure 1B). Cox4 was protease sensitive in Δ*yta10* and Δ*yta12* cells, indicating that it was present in an unassembled state (Figure 1B). Upon expression of Yta10$^{E559Q}$p in Δ*yta*10 cells or Yta12$^{E614Q}$p in Δ*yta12* cells, Cox4 exhibited a similar trypsin resistance to that found in wild-type mitochondria (Figure 1B). In contrast,

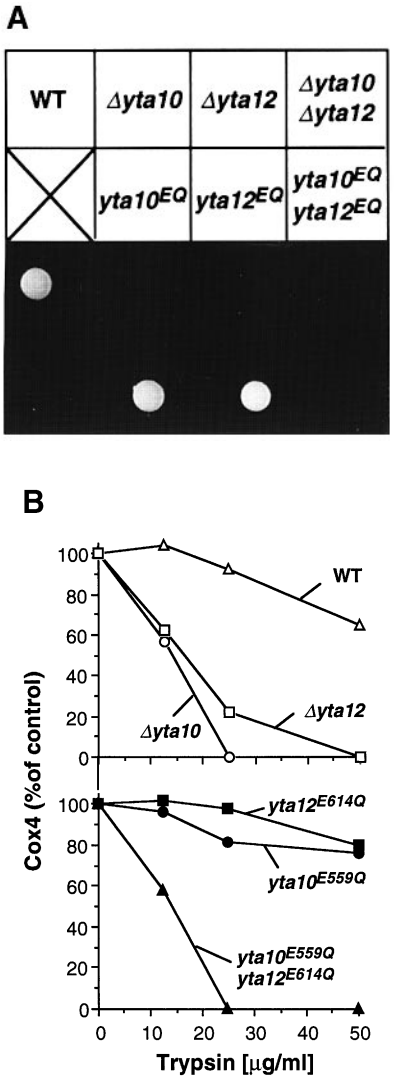


**Fig. 1.** Phenotypic analysis of *yta10*$^{E559Q}$*yta12*$^{E614Q}$ cells. (**A**) Expression of Yta10$^{E559Q}$p and Yta12$^{E614Q}$p in Δ*yta10*Δ*yta12* cells does not restore growth on non-fermentable carbon sources. Wild-type ('WT'), Δ*yta10* (YHA101), Δ*yta12* (YHA201), Δ*yta10*Δ*yta12* (YHA301), *yta10*$^{E559Q}$ ('*yta10*$^{EQ}$'; YHA103), *yta12*$^{E614Q}$ ('*yta12*$^{EQ}$'; YHA203) and *yta10*$^{E559Q}$*yta12*$^{E614Q}$ cells ('*yta10*$^{EQ}$*yta12*$^{EQ}$'; YGS302) were grown at 30°C to logarithmic phase on selective medium supplemented with the respective auxotrophic markers and 2% glucose. Cells were isolated by centrifugation, resuspended in $H_2O$ and spotted onto YP medium containing 3% glycerol. The plates were incubated for 3 days at 30°C. (**B**) Inactivation of the *m*-AAA protease impairs assembly of cytochrome *c* oxidase. The sensitivity of Cox4 to externally added trypsin was determined in mitochondria harbouring mutant *m*-AAA protease as described in Materials and methods. Cox4 was present at low levels in Δ*yta10*, Δ*yta12* and *yta10*$^{E559Q}$*yta12*$^{E614Q}$ mitochondria (see Figure 3A).

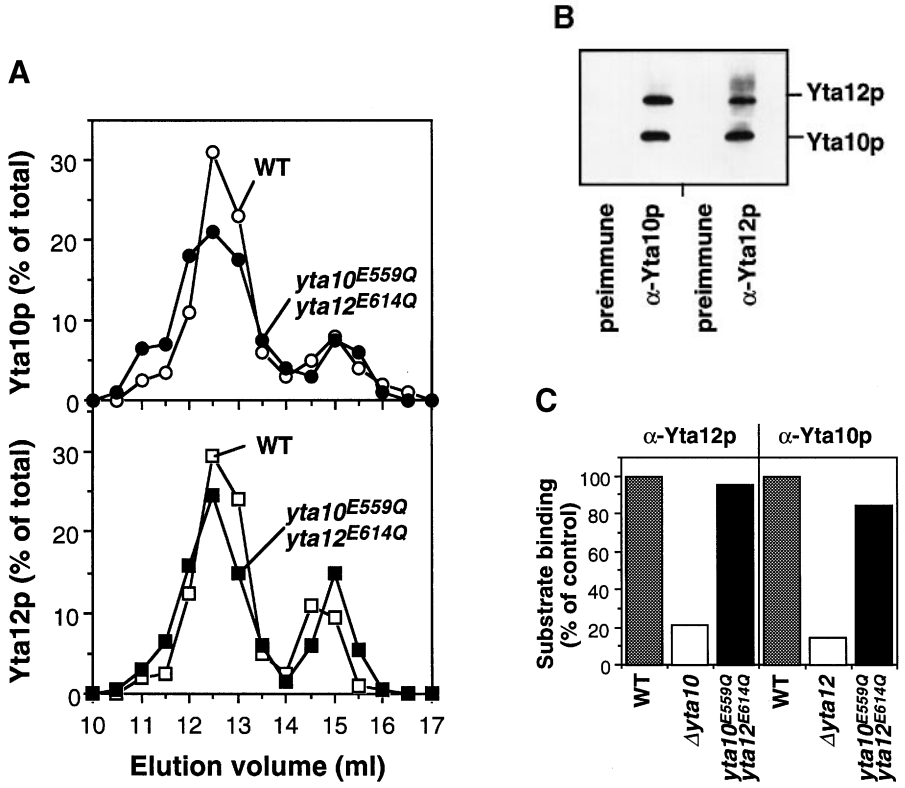


**Fig. 2.** Assembly and substrate binding of proteolytically inactive *m*-AAA protease. (**A**) Yta10$^{E559Q}$p and Yta12$^{E614Q}$p form a high molecular weight complex in the inner membrane. Wild-type ('WT') and *yta10*$^{E559Q}$*yta12*$^{E614Q}$ (YGS302) cells were solubilized and mitochondrial extracts were fractionated by Superose 6 gel chromatography as described (Arlt *et al.*, 1996). Yta10p and Yta12p were detected in the eluate fractions by immunoblotting using a chemiluminescence detection system. Protein amounts, determined by laser densitometry, are given as a percentage of total Yta10p or Yta12p in the eluate. Hsp60 (840 kDa), thyroglobulin (670 kDa), apoferritin (443 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa) and carboanhydrase (29 kDa) were used as standards for calibration. (**B**) Co-immunoprecipitation of Yta10$^{E559Q}$p and Yta12$^{E614Q}$p. Mitochondria (200 μg) isolated from *yta10*$^{E559Q}$*yta12*$^{E614Q}$ (YGS302) cells were solubilized and, after a clarifying spin, co-immunoprecipitations were performed with pre-immune and Yta10p- and Yta12p-specific antisera as described (Arlt *et al.*, 1996). The precipitates were analysed by SDS–PAGE and immunoblotting with Yta10p- and Yta12p-specific antisera. (**C**) Cross-linking of substrate polypeptides to proteolytically inactive *m*-AAA protease. Mitochondrially encoded polypeptides were labelled with [$^{35}$S]methionine for 20 min at 25°C in wild-type ('WT'), Δ*yta10* (YHA101), Δ*yta12* (YHA201) and *yta10*$^{E559Q}$*yta12*$^{E614Q}$ (YGS302) mitochondria and subjected to chemical cross-linking using 1,5-difluoro-2,4-dinitrobenzene (100 μM) as previously described (Arlt *et al.*, 1996). Association with Yta10p or Yta12p was determined by immunoprecipitation with Yta10p- or Yta12p-specific antisera, respectively. After SDS–PAGE, radioactivity in the precipitate was quantified using a phosphorimaging system to determine the translation efficiency. The amount of protein that was used in the immunoprecipitation was adjusted according to the incorporated radioactivity. $^{35}$S-Labelled polypeptides bound to Yta10p or Yta12p in wild-type mitochondria were set to 100% of control.

Cox4 was sensitive to degradation by trypsin added to detergent-lysed mitochondria harbouring proteolytically inactive forms of both Yta10p and Yta12p (Figure 1B). Thus, similarly to cells lacking Yta10p and Yta12p, the assembly of the cytochrome *c* oxidase is impaired in *yta10*$^{E559Q}$*yta12*$^{E614Q}$ mutant cells.

