# Afg3p, a mitochondrial atp-dependent metalloprotease, is involved in degradation of mitochondrially-encoded cox1, cox3, cob, su6, su8 and su9 subunits of the inner membrane complex-III, complex-IV and complex-V

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# Afg3p, a mitochondrial ATP-dependent metalloprotease, is involved in degradation of mitochondrially-encoded Cox1, Cox3, Cob, Su6, Su8 and Su9 subunits of the inner membrane complexes III, IV and V

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**Abstract The yeast *AFG3* gene encodes an ATP-dependent metalloprotease belonging to a subgroup of the AAA-family. This protease has been suggested to be essential for a metal- and ATP-dependent breakdown of incompletely mitochondrially synthesized polypeptides in the inner membrane, a process proposed to be important for mitochondrial function (Pajic et al. (1994) FEBS Lett. 353, 201–206). Here, we confirm the proteolytic activity by site-directed mutagenesis and demonstrate that the proteins Cox1, Cox3, Cob, Su6, Su8 and Su9 are substrates of Afg3p. Surprisingly, this proteolytic activity is not required for respiratory function and thus presumably also not essential for mitochondrial biogenesis.**

*Key words:* AFG3; RCA1; YME1; Mitochondria; AAA-family; Metalloprotease

## 1. Introduction

In yeast mitochondria, seven proteins belonging to complexes of the respiratory chain and to the F1F0-ATP synthase are encoded by the organelle genome, the other subunits of these heteromultimeric complexes being nuclear gene products [1]. This division of labour implies that mechanisms must exist for balancing cytoplasmic and mitochondrial synthesis. Several levels of control such transcription of the nuclear genes, translation of mitochondrial mRNAs or fine tuning at level of protein degradation are possible [1]. Little is known about the last aspect, although earlier work showed that mitochondria contain proteases which are capable of ATP-dependent degradation of excess amounts of unassembled mitochondrial translation products (see [2] for a review). In recent years, different components of the mitochondrial protein degradation system have been identified. In the matrix subcompartment, PIM1 has two functions: selective protein turnover and degradation of misfolded proteins [3]. The ATP-dependent protein degradation system associated with the inner membrane [4,5] appears to be composed of the three putative mitochondrial ATP-dependent metalloproteases: Afg3p (Yta10p), Rca1p (Yta12p) and Yme1p (Yta11p) recently identified [6–11]. These proteins belong to a subgroup of a novel ATPase family, the AAA family (see [12] for a recent review). These AAA proteins have been proposed to function in specific degradation of unassembled subunits as demonstrated for Yme1p (its substrate is the unassembled subunit 2 of the cytochrome *c* oxidase) [11,13] and/or degradation of incompletely mitochondrial synthesized polypeptides in the inner membrane as suggested for Afg3p [14]. However, lack of Afg3p or Rca1p also affects the level of functional respiratory chain complexes and the assembly of the F1F0-ATP synthase [7,9,15]. Yme1p also affects the respiratory chain, but mainly at 37°C [11]. Taken together, these ATP-dependent metalloproteases seem to be required for functions important for biogenesis of the organelle.

We isolated the *AFG3* gene by genetic complementation of a temperature-sensitive respiratory-deficient mutant [6] and have started a functional analysis of the protein to obtain more insight into its role in mitochondrial biogenesis. Here, we demonstrate that the proteins Cox1, Cox3, Cob, Su6, Su8 and Su9 are substrates for the proteolytic activity displayed by Afg3p. Surprisingly, a yeast mutant lacking Afg3p's proteolytic activity is still capable of non-fermentative growth. This activity is thus presumably also not essential for mitochondrial biogenesis.

