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Review

ATP-dependent proteases controlling mitochondrial function in the yeast *Saccharomyces cerevisiae*

L. Van Dyck and T. Langer*

Institut für Physiologische Chemie, Ludwig-Maximilians-Universität München, Goethestrasse 33, D-80336 München (Germany), Fax + 49 89 5996270, e-mail: langer@bio.med.uni-muenchen.de

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Abstract. Regulated protein degradation by ATP-dependent proteases plays a fundamental role in the biogenesis of mitochondria. Membrane-bound and soluble ATP-dependent proteases have been identified in various subcompartments of this organelle. Subunits composing these proteases are evolutionarily conserved from yeast to humans and, in support of an endosymbiotic origin of mitochondria, evolved from prokaryotic ancestors: the PIM1/Lon protease is active in the matrix of mitochondria, while the *i*-AAA protease and the *m*-AAA protease mediate the turnover of inner membrane proteins. Most of the knowledge concerning the biogenesis and the physiological role of ATP-dependent proteases comes from studies in the yeast *Saccharomyces cerevisiae*. Proteases were found to be required for mitochondrial stasis, for the maintenance of the morphology of the organelle and for mitochondrial genome integrity. ATP-dependent proteolysis is crucial for the expression of mitochondrially encoded subunits of respiratory chain complexes and for the assembly of these complexes. Hence, mitochondrial ATP-dependent proteases exert multiple roles which are essential for the maintenance of cellular respiratory competence.

Key words. *PIM1*; *LON*; *YTA10* (*AFG3*); *YTA12* (*RCA1*); *YME1*; *m*-AAA protease; *i*-AAA protease; yeast; mitochondria; ATP-dependent protease; proteolysis; respiratory chain; respiration; intron splicing; complex assembly.

Introduction

Mitochondria carry out the process of respiration and use the energy released to synthesize ATP, fulfilling thereby most of the energy requirements of aerobic cells. They are also required for the synthesis of a number of important biological compounds such as lipids, heme, amino acids and nucleotides. Hence, mitochondria are essential for cell viability.

Mitochondrial homeostasis depends on the activity of various proteases present in the organelle (fig. 1). The proteolytic system of mitochondria consist of specific processing peptidases which remove N-terminal targeting signals from nuclear-encoded mitochondrial preproteins [1–6], and ATP-dependent proteases which exert regulatory functions crucial for the biogenesis of respiratory chain complexes [7–11]. These ATP-dependent proteases also prevent the potentially harmful accumulation of nonassembled subunits of multienzyme complexes and, thereby, ensure their proper stoichiometry.

The yeast *Saccharomyces cerevisiae* has proven to be a unique and powerful tool in analysing the biochemistry and the function of mitochondrial ATP-dependent proteases. As a facultative anaerobic organism, it can selectively repress respiratory activity during fermenta-

* Corresponding author.

tive and anaerobic growth, and live by glycolysis alone. Thus, mutations leading to the loss of respiratory function can be readily investigated in yeast, and, mitochondrial ATP-dependent proteases are best characterized in this organism. Recent studies, however, also highlight the crucial role of these conserved and ubiquitous enzymes in higher eukaryotes. The present paper reviews the current knowledge on mitochondrial ATP-dependent proteases as well as their role in mitochondrial gene expression and in the biogenesis of respiratory chain complexes.

Mitochondrial ATP-dependent proteases

Part of the proteolytic system in mitochondria is composed of energy-dependent proteases which all are derived from prokaryotic ancestors (fig. 1). The *i*-AAA protease and the *m*-AAA protease are embedded in the inner membrane with their proteolytic domains facing the *i*ntermembrane space and the *m*atrix, respectively [12, 13]. A Lon-like protease, termed PIM1 protease in *S. cerevisiae*, controls protein turnover in the matrix space of mitochondria [14, 15]. These three proteases are highly conserved from yeast to humans. In contrast, Clp-like proteases are only present in organelles of higher eukaryotes [16–20]. In *Escherichia coli*, Clp proteases are composed of either of two regulatory ATPase subunits, ClpA and ClpX, and a proteolytic subunit, ClpP [21–27]. Despite the identification of a ClpX homologue in yeast mitochondria [28], no ClpP-encoding gene was identified in the complete genome of *S. cerevisiae*. Yeast, however, could be an exception, as organellar ClpP homologues from mouse, human, *Caenorhabditis elegans*, and plants are found in the databases. Finally, an ATP-dependent protease activity distinct from the *i*-AAA protease has been characterized in the intermembrane space of rat liver mitochondria [29]. The protease, termed MISP I (mitochondrial intermembrane space protease), appears to be an oligomeric enzyme complex with a molecular mass of 200 kDa which is able to degrade fluorogenic peptide substrates and radiolabeled model proteins.