These experiments reveal strikingly different effects of mutations in the proteolytic sites of Yta10p and Yta12p on mitochondrial functions: the respiratory activity of yeast cells and the assembly of respiratory chain complexes remain unaffected in the presence of either proteolytically inactive Yta10p or Yta12p. In contrast, mutations in the proteolytic sites of both Yta10p and Yta12p impair respiration and the assembly of respiratory complexes, a phenotype also observed with *yta10*- and *yta12*-null mutant strains. These results point to essential proteolytic functions of the *m*-AAA protease in mitochondrial biogenesis, and suggest that the protease exerts residual activity after mutating the proteolytic site of one subunit.

### *Mutations in the proteolytic sites of Yta10p and Yta12p do not affect m-AAA protease assembly or substrate binding*

Yta10p and Yta12p appear to perform their proteolytic function only after assembly into a high molecular weight complex in the inner membrane (Arlt *et al.*, 1996). To exclude indirect effects of mutations in the proteolytic sites, complex formation of Yta10$^{E559Q}$p and Yta12$^{E614Q}$p was examined. Mitochondria were isolated from wild-type and *yta10*$^{E559Q}$*yta12*$^{E614Q}$ cells and gel filtration experiments were performed after solubilization of mitochondrial membranes. Proteolytically inactive Yta10p or Yta12p, like the wild-type proteins, co-eluted from the sizing column in two peaks corresponding to apparent molecular masses of ~1000 and 250 kDa (Figure 2A). The large complex contains assembled Yta10p and Yta12p, while the 250 kDa form may correspond to a homooligomeric assembly intermediate of both proteins (Arlt *et al.*, 1996).

Assembly of Yta10$^{E559Q}$p and Yta12$^{E614Q}$p was demonstrated directly by co-immunoprecipitation in further experiments. After solubilization of *yta10$^{E559Q}$yta12$^{E614Q}$* mitochondria with Triton X-100, extracts were incubated with pre-immune, Yta10p- and Yta12p-specific antisera. The precipitates were analysed by Western blotting. Immunoprecipitation of Yta12$^{E614Q}$p with Yta10p-specific antiserum and of Yta10$^{E559Q}$p with Yta12p-specific antiserum was observed (Figure 2B). In contrast, Yta10$^{E559Q}$p and Yta12$^{E614Q}$p were not precipitated with pre-immune serum (Figure 2B). These results demonstrate that the assembly of the *m*-AAA protease is not affected by the mutations in the proteolytic sites of its subunits.

The binding of substrate polypeptides to the proteolytically inactive *m*-AAA protease was examined by chemical cross-linking (Figure 2C). Mitochondrially encoded polypeptides were synthesized in the presence of [$^{35}$S]methionine in wild-type and *yta10$^{E559Q}$yta12$^{E614Q}$* mitochondria which were then subjected to chemical cross-linking using 1,5-difluoro-2,4-dinitrobenzene. After solubilization of the mitochondria, Yta10$^{E559Q}$p and Yta12$^{E614Q}$p were immunoprecipitated with Yta10p- and Yta12p-specific antisera, respectively. Binding of substrate polypeptides occurred with similar efficiency to the proteolytically active and inactive forms of Yta10p and Yta12p (Figure 2C). This finding also substantiates the association of Yta10$^{E559Q}$p with Yta12$^{E614Q}$p, as substrate binding was strongly reduced in Δ*yta10* and Δ*yta12* mitochondria and thus occurs only to the assembled *m*-AAA protease (Figure 2C).

We conclude from these results that the replacement of the glutamic acid residues in the consensus metal-binding sites of Yta10p or Yta12p by glutamine does not interfere with the assembly of the *m*-AAA protease nor with the binding of substrate polypeptides to the complex. Thus, the impaired respiratory competence of *yta10$^{E559Q}$yta12$^{E614Q}$* cells suggests crucial proteolytic functions of the *m*-AAA protease for the assembly of the respiratory chain.

### *Mitochondrially encoded subunits of the cytochrome c oxidase do not accumulate in mitochondria lacking m-AAA protease*

In order to identify processes in mitochondria which are under the proteolytic control of the *m*-AAA protease, we further determined its role in the biogenesis of the cytochrome *c* oxidase. Cox4 accumulated at strongly reduced levels in Δ*yta10*, Δ*yta12* and *yta10$^{E559Q}$yta12$^{E614Q}$* mitochondria (Figure 3A). Similarly, the mitochondrially encoded subunits Cox1, Cox2 and Cox3 were not detectable in mitochondria lacking active *m*-AAA protease (Figure 3A). In agreement with the growth phenotype and the cytochrome spectra, however, subunits of the cytochrome *c* oxidase were present at wild-type levels in *yta10$^{E559Q}$* and *yta12$^{E614Q}$* mitochondria (Figure 3A).

If the *m*-AAA protease act only post-translationally, non-assembled subunits might be subject to rapid proteolysis. Alternatively, defects in the synthesis of mitochondrially encoded subunits in the absence of the *m*-AAA protease have to be considered. To examine the latter possibility, labelling of mitochondrial translation products with [$^{35}$S]methionine was performed in cells lacking Yta10p and Yta12p (Figure 3B). Cox1, Cox2 and Cox3 were synthesized at greatly reduced levels in Δ*yta10* and Δ*yta12*

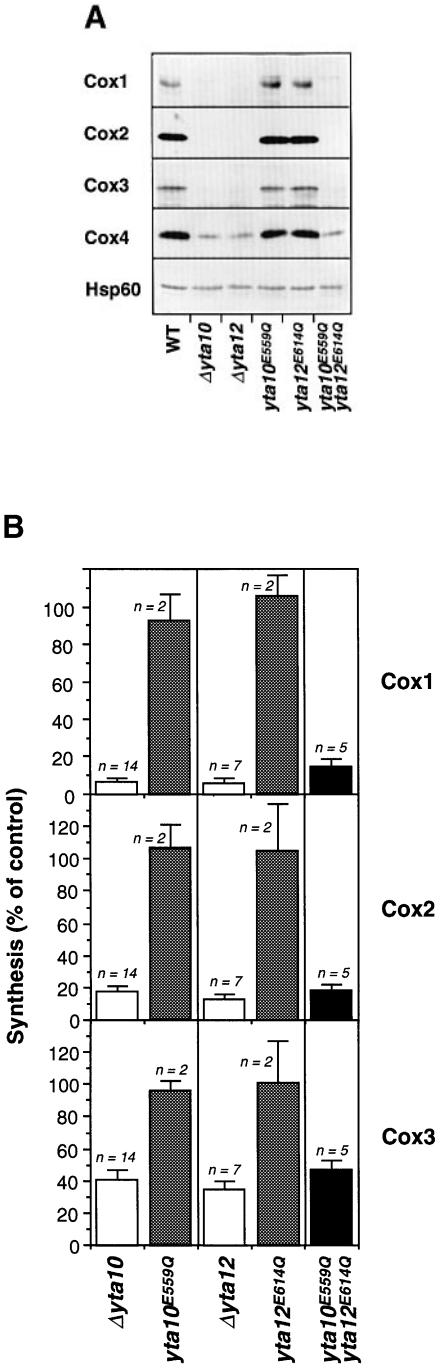


**Fig. 3.** Mitochondrially encoded subunits of the cytochrome *c* oxidase do not accumulate in the absence of *m*-AAA protease activity. (**A**) Steady-state levels of cytochrome *c* oxidase subunits in *yta10* and *yta12* mutant mitochondria. Mitochondrial proteins (30 μg) were analysed by SDS–PAGE and immunoblotting. (**B**) Labelling of mitochondrially encoded subunits in cells harbouring mutant *m*-AAA protease. Mitochondrial translation products were labelled *in vivo* in wild-type ('WT'), Δ*yta10* (YHA101), *yta10$^{E559Q}$* (YHA103), Δ*yta12* (YHA201), *yta12$^{E614Q}$* (YHA203) and *yta10$^{E559Q}$yta12$^{E614Q}$* (YGS302) cells for 20 min at 30°C as described (Douglas *et al.*, 1979; McKee and Poyton, 1984; Langer *et al.*, 1995). Incorporation of [$^{35}$S]methionine was determined using a phosphoimaging system. The translation efficiency in Δ*yta10*, Δ*yta12* and *yta10$^{E559Q}$yta12$^{E614Q}$* cells was on average reduced to ~30, 25 and 20%, respectively, when compared with wild-type cells. Synthesis of Cox1, Cox2 and Cox3 was quantified and corrected for the translation efficiency in the various strains. Labelling of Cox1, Cox2 and Cox3 in wild-type cells was set to 100% of control. The average of several experiments is given (mean ± SEM). *n* indicates the number of experiments.

cells when compared with wild-type cells (Figure 3B). Although a decreased translation efficiency of all mitochondrially encoded proteins was observed in these cells, this did not account for the reduced labelling of Cox1, Cox2 and Cox3 in the absence of either Yta10p or Yta12p (see quantification in Figure 3B). The expression of the mitochondrially encoded subunits of the cytochrome *c* oxidase was also impaired in *yta10*$^{E559Q}$*yta12*$^{E614Q}$ cells and, therefore, depends on the proteolytic activity of the *m*-AAA protease. The enzymatic activity of one subunit of the *m*-AAA protease, however, is sufficient to ensure the accumulation of Cox1, Cox2 and Cox3 as they were synthesized at wild-type levels in Δ*yta10* or Δ*yta12* mitochondria harbouring Yta10$^{E559Q}$p or Yta12$^{E614Q}$p, respectively (Figure 3A).