## 2. Materials and methods

### 2.1. Strains and growth media

The DW167-1C yeast strain (*MATa*, $\rho^+$ Δintrons, *his3-11,15*, *leu2-3,112*, *ura3-1*) is a spore derived from a cross between the strains D273K(167)(*MATα*, $\rho^+$ Δintrons, *met6*, *lys2*) (the mitochondrial DNA of this strain, which lacks introns, comes from strain KAR(167)(*MATa*, $\rho^+$ Δintrons, *trp5*, *kar1-1*) [16] and was introduced by cytoduction) and W303/1Aρ° (*MATa*, ρ°, *ade2-1*, *his3-11,15*, *leu2-3,112*, *ura3-1*, *trp1-1*, *can1-100*). The strain DAY6 (*MATa*, $\rho^+$ Δintrons, *his3-11,15*, *leu2-3,112*, *ura3-1*, *afg3::URA3*) was obtained by transforming DW167-1C with a 2.3 kb *Hin*dIII/*Eco*RI fragment from pNG85.1 and selected on minimal medium minus uracil. The disruption of *AFG3* in the mutant strain DAY6 was confirmed by Southern blot analysis (using DNA probes carrying either the *AFG3* gene or the *URA3* gene) and tetrad dissection after crossing with strain D273UK (*MATα*, $\rho^+$, *met6*, *ura3-1*, *lys2*). The strains RAY1 (*MATa*, $\rho^+$ Δintrons, *his3-11,15*, *leu2-3,112*, *ura3-1*, *AFG3-c-myc*) and PMY2 (*MATa*, $\rho^+$ Δintrons, *his3-11,15*, *leu2-3,112*, *ura3-1*, *afg3-2-c-myc*)(*afg3-2* allele carries the mutation E558Q) were obtained by transforming DAY6 with a 3.5kb *Pst*I fragment from pCB1 and a 3.5 kb *Hin*dIII/*Eco*RI fragment from pPN2, respectively, and selected on YPGly. Correctness of gene substitution was confirmed through Southern blot analysis (using DNA probes carrying either the *AFG3* gene or the *URA3* gene). Minimal medium contained 0.67% yeast nitrogen base without amino acids, 2% glucose, supplemented with histidine, leucine (each at 30 μg/ml). YPGal or YPGly contains 1% yeast extract, 1% bacto-peptone and 2% galactose or 2% glycerol, respectively. Lactate medium contains 1.5% lactic acid, 2% sodium lactate, 8 mM $MgSO_4.7H_2O$, 45 mM $(NH_4)_2HPO_4$, 0.5% yeast extract, pH 4.5.

### 2.2. Preparation of mitochondria and proteolysis of mitochondrially-encoded proteins

Cells of wild-type and mutant strains were grown aerobically on

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*Abbreviations:* EGTA, ethyleneglycolbis(aminoethylether)-tetra-acetic acid; TCA, trichloroacetic acid; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electropheresis.


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Table 1
Generation time of the strains DW167-1C, DAY6, RAY1 and PMY2 in medium containing either galactose or lactate

| Strain | Generation time | |
|---|---|---|
| | Galactose | Lactate |
| DW167-1C | 2 h | 2 h 25 min |
| DAY6 | 3 h | 20 h |
| RAY1 | 2 h | 2 h 25 min |
| PMY2 | 2 h | 2 h 25 min |

The generation time of each strain was determined by measuring the turbidity at 600 nm in the exponential phase of cells growing at 30°C on a complete medium containing galactose or lactate as carbon source.

YPGal, harvested in the exponential growth phase and mitochondria were isolated from protoplasts as described in [17], except that zymolyase was used instead of cytohelicase. The synthesis of labelled mitochondrial translation products and proteolysis reaction were carried out according to [14] except that, after the in vitro translation reaction, mitochondria were washed three times with 0.6 M mannitol, 2 mM EGTA, 10 mM Tris/maleate, pH 6.8.