Like other ATP-dependent proteases, mitochondrial proteases form high molecular mass complexes and thus might be self-compartmentalizing enzymes mediating proteolysis in a protected environment [30]. ATPase

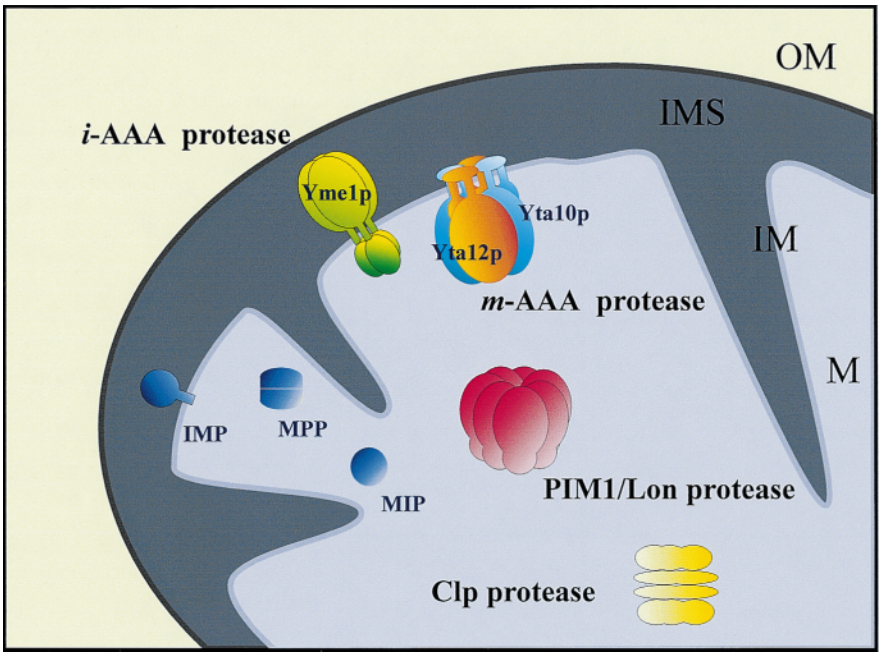

Figure 1. Proteolytic system of mitochondria. Several ATP-dependent proteases have been identified in mitochondria. The matrix space enzyme PIM1/Lon protease as well as the *i*-AAA and *m*-AAA proteases, oligomeric complexes embedded in the inner membrane with their catalytic sites facing the intermembrane space and the matrix, respectively, seem to be present in mitochondria of all eukaryotic cells. Hetero-oligomeric Clp proteases, composed of a peptidase and an ATPase subunit, have been identified in organelles of higher eukaryotes but not in the yeast *S. cerevisiae*. Three non-ATP-dependent peptidases are involved in mitochondrial protein precursor processing: the matrix-processing peptidase (MPP) [1], the inner membrane peptidase (IMP) [2], and the mitochondrial intermediate peptidase (MIP) [3]. OM, outer membrane; IMS, intermembrane space; IM, inner membrane; M, matrix space.

and proteolytic activities are associated within well-defined domains of a single polypeptide chain, as in the case of Lon-like proteases and AAA proteases, or reside in different subunits of a hetero-oligomeric complex such as in Clp proteases. The ATPase domains of these families of proteases display a common fold and belong to the same superfamily of proteins [30, 31]. They constitute the regulatory elements of energy-dependent proteases. ATP-dependent conformational changes of the proteases presumably determine the accessibility of the proteolytic sites and, at the same time, trigger unfolding of bound substrate polypeptides, thereby facilitating their degradation. ATPase activity and proteolysis are thus tightly coupled processes.