### *The m-AAA protease is required for the expression of COX1 in mitochondria*

As degradation of Cox2 and Cox3 has been observed in the absence of Cox1 (McEwen *et al.*, 1986; Van Dyck *et al.*, 1998), it is conceivable that the *m*-AAA protease is required primarily for efficient synthesis of Cox1. Therefore, the expression of the *COX1* gene was examined in more detail. The *m*-AAA protease may affect transcription or translation. As *COX1* represents a mosaic gene harbouring several introns (Costanzo and Fox, 1990; Grivell, 1995), defects in pre-mRNA processing in the absence of the protease also have to be considered.

Mitochondrial RNA (mtRNA) was isolated from cells containing mutations in *YTA10*, *YTA12* or both and analysed by Northern hybridization with probes specific for *COX1* and *COX2*. Inactivation of the *m*-AAA protease did not affect the accumulation of *COX2* mRNA which was present at wild-type levels in mitochondria lacking *m*-AAA protease (Figure 4A). In contrast, transcripts of ~2.1 kb corresponding to mature *COX1* mRNA were hardly detectable in Δ*yta10* and Δ*yta12* mitochondria (Figure 4A). Although wild-type levels accumulated in mitochondria harbouring either Yta10$^{E559Q}$p or Yta12$^{E614Q}$p, mature *COX1* transcripts were almost completely absent in *yta10*$^{E559Q}$-*yta12*$^{E614Q}$ cells (Figure 4A). Thus, the deficiency in the formation of mature *COX1* mRNA results from the loss of the proteolytic activity of the *m*-AAA protease.

To examine whether the function of the *m*-AAA protease is related to the presence of introns in the *COX1* gene, we generated Δ*yta10* and Δ*yta12* cells harbouring an intronless mitochondrial genome. mtRNA was isolated and analysed by Northern blotting with a *COX1* exon probe (Figure 4B). Mature *COX1* transcripts accumulated in these cells at levels similar to wild-type cells. Thus, transcription could proceed normally in the absence of the *m*-AAA protease and only the maturation of intron-containing *COX1* transcripts was impaired upon inactivation of the protease. Moreover, labelling of mitochondrial translation products revealed that the synthesis of Cox1 was at least partially restored in Δ*yta10* and Δ*yta12* cells carrying an intronless mitochondrial genome (Figure 4C). Apparently, translation of mature *COX1* mRNA can also occur in the absence of the *m*-AAA protease.

Taken together, these results establish the requirement for the *m*-AAA protease for the expression of the mitochondrially encoded *COX1* gene. The apparent absence of precursor and mature forms of *COX1* mRNA in mitochondria lacking the *m*-AAA protease may reflect a crucial role for the protease in the stabilization of intron-containing *COX1* transcripts. However, it is also conceivable that the *m*-AAA protease directly regulates processing of *COX1* pre-mRNAs. Splicing defects in the absence of the protease

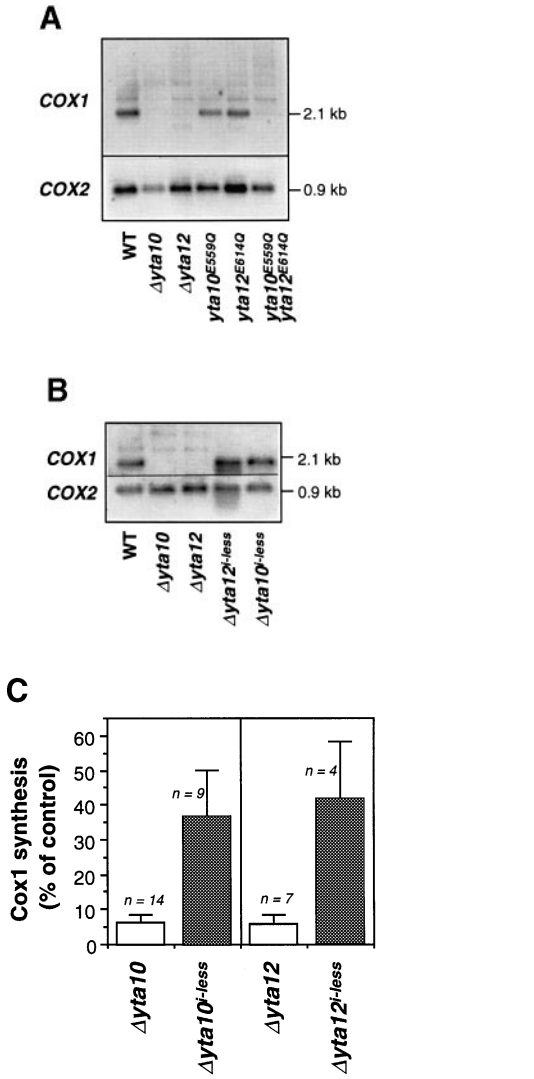


**Fig. 4.** Inactivation of the *m*-AAA protease impairs the maturation of *COX1* pre-mRNA. (**A**) Northern blot analysis of *COX1* and *COX2* transcripts in *yta10* and *yta12* mutant mitochondria. mtRNA was extracted from wild-type ('WT'), Δ*yta10* (YGS101), Δ*yta12* (YGS201), *yta10*$^{E559Q}$ (YGS103), *yta12*$^{E614Q}$ (YGS203) and *yta10*$^{E559Q}$*yta12*$^{E614Q}$ (YGS302) mitochondria and analysed by Northern hybridization with a *COX1*-specific exon probe and with a *COX2* probe as a gel loading control. (**B**) Northern blot analysis of *COX1* and *COX2* mRNAs in Δ*yta10* and Δ*yta12* mitochondria harbouring an intronless mitochondrial genome. mtRNA was isolated from wild-type ('WT') and from Δ*yta10* and Δ*yta12* strains containing ('Δ*yta10*', YGS101; 'Δ*yta12*', YGS201) or lacking ('Δ*yta10*$^{i\text{-}less}$', YGS108; 'Δ*yta12*$^{i\text{-}less}$', YGS208) introns and analysed as in (A). (**C**) Synthesis of Cox1 in Δ*yta10* and Δ*yta12* mitochondria harbouring an intronless mitochondrial genome. Labelling of mitochondrial translation products was performed in wild-type and Δ*yta10* and Δ*yta12* cells containing ('Δ*yta10*', YGS101; 'Δ*yta12*', YGS201) or lacking ('Δ*yta10*$^{i\text{-}less}$', YGS108; 'Δ*yta12*$^{i\text{-}less}$', YGS208) introns as in Figure 3B. Cox1 synthesis was quantified and corrected for differences in the translation efficiency as in Figure 3B. Cox1 synthesis in wild-type cells was set to 100% of control. The average of several experiments is given (mean ± SEM). *n* indicates the number of experiments.