*2.3. Analytical procedures*

SDS-polyacrylamide gel electrophoresis was done according to [18]. Protein concentration was measured by the method described in [19]. Bovine serum albumin was used as standard. Protein gels were dried and exposed to Hyperfilm-Bmax (Amersham). The autoradiograms were scanned using the programs MacImage (Xerox Imaging Systems Inc.) and Scan Analysis (Biosoft).

*2.4. DNA manipulations*

Plasmid pP4 (a pUC18 vector containing the 2.9 kb *Pst*I fragment carrying the *AFG3* gene [6]) was cut with *Bgl*II and *Bam*HI. The 1170 bp *Bgl*II fragment of plasmid pFL38 containing the *URA3* gene was inserted into the 3.6 kb *Bgl*II/*Bam*HI fragment from plasmid pP4. The resulting plasmid pAURO was cut with *Nhe*I and *Bgl*II and blunted. After ligation, the 4.9 kb *Nhe*I°/*Bgl*II° fragment yielded the plasmid pNG8. A M13mp8 phage carrying the 0.5 kb *Bam*HI fragment located in the 3′-part of the *AFG3* gene [6] was cut with *Hin*dIII and *Eco*RI. This fragment was blunted and subcloned into pNG8 cut with *Sma*I, giving the plasmid pNG85.1. To construct the *AFG3-c-myc* fusion gene, plasmid pHH6.8 (a pUC18 plasmid containing the 6.8 kb *Hin*dIII fragment from pHB9 [6] was cut with *Bsu*36I and blunted before *Xba*I cut. The resulting 2.8 kb *Xba*I/*Bsu*36I° fragment was subcloned into YCpmyc181 [20] (*Xma*I°/*Xba*I) yielding the plasmid pCAM. For mutagenesis of the metalloprotease HEXXH active site motif, a 2.6 kb *Hin*dIII/*Eco*RI fragment from p44 (this plasmid contains a shorter form of the 2.9 kb *Pst*I fragment generated after a treatment with *Exo*III and S1 [21]) was subcloned into the multicloning site of pSELECT (Promega Corp.). The mutation E558Q was introduced with the following synthetic oligonucleotide: 5′-GCATGTCCTGCCTGATGATAGGCCACTG-3′. The complementary sequence corresponding to the glutamine codon is underlined.