Structure and biogenesis of PIM1 protease

Lon-like ATP-dependent serine proteases have been identified in the mitochondrial matrix space of yeast and mammalian cells [14, 15, 32, 33]. Furthermore, homologues are encoded in the genomes of several plant species and of the nematode *C. elegans*, as well as in the genomes of eu- and archaebacteria. Members of the Lon-like protease family are extremely well conserved but lack any sequence similarity to classic serine proteases. In all Lon-like proteins, an ATPase domain can be distinguished from a C-terminal proteolytic domain containing the catalytic serine residue (fig. 2). Sequence divergences localize in the N-terminal third of the proteins and in a spacer region between the two catalytic sites. These differences may be related to the specificity of the proteases.

PIM1 protease, the Lon-like enzyme in mitochondria of *S. cerevisiae*, assembles in a high molecular mass, and presumably homo-oligomeric structure in the matrix space of the organelle [14, 34, 35]. The purified enzyme forms a complex of approximately 800 kDa with a heptameric stoichiometry [35–37]. The analysis of mitochondrial extracts revealed, however, a native molecular mass of about 1600 kDa for PIM1 protease [34]. It is, therefore, conceivable that the protease is part of a high molecular mass structure in mitochondria, formed either by assembly of two homo-oligomeric subcomplexes or by interacting with other, unidentified mitochondrial proteins.

The assembly of PIM1 protease requires both binding and hydrolysis of ATP [34]. Impaired binding of the nucleotide resulting from mutation of the conserved lysine residue at position 638 to an asparagine in the P-loop of Pim1p abolishes the formation of the 1600-kDa complex and inactivates the protease. Similarly, the high molecular mass structure of PIM1 protease is not detectable in mitochondrial extracts lacking ATP. Nonhydrolysable nucleotide analogs cannot substitute for ATP in these experiments, suggesting that ATP hydrolysis is a prerequisite for oligomerization of the protease.

Pim1p subunits are synthesized as a pre-pro-enzyme of 1133 residues [34]. The first 37 amino acids of the polypeptide constitute a mitochondrial targeting sequence which is removed by the matrix-processing peptidase upon import in the mitochondrial matrix space. Residues 38–98 constitute a pro-region which is autocatalytically processed upon assembly of newly imported Pim1p subunits. Cleavage of the pro-region can occur in an inter- as well as in an intramolecular reaction [34]. The existence of a pro-region is a common feature of many serine and aspartate proteases [38]. In the case of Pim1p, the pro-region is crucial for efficient sorting to mitochondria: Pim1p mutant variants lacking the pro-region mostly localize in the cytosol [34]. It is conceivable that the pro-region exerts chaperone-like properties and stabilizes newly synthesized Pim1p subunits in an import-competent conformation. A direct role of the pro-region in targeting Pim1p precursor to mitochondria, however, cannot be excluded presently.

PIM1 protease is required for mitochondrial genome integrity

Cells lacking PIM1 protease are unable to maintain functional mitochondrial DNA (mtDNA) [14, 15]. Destabilization of the mitochondrial genome results in mutants called ρ^-, or cytoplasmic *petite*, which harbor mtDNA with extensive deletions and repetition of the conserved sequence [39]. As mtDNA specifies genes for respiratory chain subunits as well as products required for mitochondrial gene expression (see below), loss of mitochondrial information results in the absence of mitochondrially encoded proteins. Consequently, *pim1* null mutants are unable to grow on nonfermentable carbon sources.

Mutation of the conserved catalytic serine residue 1015 of Pim1p to alanine completely abolishes proteolytic activity but does not impair the assembly of the protease [34, 40]. Cells harboring a disrupted allele of the *PIM1* gene and expressing the mutant variant $Pim1p^{S1015A}$ are unable to preserve the integrity of the mitochondrial genome, indicating that the respiratory competence of yeast cells depends on the proteolytic function of PIM1 protease [34]. Similarly, mtDNA integrity is affected when the conserved lysine residue 638 of the ATP binding domain of Pim1p is changed to asparagine [34]. The mutant variants $Pim1p^{S1015A}$ and $Pim1p^{K638N}$ thus display the same *petite* phenotype which reflects the requirements of both ATPase and proteolytic activities for PIM1 function.