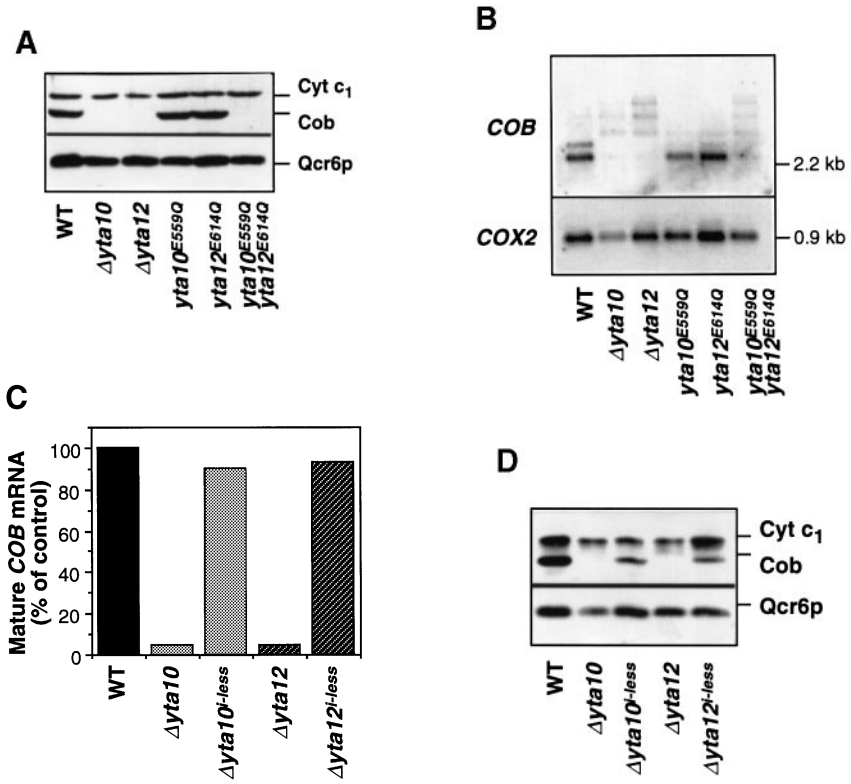


**Fig. 5.** Defective expression of *COB* in mitochondria lacking *m*-AAA protease activity. (**A**) Steady-state level of Cob in *yta10* and *yta12* mutant mitochondria. Mitochondrial proteins (60 μg) were analysed by SDS–PAGE and immunoblotting. (**B**) Northern blot analysis of *COB* and *COX2* transcripts in mitochondria harbouring mutant *m*-AAA protease. mtRNA was extracted from wild-type ('WT'), Δ*yta10* (YGS101), Δ*yta12* (YGS201), $yta10^{E559Q}$ (YGS103), $yta12^{E614Q}$ (YGS203) and $yta10^{E559Q}yta12^{E614Q}$ (YGS302) mitochondria and analysed by Northern hybridization with a *COB*-specific exon probe and with a *COX2* probe as a gel loading control. (**C**) Northern blot analysis of *COB* transcripts in Δ*yta10* and Δ*yta12* mitochondria containing intronless mtDNA. mtRNA was isolated from wild-type ('WT') and from Δ*yta10* and Δ*yta12* strains containing ('Δ*yta10*', YGS101; 'Δ*yta12*', YGS201) or lacking ('$\Delta yta10^{i\text{-}less}$', YGS108; '$\Delta yta12^{i\text{-}less}$', YGS208) introns and analysed with a *COB*-specific probe as in (A). Mature *COB* mRNA was quantified using a phosphorimaging system and is given as a percentage of wild-type of control. (**D**) Steady-state level of Cob in Δ*yta10* and Δ*yta12* cells harbouring an intronless mtDNA. Mitochondria (60 μg), isolated from cells described in (B), were subjected to SDS–PAGE and analysed by immunoblotting.

may result in the degradation of *COX1* pre-mRNAs, thereby preventing the accumulation of non-processed transcripts in these mitochondria.

### *Inactivation of the m-AAA protease impairs the synthesis of Cob in mitochondria*

In addition to the cytochrome *c* oxidase, a deficiency in the assembly of the $bc_1$ complex has been observed in the absence of either Yta10p or Yta12p (Tauer *et al.*, 1994; Tzagoloff *et al.*, 1994). Mitochondrially encoded cytochrome *b* (Cob), a subunit of the $bc_1$ complex, is encoded by an intron-containing gene in mitochondria, as is Cox1. Therefore, a role for the *m*-AAA protease in the expression of *COB* in mitochondria had to be considered. Indeed, Cob was not detectable by immunoblotting in Δ*yta10* or Δ*yta12* mitochondria, while two other subunits of the $bc_1$ complex, cytochrome $c_1$ and Qcr6p, were present at wild-type levels (Figure 5A). The dependence on the proteolytic activity of the *m*-AAA protease was examined in cells harbouring $Yta10^{E559Q}$p, $Yta12^{E614Q}$p or both (Figure 5A). Wild-type levels of Cob accumulated in $yta10^{E559Q}$ and $yta12^{E614Q}$ mitochondria, whereas Cob was not detectable in $yta10^{E559Q}yta12^{E614Q}$ mitochondria (Figure 5A). Thus, the accumulation of Cob depends on the proteolytic activity of the *m*-AAA protease, suggesting an impaired synthesis of Cob in the absence of the protease.

mtRNA was isolated from cells carrying mutations in *YTA10*, *YTA12* or both and analysed with a probe specific for *COB*. With wild-type cells and cells harbouring $Yta10^{E559Q}$p or $Yta12^{E614Q}$p, the probe hybridized with transcripts of 2.2 kb corresponding to mature *COB* mRNA (Figure 5B). In contrast, mature *COB* transcripts accumulated at greatly reduced levels in mitochondria of Δ*yta10*, Δ*yta12* or $yta10^{E559Q}yta12^{E614Q}$ cells (Figure 5B). These results demonstrate that the efficient formation of mature *COB* transcripts depends on proteolysis by the *m*-AAA protease. Similarly to *COX1* transcripts, the requirement for the *m*-AAA protease for the stabilization of *COB* transcripts is restricted to intron-containing *COB* pre-mRNAs. mtRNA was isolated from Δ*yta10* and Δ*yta12* cells harbouring an intronless mitochondrial genome and analysed by Northern hybridization with a *COB*-specific probe. Similar levels of mature *COB* transcripts were detected in wild-type, Δ*yta10* and Δ*yta12* cells (Figure 5C).

To examine whether expression of *COB* is restored in Δ*yta10* and Δ*yta12* mitochondria containing an intronless mitochondrial genome, steady-state levels of Cob were determined (Figure 5D). Cob accumulated in mitochondria which lack Yta10p or Yta12p but harbour intronless mtDNA (Figure 5D). We conclude from these results that removal of introns from the mitochondrial genome alleviates the dependence of *COB* expression on the

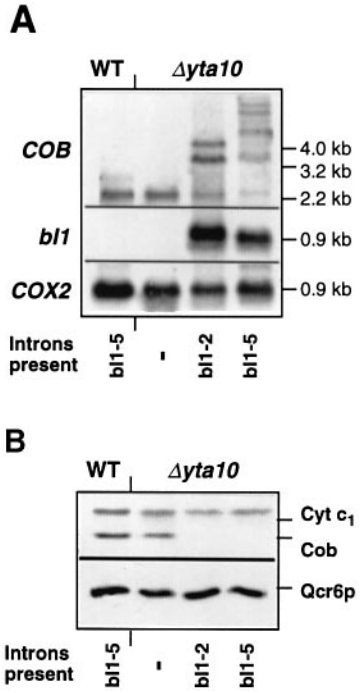


**Fig. 6.** Splicing of the maturase-encoding *COB* intron bI2 depends on the *m*-AAA protease. (**A**) Northern blot analysis of *COB* transcripts in Δ*yta10* strains harbouring different numbers of introns in the *COB* gene. mtRNA was extracted from mitochondria of wild-type ('WT'), Δ*yta10* (YGS101), Δ*yta10*$^{i\text{-}less}$ (YGS108) and Δ*yta10* mitochondria harbouring a *COB* gene with introns bI1 and bI2 only (YGS110). The introns present in the *COB* gene in the various strains are indicated. mtRNA was analysed with a *COB*-specific exon probe ('*COB*'), a probe specific for intron bI1 ('*bI1*') of the *COB* gene and with a *COX2*-specific probe as a gel loading control. The excised intron bI1 was detectable in wild-type mitochondria only upon prolonged exposure of the membrane. (**B**) Steady-state levels of Cob in Δ*yta10* mitochondria harbouring different numbers of introns in the *COB* gene. Mitochondria isolated from wild-type and Δ*yta10* strains described in (A) were subjected to SDS–PAGE and analysed by immunoblotting. Cob protein was detectable at very low levels in Δ*yta10* harbouring a *COB* gene with introns bI1 and bI2 upon prolonged exposure of the membrane.

*m*-AAA protease. Thus, the function of the protease is linked directly to the presence of introns in this gene.