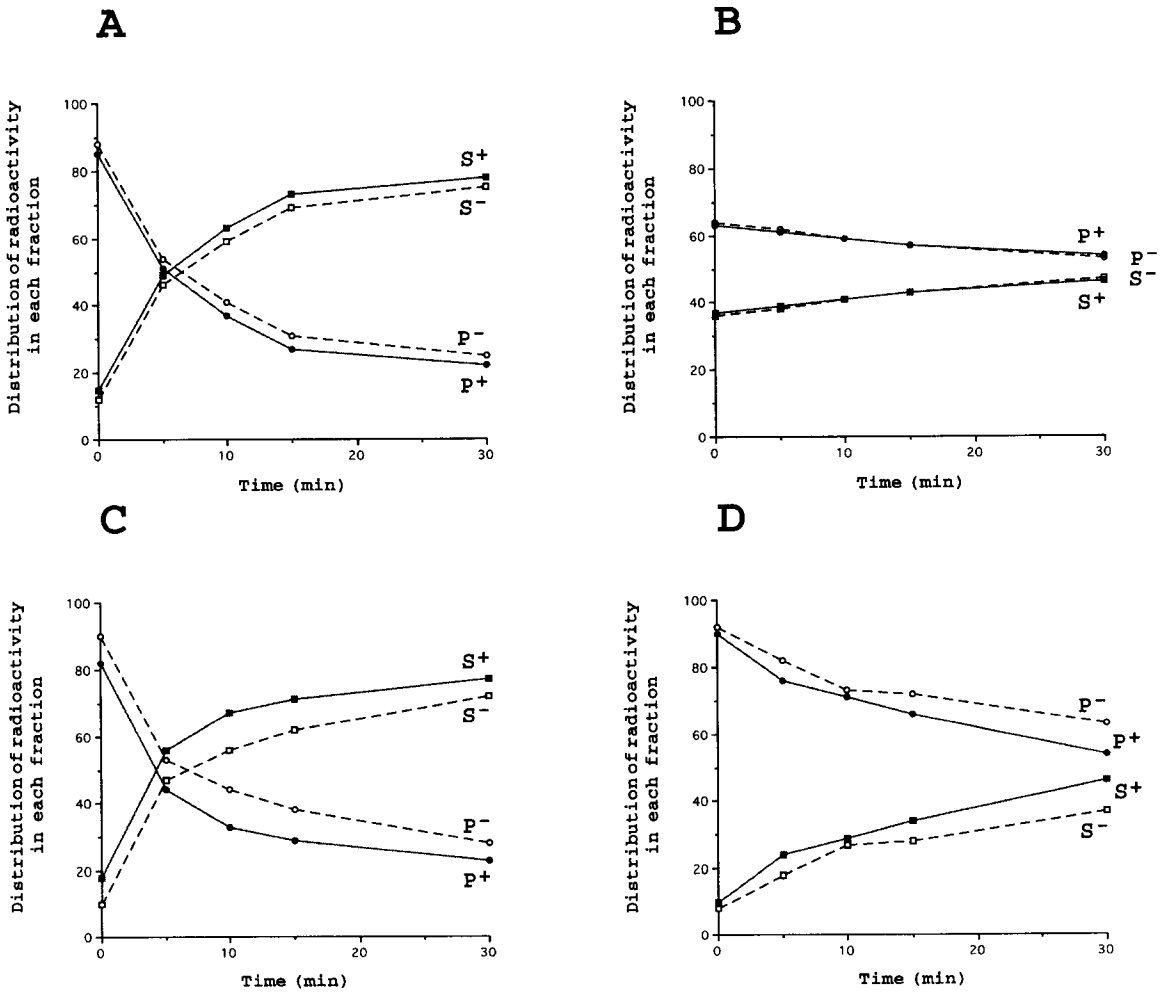


Fig. 1. Degradation of mitochondrial translation products synthesized in presence or in absence of puromycin. The in vitro labelling of mitochondrial translation products was carried out with (+) or without puromycin (−). Proteolysis of mitochondrially translated products in each strain, (A) DW167-1C, (B) DAY6, (C) RAY1 and (D) PMY2, was monitored. After TCA-precipitation, the radioactivity of supernatant (S) and pellet (P) fractions was measured at different time points of the proteolytic reaction.

The mutation introduced and the surrounding sequences were checked by sequencing with appropriate synthetic oligonucleotides as primers. The *Bsg*I/*Nco*I fragment carrying the mutation E558Q was exchanged with the corresponding fragment in pCAM giving the plasmid pCAM-EQ. Verification of fragment substitution was carried out by sequencing. To construct the plasmid pCB1, the 0.8 kb *Bam*HI fragment from pHB14 [6] (this DNA fragment carries the 3′ downstream sequence of *AFG3*) was blunted and subcloned into pCAM-EQ cut with *Nar*I and blunted. To construct the plasmid pPN2, the 2.9 kb *Hin*dIII/*Nar*I fragment from pCAM-EQ was blunted and subcloned into pP4