The mechanism underlying the loss of mtDNA in *pim1* mutants remains an open question. A direct involvement of PIM1 protease in nucleic acid metabolism

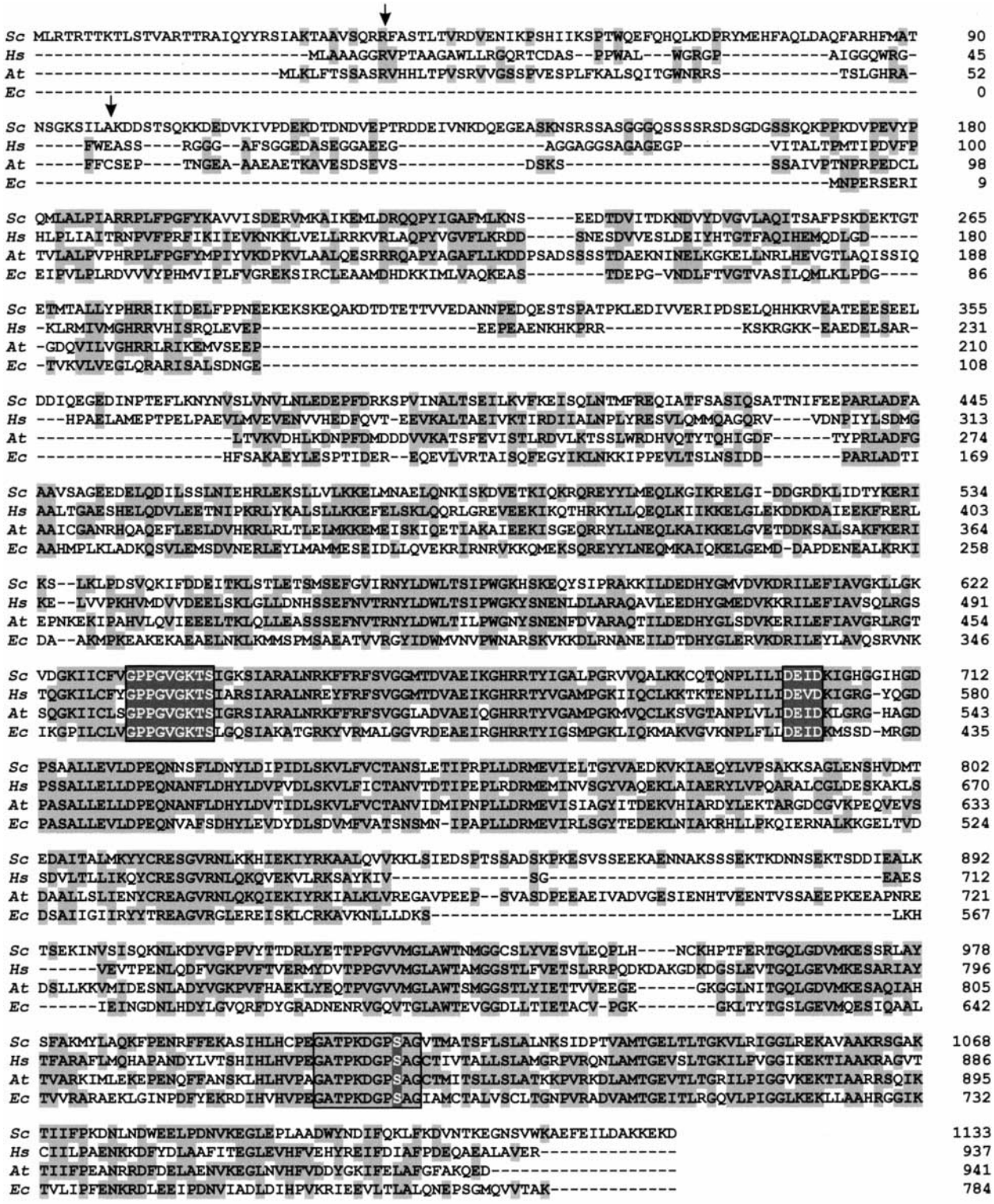

Figure 2. Amino acid sequence alignment of ATP-dependent Lon-like proteases from eukaryotes and prokaryotes. The following sequences have been aligned using the CLUSTAL program: Pim1p/Lon from *S. cerevisiae* (*Sc*; accession number X74544); the Lon proteases from human (*Hs*; S42366), from *A. thaliana* (*At*; U88087) and from *E. coli* (*Ec*; J03896). Identical residues in at least two sequences are indicated with a grey background. ATP-binding site Walker A and B motifs are in white and indicated with a dark background. Conserved residues surrounding the catalytic site serine (indicated in white) are boxed. The processing sites of the mitochondrial presequence (after residue 37) and of the pro-region of PIM1 protease (after residue 98) are indicated with arrows.

seems conceivable. Indeed, one of the most peculiar features of *E. coli* and human Lon proteases is their high affinity for DNA. *E. coli* Lon can bind to a TG-rich DNA promoter element of HIV2 virus in a sequence-specific manner [41]. DNA but not RNA stimulates the proteolytic activity of *E. coli* Lon in vitro [42]. Furthermore, protein substrates promote the dissociation of Lon from DNA. Human Lon binds to mitochondrial promoters in a single-stranded, site- and strand-specific manner [43]. It has, therefore, been speculated that Lon-like proteases might regulate mtDNA replication and/or transcription using DNA binding to degrade regulatory proteins at sites adjacent to promoters. A possible DNA-binding property of yeast PIM1 protease, however, still has to be established.

The isolation of *SSS1* as a suppressor gene which, upon overexpression, maintained mtDNA integrity in the absence of PIM1 protease did not provide direct information on the role of the protease in mtDNA metabolism [11]. Sss1p is a subunit of Sec61p-complexes mediating protein translocation across the endoplasmic reticulum membrane [44, Esnault, 1994 # 92]. The effect of Sss1p on mtDNA integrity seems, therefore, to be indirect. Notably, preservation of mtDNA integrity in *pim1* null mutants by overproducing Sss1p did not lead to the formation of functional respiratory chain complexes [11]. This observation provided the very first evidence that PIM1 protease also directly affects the biogenesis of respiratory chain complexes and thus the respiratory competence of yeast cells (see below).