### *The m-AAA protease affects the splicing of a maturase-encoding COB intron*

To characterize further the role of the *m*-AAA protease in the maturation of *COB* transcripts, we analysed mtRNA from wild-type and Δ*yta10* cells with a probe specific for intron bI1 of *COB*, a group II intron which forms a stable lariat structure upon splicing (Costanzo and Fox, 1990; Perlman, 1990; Grivell, 1995). The probe hybridized with a transcript of ~0.8 kb corresponding to the excised lariat regardless of the presence of Yta10p in mitochondria (Figure 6A). Thus, the *m*-AAA protease is not required for the splicing of this intron. Notably, the excised intron accumulated at greatly increased levels in Δ*yta10* mitochondria when compared with wild-type cells (Figure 6A). The impaired synthesis of Cob protein in the absence of the *m*-AAA protease most likely causes an upregulation of transcription of *COB* resulting in the accumulation of the excised intron in these cells.

The splicing of many mitochondrial introns depends on mRNA maturases which are encoded within the same intron (Grivell and Schweyen, 1989; Costanzo and Fox, 1990; Pel and Grivell, 1993). To examine the role of the *m*-AAA protease in the splicing of a maturase-encoding intron, *YTA10* was disrupted in a yeast strain with mtDNA containing only introns bI1 and bI2 in the *COB* gene. In contrast to intron bI1, an mRNA maturase is encoded by intron bI2 (Lazowska *et al.*, 1980). mtRNA was isolated from these cells and analysed with an exon-specific *COB* probe and a probe specific for the intron bI1 (Figure 6A). Similarly to Δ*yta10* cells harbouring the complete *COB* gene, the excised intron bI1 accumulated in these cells, demonstrating efficient splicing of this intron (Figure 6A). In contrast, mature *COB* mRNA accumulated at strongly reduced levels in mitochondria lacking Yta10p, while precursor transcripts of ~4.0 and 3.2 kb were detected in these cells (Figure 6A). Furthermore, Cob protein was hardly detectable by immunoblotting of mitochondria isolated from these cells (Figure 6B). Thus, maturation of *COB* transcripts harbouring only introns bI1 and bI2 still depends on the *m*-AAA protease. As intron bI1 is excised normally in the absence of the protease, these results demonstrate the requirement for the *m*-AAA protease for the efficient splicing of the maturase-encoding *COB* intron bI2.

### *Proteolytically inactive m-AAA protease does not maintain the respiratory competence of cells harbouring an intronless mitochondrial genome*

The synthesis of both Cox1 and Cob was restored in Δ*yta10* and Δ*yta12* cells upon removal of introns from the corresponding genes. Still, these cells were respiratory deficient and cytochromes $aa_3$ and *b* were absent, indicating impaired complex assembly (data not shown). This finding is reminiscent of other proteins involved in mitochondrial mRNA splicing whose inactivation was found to impair the respiratory competence of cells containing intronless mtDNA (Seraphin *et al.*, 1989; Wiesenberger *et al.*, 1992). Expression of proteolytically inactive Yta10p and Yta12p in Δ*yta10*Δ*yta12* cells did not maintain respiration nor were cytochromes detectable in spectra of these cells (data not shown). Thus, in addition to the synthesis of Cox1 and Cob, the assembly of respiratory chain complexes appears to be under the proteolytic control of the *m*-AAA protease.

## Discussion

Cells lacking Yta10p or Yta12p are respiratory deficient and exhibit defects in the proteolysis of non-assembled inner membrane proteins and the assembly of respiratory chain complexes (Guélin *et al.*, 1994; Pajic *et al.*, 1994; Tauer *et al.*, 1994; Tzagoloff *et al.*, 1994). Our results explain this pleiotropic phenotype by the loss of essential proteolytic functions of the *m*-AAA protease. Mutations in the proteolytic centre of both Yta10p and Yta12p, similarly to deletions of either *YTA10* or *YTA12*, lead to loss of the respiratory competence of the cells. Inactivation of the *m*-AAA protease impairs the synthesis of the mitochondrially encoded respiratory chain subunits Cox1 and Cob and, thereby, the assembly of the cytochrome *c* oxidase and the $bc_1$ complex in the inner membrane. Thus, the biogenesis of the respiratory chain is under the proteolytic control of the ATP-dependent *m*-AAA protease.

The *m*-AAA protease represents a large proteolytic complex composed of Yta10p and Yta12p. Cells carrying proteolytically inactive variants of both Yta10p and Yta12p are respiratory deficient, while mutating either protein alone did not interfere with the respiratory competence of the cells. The genetic interaction of mutations in the proteolytic sites of Yta10p and Yta12p allows several conclusions on the function of both proteins in mitochondria. First, the complex of Yta10p and Yta12p represents the proteolytically active structure which ensures cellular respiration. In agreement with this finding, substrate polypeptides have been found exclusively in association with assembled Yta10p and Yta12p (Arlt *et al.*, 1996). Secondly, the *m*-AAA protease exerts residual proteolytic activity after inactivation of either Yta10p and Yta12p, which is sufficient to maintain respiratory competence but not to mediate the degradation of non-assembled inner membrane proteins (Arlt *et al.*, 1996; Guélin *et al.*, 1996). It is conceivable that the proteolysis of polypeptides by the *m*-AAA protease requires multiple cleavage events which may depend on the coordinated activity of both subunits. The activity of one subunit of the *m*-AAA protease, on the other hand, may allow the processing or clipping of polypeptides, apparently sufficient to maintain respiration. Indeed, Yta10p was shown to mediate the cleavage of a model substrate if assembled with proteolytically inactive Yta12p (Leonhard *et al.*, 1996). Thirdly, the subunits of the *m*-AAA protease, Yta10p and Yta12p, exert overlapping substrate specificity. The respiratory competence of the cells is maintained regardless of which subunit of the protease exerts proteolytic activity. The specificities of the two subunits of the *m*-AAA protease, however, are not identical. Differential effects of mutations in Yta10p and Yta12p on the degradation of a model substrate protein have been observed (Leonhard *et al.*, 1996).

The proteolytic activity of the *m*-AAA protease is crucial for the expression of the intron-containing *COX1* and *COB* genes in mitochondria. While transcription proceeds normally in the absence of the protease, mature-sized transcripts of *COX1* and *COB* are present at strongly decreased levels in cells lacking Yta10p or Yta12p. The dependence on the *m*-AAA protease is alleviated in cells harbouring an intronless mitochondrial genome, indicating that the protease regulates the processing and/or stability of *COX1* and *COB* precursor transcripts. Unspliced species of *COX1* and *COB* transcripts did not accumulate in the absence of *m*-AAA protease activity, suggesting that the stability of transcripts harbouring several introns is affected in these mitochondria. Intron-containing *COB* precursor RNAs over-accumulate, however, in the absence of the *m*-AAA protease in mitochondria containing only intron bI1 and bI2 in the *COB* gene. The same phenomenon has been observed previously upon inactivation of other nuclear genes involved in the splicing of *COB* or *COX1* transcripts (Seraphin *et al.*, 1988; Pel *et al.*, 1990; Wiesenberger *et al.*, 1992). These results thus demonstrate that the *m*-AAA protease is required for the splicing of intron bI2 encoding an mRNA maturase. The stability or activity of a protein involved in the splicing of a maturase-encoding intron is apparently under the proteolytic control of the *m*-AAA protease in mitochondria. mRNA maturases are synthesized as fusion proteins with the peptide products of the preceding exons and, presumably after proteolytic activation, mediate the splicing of the intron by which they are encoded (Costanzo and Fox, 1990; Perlman, 1990; Grivell, 1995). The *m*-AAA protease may catalyse the processing of at least some of these fusion proteins, thereby releasing active maturase.

The experiments described here establish the requirement for the *m*-AAA protease for the splicing of the intron bI2 of the *COB* gene and thereby also for the removal of subsequent *COB* introns. The impaired splicing of *COB* transcripts is sufficient to explain the deficiency in the maturation of *COXI* pre-mRNA as the maturase encoded by the intron bI4 of the *COB* gene is required for the splicing of both intron bI4 itself and intron aI4 of the *COXI* gene (Dhawale *et al.*, 1981; Banroques *et al.*, 1987). A more general role for the protease during the splicing of maturase-encoding introns remains, therefore, to be demonstrated. Inactivation of the *m*-AAA protease did not result in the over-accumulation of specific *COB* and *COX1* precursor transcripts in cells with a *COB* gene containing several introns. The requirement for the protease is apparently not confined to one particular intron. Rather, a cumulative effect on the splicing of several introns has to be envisaged.