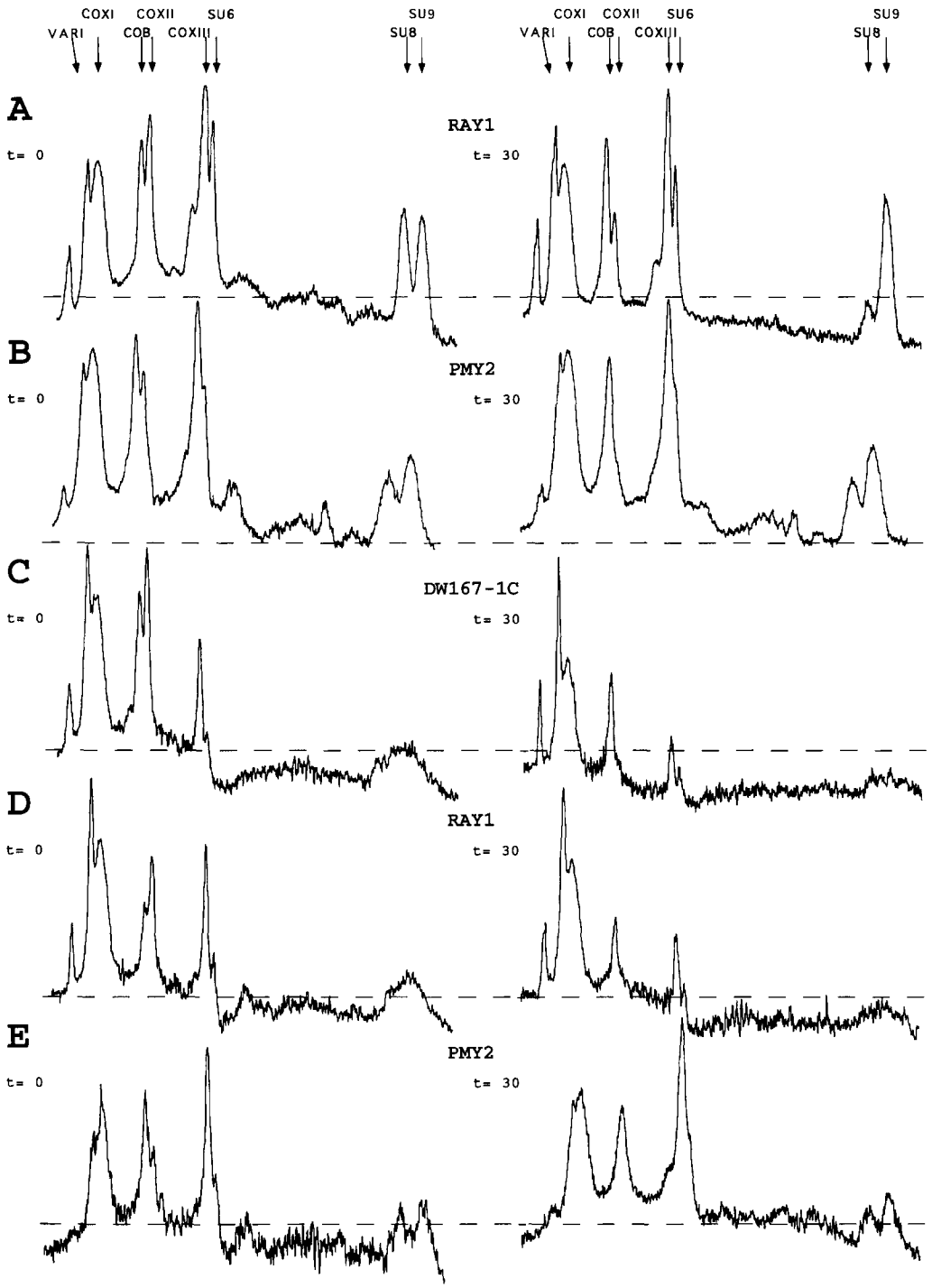


Fig. 2. Analysis of the mitochondrial translation products after 0 and 30 min of the proteolytic reaction. Isolated mitochondria were used to synthesize proteins in the absence of puromycin. The TCA-pellets at time points 0 and 30 min were resuspended in 50 μl of 5% SDS. An identical volume of these both fractions was analysed by SDS-PAGE. The autoradiograms were analysed as described in section 2. (A and D) and (B and E) are autoradiograms obtained at two different times of exposure of samples from strain RAY1 and PMY2, respectively. (C) is an autoradiogram obtained in strain DW167-1C. For each proteolytic reaction, the radioactivity present in the supernatant and in the pellet at each time point, was measured and summed. The value for each time point was within a range of 3–5% around the average of the values obtained from the five time points. VarI = protein associated with mitoribosomes; COXI, -II and -III = subunits I, II and III of the cytochrome *c* oxidase; COB = apocytochrome *b*; SU6, -8 and -9, subunits 6, 8 and 9 of the ATP synthase. Dashed lines indicate the same level of pixels of the scan at time: 0 and 30 min for each strain. The peak on the left side of VarI may correspond to the oligomer of SU9.

cut with *Hin*dIII and *Nco*I and blunted before the subcloning. Southern blotting and hybridization were as described in [22].

## 3. Results

### 3.1. Disruption of the AFG3 gene and phenotype of the resultant mutant

To construct an *AFG3* null strain, we replaced 73% of the coding region with a 1.2 kb *Bgl*II fragment containing the *URA3* gene. This large deletion was necessary to prevent recombination between plasmids carrying a mutated *afg3* gene and the previously disrupted chromosomal *AFG3* locus (substitution of the 0.3kb *Bgl*II fragment [6] by the *URA3* marker).

The AFG3° strain (DAY6) has a generation time of 20 h on a medium containing lactate as a carbon source, compared with 2 h 25 min for the wild-type parental strain (Table 1).