Low levels of mature *COX1* and *COB* transcripts were detected in mitochondria in the absence of the *m*-AAA protease. Other proteins may partially substitute for the *m*-AAA protease in this case. PIM1 protease, an ATP-dependent Lon-like protease in the mitochondrial matrix space (Suzuki *et al.*, 1994; Van Dyck *et al.*, 1994), is a likely candidate; its overexpression was found to restore the respiratory competence of cells lacking *m*-AAA protease (Rep *et al.*, 1996a). The analysis of the proteolytic breakdown of misfolded polypeptides in mitochondria provided biochemical evidence for overlapping substrate specificities of both proteases (Savel'ev *et al.*, 1998). *pim1* mutant cells, similarly to Δ*yta10* and Δ*yta12* cells, exhibit deficiencies in the synthesis of Cox1 and Cob (Van Dyck *et al.*, 1998). PIM1 protease is required for the stability and splicing of intron-containing pre-mRNAs of either gene and, in addition, for the translation of mature *COX1* transcripts (Van Dyck *et al.*, 1998). Thus, the expression of the intron-containing *COX1* and *COB* genes is under the proteolytic control of two ATP-dependent proteases in mitochondria.

Similarly to PIM1 protease, overexpression of Oxa1p has been reported to suppress the respiratory deficiency of cells lacking *m*-AAA protease (Rep *et al.*, 1996a). Oxa1p facilitates the membrane insertion of integral inner membrane proteins from the matrix side, but evidently does not exert proteolytic activity (He *et al.*, 1997; Hell *et al.*, 1997). Our results explain impaired respiration in the absence of the *m*-AAA protease by the loss of essential proteolytic functions and, therefore, suggest an indirect suppressive effect of Oxa1p. Further studies are required to elucidate the molecular consequences of an Oxa1p overproduction.

The deficiency in the assembly of the cytochrome *c* oxidase and the $bc_1$ complex in the absence of Yta10p or Yta12p can be explained satisfactorily by defects in the synthesis of Cox1 and Cob. The respiratory competence of cells harbouring an intronless mitochondrial genome, however, was not maintained upon inactivation of the *m*-AAA protease, pointing to additional post-translational

**Table I.** Yeast strains

| Strain | Genotype | Reference |
|---|---|---|
| YHA101 | *MAT***a** *ade2-1 his3-11,15 trp1-1 leu2,112 ura3-52 yta10::URA3* | Tauer *et al*. (1994) |
| YHA103 | *MAT***a** *ade2-1 his3-11,15 trp1-1 leu2,112* YCplac111 *(LEU2, CEN):ADH1-YTA10$^{E559Q}$ ura3-52 yta10::URA3* | Arlt *et al*. (1996) |
| YGS101 | *MAT***a** *ade2-1 his3-11,15 yta10::HIS3MX6 trp1-1 leu2,112 ura3-52* | this study |
| YGS103 | *MAT***a** *ade2-1 his3-11,15 yta10::HIS3MX6 trp1-1* YCplac22 (*TRP1, CEN*):*ADH1-YTA10$^{E559Q}$ leu2,112 ura3-52* | this study |
| YGS108 | *MAT***a** *ade2-1 his3-11,15 yta10::HIS3MX6 trp1-1* YCplac22 (*TRP1, CEN*):*ADH1 leu2,112 ura3-52* ($\rho^+$ intronless) | this study |
| YGS110 | *MAT***a** *kar1-1 leu1 yta10::KanMX4* ($\rho^+$ bI1,bI2) | Golik *et al*. (1995) |
| YHA301 | *MAT***a** *ade2-1 his3-11,15 yta12::HIS3 trp1-1 leu2,112 ura3-52 yta10::URA3* | |
| YGS302 | *MAT***a** *ade2-1 his3-11,15 yta10::HIS3MX6 trp1-1* YCplac22 (*TRP1, CEN*):*ADH1-YTA10$^{E559Q}$ leu2,112* YCplac111 (*LEU2, CEN*):*ADH1-YTA12$^{E614Q}$ ura3-52 yta12::KanMX4* | this study |
| YGS305 | *MAT***a** *ade2-1 his3-11,15 yta12::HIS3MX6 trp1-1* YCplac22 (*TRP1, CEN*):*ADH1-YTA10$^{E559Q}$ leu2,112* YCplac111 (*LEU2, CEN*):*ADH1-YTA12$^{E614Q}$ ura3-52 yta10::KanMX4* ($\rho^+$ intronless) | this study |
| YHA201 | *MAT***a** *ade2-1 his3-11,15 yta12::HIS3 trp1-1 leu2,112 ura3-52* | Arlt *et al*. (1996) |
| YHA203 | *MAT***a** *ade2-1 his3-11,15 yta12::HIS3 trp1-1 leu2,112* YCplac111 (*LEU2, CEN*):*ADH1-YTA12$^{E614Q}$ ura3-52* | Arlt *et al*. (1996) |
| YGS201 | *MAT***a** *ade2-1 his3-11,15 yta12::HIS3MX6 trp1-1 leu2,112 ura3-52* | this study |
| YGS203 | *MAT***a** *ade2-1 his3-11,15 trp1-1 leu2,112* YCplac111 (LEU2, *CEN*):*ADH1-YTA12$^{E614Q}$ ura3-52 yta12::KanMX4* | this study |
| YGS208 | *MAT***a** *ade2-1 his3-11,15 yta12::HIS3MX6 trp1-1* YCplac22 (*TRP1, CEN*):*ADH1 leu2,112 ura3-52* ($\rho^+$ intronless) | this study |

functions of the *m*-AAA protease. While expression of *COB* and *COX1* was restored in Δ*yta10* or Δ*yta12* mitochondria harbouring intronless mtDNA, the cytochrome spectra of these cells indicate impaired complex assembly. Moreover, the $F_1F_0$-ATPase did not assemble in cells lacking *m*-AAA protease despite the synthesis of mitochondrially encoded subunits of this complex (Tzagoloff *et al.*, 1994; Paul and Tzagoloff, 1995; Arlt *et al.*, 1996). Although its precise role remains to be characterized, our results point to essential proteolytic functions of the *m*-AAA protease also in these processes.

## Materials and methods

### Yeast strains and growth conditions

Yeast strains used in this study are derivatives of W303 and are summarized in Table I. Cells were grown at 30°C on YP medium (1% yeast extract, 2% peptone) containing 2% galactose and 0.5% lactate or on minimal medium (0.7% yeast nitrogen base containing ammonium sulfate) supplemented with the auxotrophic requirements.

*YTA10* (YGS101) and *YTA12* (YGS201) were disrupted by PCR-targeted homologous recombination using the heterologous markers *HIS3MX6* or *KanMX4* (Wach *et al.*, 1994). The complete open reading frame of *YTA12* or an internal fragment of *YTA10* (bp 150–2170) were replaced by the disruption cassettes. Homologous recombination was verified by PCR. To generate a W303 strain containing an intronless mitochondrial genome (YGS6), a $\rho^0$ derivative of W303-1B was generated by ethidium bromide treatment (Fox *et al.*, 1991) and transformed with the plasmid YCplac22 (CEN, *TRP1*). This strain was crossed with kar167-1 cells harbouring intronless mtDNA (Seraphin *et al.*, 1987), and haploid cytoductants were isolated on selective medium lacking tryptophan (YGS6) (Conde and Fink, 1976; Berlin *et al.*, 1991). The strains YGS108 and YGS208 were generated by PCR-targeted deletion of *YTA10* or *YTA12* in these cells using the *HIS3MX6* disruption cassette. The Δ*yta10* strain harbouring only intron bI1 and bI2 (YGS110) is a derivative of the strain CKST9 (Golik *et al.*, 1995).

To allow co-expression of Yta10$^{E559Q}$p and Yta12$^{E614Q}$p, a 4 kb DNA fragment containing the *ADH1* promotor and *YTA10$^{E559Q}$* was isolated from YCplac111/*ADH1*/*YTA10$^{E559Q}$* (Arlt *et al.*, 1996) by restriction digestion with *Spe*I and *Hin*dIII and cloned into the centromere-based plasmid YCplac22 (Gietz and Sugino, 1988). To generate *yta10$^{E559Q}$-yta12$^{E614Q}$* cells (YGS302) or *yta10$^{E559Q}$yta12$^{E614Q}$* cells containing intronless mtDNA (YGS305), plasmids encoding Yta10$^{E559Q}$p or Yta12$^{E614Q}$p were transformed into the Δ*yta12* strains YGS203 or YGS208, respectively. *YTA10* was then disrupted using the *HIS3MX6* or the *KanMX4* cassette by PCR-targeted homologous recombination. The maintenance of mtDNA was examined by mating with *mit*$^-$ tester strains and by labelling of mitochondrial translation products *in vivo*.

### Assessing assembly of cytochrome c oxidase

Mitochondria (300 μg) were resuspended at a concentration of 5 mg/ml in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS–KOH pH 7.2) containing 0.8% (v/v) laurylmaltoside and solubilized by vigorous mixing for 30 min at 4°C. After a clarifying spin for 30 min at 110 000 *g*, the sample was divided. Mitochondria (50 μg) were diluted 3-fold with SEM buffer, and trypsin was added at the concentrations indicated. Trypsin digestion was performed for 20 min at 4°C and then inhibited by incubating with soybean trypsin inhibitor (600 μg/ml) for 5 min at 4°C. The trichloroacetic acid (TCA)-precipitated samples were analysed by SDS–PAGE and immunoblotting with a polyclonal antiserum directed against Cox4. Cox4 present in solubilized mitochondria without adding trypsin was set to 100%.

### Gel electrophoresis and Western blot analysis

Standard procedures were employed for SDS–PAGE and immunoblotting. For denaturation prior to electrophoresis, mitochondrial proteins were incubated for 30 min at 4°C under vigorous mixing in SDS sample buffer. Goat anti-rabbit antibodies conjugated to horseradish peroxidase (Sigma) and a chemiluminescence kit (Amersham) were used for immunodetection. Polyclonal antisera directed against Cob and Cox3 were raised against purified proteins kindly provided by U.Brandt (University of Frankfurt). The Cob antiserum cross-reacted with cytochrome $c_1$ in mitochondria. Cox1-specific antiserum was obtained from Molecular Probes.

### Miscellaneous

The following procedures were performed as previously described: determination of cytochrome spectra (Claisse *et al.*, 1970); isolation of mitochondria (Herrmann *et al.*, 1994; Zinser and Daum, 1995); blue native gel electrophoresis (Schägger and von Jagow, 1991; Schägger, 1995); gel filtration analysis of mitochondrial extracts (Arlt *et al.*, 1996); co-immunoprecipitation of Yta10p and Yta12p (Arlt *et al.*, 1996); labelling of mitochondrial translation products (Douglas *et al.*, 1979; McKee and Poyton, 1984; Langer *et al.*, 1995); chemical cross-linking of substrate polypeptides to the *m*-AAA protease (Arlt *et al.*, 1996); and Northern hybridization analysis (Van Dyck *et al.*, 1998).

The following DNA fragments were used as probes for the Northern blot analysis: *COB* exon probe, pA12/Mb2 (Nobrega and Tzagoloff, 1980); *COXI* exon probe, pCOX1/A4-I corresponding to a DNA fragment from *COXI* containing exon A4 and part of intron aI4 (kindly provided by A.Tzagoloff); *COXII* probe, PCR-amplified 689 bp internal DNA fragment of *COXII*; and *COB* intron probes, bI1 and pYJL12 (Lazowska *et al.*, 1989).

## Acknowledgements

We thank Drs J.Lazowska and A.Tzagoloff for plasmids and yeast strains, and Dr U.Brandt for purified Cox3 and Cob protein. The excellent technical assistance of Petra Robisch and Alexandra Weinzierl is gratefully acknowledged. R.P. was a recipient of a Senior Research Fellowship of the European Union. The work was supported by grants from the Deutsche Forschungsgemeinschaft (La918/1-2; SFB 184/B21) to T.L.

## References


Arlt,H., Tauer,R., Feldmann,H., Neupert,W. and Langer,T. (1996) The YTA10–12-complex, an AAA protease with chaperone-like activity in the inner membrane of mitochondria. *Cell*, **85**, 875–885.

Banroques,J., Perea,J. and Jacq,C. (1987) Efficient splicing of two yeast mitochondrial introns controlled by a nuclear-encoded maturase. *EMBO J.*, **6**, 1085–1091.

Barrett,A.J. (1997) Evolution and structural classification of peptidases. In Hopsu-Havu,V.K., Järvinen,M. and Kirschke,H. (eds), *Proteolysis in Cell Functions.* IOS Press, Amsterdam, pp. 3–12.

Berlin,V., Brill,J.A., Trueheart,J., Boeke,J.D. and Fink,G.R. (1991) Genetic screens and selections for cell and nuclear fusion mutants. *Methods Enzymol.*, **194**, 774–792.

Beyer,A. (1997) Sequence analysis of the AAA protein family. *Protein Sci.*, **6**, 2043–2058.

Brunner,M. and Neupert,W. (1995) Purification and characterization of the mitochondrial processing peptidase of *Neurospora crassa*. *Methods Enzymol.*, **248**, 325–342.

Claisse,M.L., Pere-Aubert,G.A., Clavilier,L.P. and Slonimski,P.P. (1970) Method for the determination of cytochrome concentrations in whole yeast cells. *Eur. J. Biochem.*, **16**, 430–438.

Conde,J. and Fink,G.R. (1976) A mutant of *Saccharomyces cerevisiae* defective for nuclear fusion. *Proc. Natl Acad. Sci. USA*, **73**, 3651–3655.

Costanzo,M.C. and Fox,T.D. (1990) Control of mitochondrial gene expression in *Saccharomyces cerevisiae*. *Annu. Rev. Genet.*, **24**, 91–113.

Dhawale,S., Hanson,D.K., Alexander,N.J., Perlman,P.S. and Mahler,H.R. (1981) Regulatory interactions between mitochondrial genes: interactions between two mosaic genes. *Proc. Natl Acad. Sci. USA*, **78**, 1778–1782.

Douglas,M., Finkelstein,D. and Butow,R.A. (1979) Analysis of products of mitochondrial protein synthesis in yeast: genetic and biochemical aspects. *Methods Enzymol.*, **56**, 58–66.

Fox,T.D., Folley,L.S., Mulero,J.J., McMullin,T.W., Thorsness,P.E., Hedin,L.O. and Costanzo,M.C. (1991) Analysis and manipulation of yeast mitochondrial genes. *Methods Enzymol.*, **194**, 149–165.

Gietz,R.D. and Sugino,A. (1988) New yeast–*Escherichia coli* shuttle vectors constructed with *in vitro* mutagenized yeast genes lacking six-base pair restriction sites. *Gene*, **74**, 527–534.

Golik,P., Szczepanek,T., Bartnik,E., Stepien,P.P. and Lazowska,J. (1995) The *S.cerevisiae* nuclear gene *SUV3* encoding a putative RNA helicase is necessary for the stability of mitochondrial transcripts containing multiple introns. *Curr. Genet.*, **28**, 217–224.

Grivell,L.A. (1995) Nucleo-mitochondrial interactions in mitochondrial gene expression. *Crit. Rev. Biochem. Mol. Biol.*, **30**, 121–164.

Grivell,L.A. and Schweyen,R.J. (1989) RNA splicing in yeast mitochondria: taking out the twists. *Trends Genet.*, **5**, 39–41.

Guélin,E., Rep,M. and Grivell,L.A. (1994) Sequence of the *AFG3* gene encoding a new member of the FtsH/Yme1/Tma subfamily of the AAA protein family. *Yeast*, **10**, 1389–1394.

Guélin,E., Rep,M. and Grivell,L.A. (1996) Afg3p, a mitochondrial ATP-dependent metalloprotease, is involved in the degradation of mitochondrially-encoded Cox1, Cox3, Cob, Su6, Su8 and Su9 subunits of the inner membrane complexes III, IV and V. *FEBS Lett.*, **381**, 42–46.

He,S. and Fox,T.D. (1997) Membrane translocation of mitochondrially coded Cox2p: distinct requirements for export of N and C termini and dependence on the conserved protein Oxa1p. *Mol. Biol. Cell*, **8**, 1449–1460.

Hell,K., Herrmann,J., Pratje,E., Neupert,W. and Stuart,R.A. (1997) Oxa1p mediates the export of the N- and C-termini of pCoxII from the mitochondrial matrix to the intermembrane space. *FEBS Lett.*, **418**, 367–370.

Herrmann,J.M., Fölsch,H., Neupert,W. and Stuart,R.A. (1994) Isolation of yeast mitochondria and study of mitochondrial protein translation. In Celis,D.E. (ed.) *Cell Biology: A Laboratory Handbook.* Academic Press, San Diego, CA, pp. 538–544.

Hooper,N.M. (1994) Families of zinc metalloproteases. *FEBS Lett.*, **354**, 1–6.

Isaya,G. and Kalousek,F. (1995) Mitochondrial intermediate peptidase. *Methods Enzymol.*, **248**, 556–567.

Jiang,W. and Bond,J.S. (1992) Families of metalloendopeptidases and their relationships. *FEBS Lett.*, **312**, 110–114.