### 3.2. Site-directed mutagenesis of the metalloprotease HEAGH active site motif of Afg3p

A protease-deficient version of Afg3p was constructed by replacement of Glu-558 by Gln. This residue forms part of the sequence motif HEAGH. Studies with the *E. coli* protease III (non-ATP-dependent metalloprotease) which contains a metalloprotease HEXXH motif, have shown that a similar mutation eliminates protease activity, without effect on zinc-binding [23]. The mutated gene was substituted for the disrupted allele of strain DAY6 giving strain PMY2 (see section 2). Since the protease mutant protein is tagged with a c-myc epitope [20], a transformant containing a wild-type *AFG3-c-myc* fusion gene, was used as control (strain RAY1). On media containing either lactate or galactose as carbon source, the generation time of the strain RAY1 is identical to the wild-type strain DW167-1C (Table 1), showing a neutral effect of the c-myc-tag present at the C-terminus of Afg3p. Surprisingly, the generation time of the strain PMY2 is also identical to the wild-type strain DW167-1C (Table 1), suggesting that protease activity of Afg3p is not essential for respiratory function.

To verify that the E→Q mutation indeed eliminates the proteolytic activity previously attributed to Afg3p, we assayed for degradation of newly-synthesised, incomplete mitochondrial translation products as described in [14]. Proteolytic activity in strain PMY2 is strongly reduced compared to both wild-type strains (Fig. 1; panel D vs. A and C, compare $S^+$ and $P^+$ in each case).

### 3.3. Analysis of mitochondrial translation products in the protease-deficient mutant PMY2

We investigated a role of Afg3p in the protein turnover of mitochondrial translated products. Therefore, the mitochondrial in vitro translation was carried out in absence of puromycin. Under these conditions, only mitochondrially-encoded proteins are synthesized. As shown in Fig. 1 (compare for each strain, $S^+$ with $S^-$ and $P^+$ with $P^-$), proteolytic activities with or without puromycin of each strain are comparable. Electrophoretic analysis of mitochondrial translation products formed during incubation of isolated mitochondria, after in vitro translation in absence of puromycin, was carried out at increasing times of incubation. The results obtained at times 0 and 30 min, in the strains DW167-1C, RAY1 and PMY2, are shown in Fig. 2.