Kalnov,S.L., Novikova,L.A., Zubatov,A.S. and Luzikov,V.N. (1979) Proteolysis of the products of mitochondrial protein synthesis in yeast mitochondria and submitochondrial particles. *Biochem. J.*, **182**, 195–202.

Langer,T. and Neupert,W. (1996) Regulated protein degradation in mitochondria. *Experientia*, **52**, 1069–1076.

Langer,T., Pajic,A., Wagner,I. and Neupert,W. (1995) Proteolytic breakdown of membrane-associated polypeptides in mitochondria of *Saccharomyces cerevisiae*. *Methods Enzymol.*, **260**, 495–503.

Lazowska,J., Jacq,C. and Slonimski,P.P. (1980) Sequence of introns and flanking exons in wildtype and *box3* mutants of cytochrome *b* reveals an interlaced splicing protein coded by an intron. *Cell*, **22**, 333–348.

Lazowska,J., Claisse,M., Gargouri,A., Kotylak,Z., Spyridakis,A. and Slonimski,P.P. (1989) Protein encoded by the third intron of cytochrome *b* gene in *Saccharomyces cerevisiae* is an mRNA maturase. Analysis of mitochondrial mutants, RNA transcripts, proteins and evolutionary relationships. *J. Mol. Biol.*, **205**, 275–289.

Leonhard,K., Herrmann,J.M., Stuart,R.A., Mannhaupt,G., Neupert,W. and Langer,T. (1996) AAA proteases with catalytic sites on opposite membrane surfaces comprise a proteolytic system for the ATP-dependent degradation of inner membrane proteins in mitochondria. *EMBO J.*, **15**, 4218–4229.

Mason,T.L., Poyton,R.O., Wharton,D.C. and Schatz,G. (1973) Cytochrome *c* oxidase from baker's yeast: I. Isolation and properties. *J. Biol. Chem.*, **248**, 1346–1354.

McEwen,J.E., Ko,C., Kloeckner-Gruissem,B. and Poyton,R.O. (1986) Nuclear functions required for cytochrome *c* oxidase biogenesis in *Saccharomyces cerevisiae*. Characterization of mutants of 34 complementation groups. *J. Biol. Chem.*, **261**, 11872–11879.

McKee,E.E. and Poyton,P. (1984) Mitochondrial gene expression in *Saccharomyces cerevisiae*. Optimal conditions for protein synthesis in isolated mitochondria. *J. Biol. Chem.*, **259**, 9320–9331.

Nobrega,F.G. and Tzagoloff,A. (1980) Assembly of the mitochondrial membrane system. DNA sequence and organization of the cytochrome *b* gene in *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **255**, 9828–9837.

Pajic,A., Tauer,R., Feldmann,H., Neupert,W. and Langer,T. (1994) Yta10p is required for the ATP-dependent degradation of polypeptides in the inner membrane of mitochondria. *FEBS Lett.*, **353**, 201–206.

Patel,S. and Latterich,M. (1998) The AAA team: related ATPases with diverse functions. *Trends Cell Biol.*, **8**, 65–71.

Paul,M.F. and Tzagoloff,A. (1995) Mutations in *RCA1* and *AFG3* inhibit $F_1$-ATPase assembly in *Saccharomyces cerevisiae*. *FEBS Lett.*, **373**, 66–70.

Pel,H.J. and Grivell,L.A. (1993) The biology of yeast mitochondrial introns. *Mol. Biol. Rep.*, **18**, 1–13.

Pel,H.J., Tzagoloff,A. and Grivell,L.A. (1990) The identification of 18 nuclear genes required for the expression of the yeast mitochondrial gene encoding cytochrome-*c* oxidase subunit I. *Curr. Genet.*, **21**, 139–146.

Perlman,P.S. (1990) Genetic analysis of RNA splicing in yeast mitochondria. *Methods Enzymol.*, **181**, 539–558.

Pratje,E., Esser,K. and Michaelis,G. (1994) The mitochondrial inner membrane peptidase. In von Heijne,G. (ed.), *Signal Peptidases.* R.G.Landes Co., Austin, TX. pp. 105–112.

Rep,M. and Grivell,L.A. (1996) The role of protein degradation in mitochondrial function and biogenesis. *Curr. Genet.*, **30**, 367–380.

Rep,M., Nooy,J., Guélin,E. and Grivell,L.A. (1996a) Three genes for mitochondrial proteins suppress null-mutations in both *AFG3* and *RCA1* when overexpressed. *Curr. Genet.*, **30**, 206–211.

Rep,M., van Dijl,M., Suda,K., Schatz,G., Grivell,L.A. and Suzuki,C.K. (1996b) Promotion of mitochondrial membrane complex assembly by a proteolytically inactive yeast Lon. *Science*, **274**, 103–106.

Savel'ev,A.S., Novikova,L.A., Kovaleva,I.E., Luzikov,V.N., Neupert,W. and Langer,T. (1998) ATP-dependent proteolysis in mitochondria: *m*-AAA protease and PIM1 protease exert overlapping substrate specificities and cooperate with the mtHsp70 system. *J. Biol. Chem.*, in press.

Schägger,H. (1995) Native electrophoresis for isolation of mitochondrial oxidative phosphorylation protein complexes. *Methods Enzymol.*, **260**, 190–202.

Schägger,H. and von Jagow,G. (1991) Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Anal. Biochem.*, **199**, 223–231.

Seraphin,B., Boulet,A., Simon,M. and Faye,G. (1987) Construction of a yeast strain devoid of mitochondrial introns and its use to screen nuclear genes involved in mitochondrial splicing. *Proc. Natl Acad. Sci. USA*, **84**, 6810–6814.

Seraphin,B., Simon,M. and Faye,G. (1988) *MSS18*, a yest nuclear gene involved in the splicing of intron aI5b of the mitochondrial *cox1* transcript. *EMBO J.*, **7**, 1455–1464.

Seraphin,B., Simon,M., Boulet,A. and Faye,G. (1989) Mitochondrial splicing requires a protein from a novel helicase family. *Nature*, **337**, 84–87.

Suzuki,C.K., Suda,K., Wang,N. and Schatz,G. (1994) Requirement for the yeast gene LON in intramitochondrial proteolysis and maintenance of respiration. *Science*, **264**, 273–276.

Suzuki,C.K., Rep,M., Van Dijl,J.M., Suda,K., Grivell,L.A. and Schatz,G. (1997) ATP-dependent proteases that also chaperone protein biogenesis. *Trends Biochem. Sci.*, **22**, 118–123.

Tauer,R., Mannhaupt,G., Schnall,R., Pajic,A., Langer,T. and Feldmann,H. (1994) Yta10p, a member of a novel ATPase family in yeast, is essential for mitochondrial function. *FEBS Lett.*, **353**, 197–200.

Tzagoloff,A., Yue,J., Jang,J. and Paul,M.F. (1994) A new member of a family of ATPases is essential for assembly of mitochondrial respiratory chain and ATP synthetase complexes in *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **269**, 26144–26151.

Van Dyck,L., Pearce,D.A. and Sherman,F. (1994) *PIM1* encodes a mitochondrial ATP-dependent protease that is required for mitochondrial function in the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **269**, 238–242.

Van Dyck,L., Neupert,W. and Langer,T. (1998) The ATP-dependent PIM1 protease is required for the expression of intron-containing genes in mitochondria. *Genes Dev.*, **12**, 1515–1524.

Vazeux,G., Wang,J., Corvol,P. and Llorens Cortes,L. (1996) Identification of glutamate residues essential for the catalytic activity and zinc coordination in aminopeptidase A. *J. Biol. Chem.*, **271**, 9069–9074.

Wach,A., Brachat,A., Poehlmann,R. and Philippsen,P. (1994) New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast*, **10**, 1793–1808.

Wheeldon,L.W., Dianoux,A.C., Bof,M. and Vignais,P.V. (1974) Stable and labile products of mitochondrial protein synthesis *in vitro*. *Eur. J. Biochem.*, **46**, 189–199.

Wiesenberger,G., Waldherr,M. and Schweyen,R.J. (1992) The nuclear gene *MRS2* is essential for the excision of groupII introns from yeast mitochondrial transcripts *in vivo*. *J. Biol. Chem.*, **267**, 6963–6969.

Yasuhara,T., Mera,Y., Nakai,T. and Ohashi,A. (1994) ATP-dependent proteolysis in yeast mitochondria. *J. Biochem. (Tokyo)*, **115**, 1166–1171.

Zinser,E. and Daum,G. (1995) Isolation and biochemical characterization of organelles from the yeast *Saccharomyces cerevisiae*. *Yeast*, **11**, 493–536.

*Received May 15, 1998; revised and accepted June 15, 1998*