In the wild-type strain RAY1, the proteins Su6 and Su8 are markedly degraded (Fig. 1; panel A $t=0$ vs. $t=30$) and their degradation is strongly reduced in the protease mutant PMY2 (Fig. 1; panel B $t=0$ vs. $t=30$). Analysis of shorter exposed autoradiograms shows that the proteins Cox1, Cob, Cox3 and Su9 are also significantly degraded in the strain DW167-1C (Fig. 1; panel C $t=0$ vs. $t=30$). We have noted that in the strain RAY1, Cox1 and Cob display less degradation (Fig. 1; panel D $t=0$ vs. $t=30$). All these proteins are not significantly degraded in the protease mutant PMY2 (Fig. 1; panel E $t=0$ vs. $t=30$). In the strain DAY6, only Cox2 is degraded consistent with the observation that this protein is a substrate of Yme1p [11,13]. Similar degradation of Cox2 is also observed in the three other strains.

## 4. Discussion

The results presented in this study provide additional information about the role of Afg3p in mitochondrial biogenesis.

On one hand, Afg3p is capable of proteolytic breakdown of incompletely synthesized mitochondrial translation products ([14], Fig. 1; compare $S^+$ and $P^+$ of panels A and C to D). In vivo, it could function in the elimination of imcomplete or damaged proteins. At the same time, it is also capable of the degradation of a specific subset of mitochondrially translated proteins: Cox1, Cox3, Cob, Su6, Su8 and Su9. This finding suggests that, in vivo, Afg3p may be required, as recently proposed for Yme1p [11,13], for protein turnover of mitochondrial proteins which are not or only incompletely assembled into inner membrane complexes.

The proteins Cox1 and Cob of which the degradation is less in the strain RAY1 than in the strain DW167-1C (Fig. 1; panel D vs. C), could suggest that the presence of the c-myc tag at the C-terminus of Afg3p interferes somewhat with recognition of these two substrates, without affecting intrinsic proteolytic activity since the other substrates are degraded as in the wild-type mitochondria. Interestingly, two missense mutations in the C-terminus of *E. coli* FtsH, an Afg3p and Rca1p homologue, affect its substrate recognition [24,25].

Results presented in Table 1 clearly show that the proteolytic activity of Afg3p is not essential for mitochondrial function since the protease-deficient mutant confers a wild-type respiratory growth to yeast cells (Table 1). This behaviour contrasts with that of FtsH which is essential for aerobic growth of *E. coli* [24–27]. The closely-related Rca1p is obviously unable to replace Afg3p function since: (i) complementation of the strain DAY6 by the *RCA1* gene does not restore wild-type growth (unpublished result); (ii) mitochondrially-encoded proteins remain stable in the protease mutant PMY2 despite the presence of Rca1p (Fig. 1; panels B and E $t=0$ vs. $t=30$). This supports non-overlapping functions for Afg3p and Rca1p.

For Yme1p, it was suggested that it could, through a chaperone-like activity, control an assembly- or degradation-competent state of Cox2 [11], thus preventing accumulation of unassembled subunits likely to interfere with complex assembly. If Afg3p fulfils a similar function, then the absence of chaperone activity in the disruption mutant would account for a weak respiratory function, since the generation time of DAY6 is strongly increased on a lactate-containing medium (Table 1). This interpretation is also supported by the finding

of strongly reduced bc1 complex and cytochrome c oxidase activities in an *AFG3*-null mutant [9].

Recent genetic analysis has been shown that the degradation of unassembled Cox2 requires three different proteins, only one of which (Osd1p/Yme1p) has so far been characterized [11]. These proteins may form a complex which is required for the assembly/degradation of Cox2. A similar situation may hold for the remaining mitochondrial translation products, with an Afg3p-containing complex mediating assembly of newly synthesized mitochondrial translation products into the various enzymes of the inner membrane. Inside this complex, the chaperone and protease activities of Afg3p may control the precise subunit stoichiometry of these enzymes by regulating access to the assembling complex and/or by contributing to the proteolytic degradation of mitochondrially-coded subunits. Of great interest now is the question of how these different activities are balanced.

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