Structure and biogenesis of *m*-AAA protease

The *m*-AAA protease consists of two proteins, Yta10p (Afg3p) and Yta12p (Rca1p), which share 53% identity [12, 45–47]. They belong to the ubiquitous AAA family of proteins (ATPases associated with a variety of cellular activities), members of which are characterized by a conserved ATPase domain of about 230 residues [48]. AAA proteins have been identified in all organisms studied so far, including archaebacteria and mammals, and are involved in such diverse functions as proteolysis, vesicular transport, cell cycle regulation or membrane anchorage of proteins (reviewed in [49, 50]).

Homologues of Yta10p and Yta12p have been identified in eubacteria and in eukaryotic cells but not in archaebacteria. Notably, a homologue is encoded in the chloroplast genome of the red alga *Porphyra purpurea* [51]. These proteins comprise a subfamily of AAA proteins with metallopeptidase activity (fig. 3). A consensus binding motif for divalent metal ions, HEXXH, located in the C-terminal domain of the proteins and characteristic of metalloproteases of the thermolysin family [52], was identified as the proteolytic center by mutational analysis [12, 53].

Yta10p (761 amino acids) and Yta12p (825 amino acids) are nuclear-encoded and targeted to mitochondria by N-terminal cleavable presequences of a predicted length of 20 and 40 residues, respectively. Both proteins are anchored in the inner mitochondrial membrane via two hydrophobic segments located in the N-terminal third of the proteins and separated by hydrophilic loops of about 10 kDa [12, 47]. The N-termini of Yta10p and Yta12p, as well as their large C-terminal domains of approximately 520 amino acids harboring ATP- and metal-binding sites, are exposed to the matrix space.

Yta10p and Yta12p form a large complex of approximately 1000 kDa in the mitochondrial inner membrane [12]. This complex represents the proteolytically active structure of the *m*-AAA protease. The integrity of the complex requires the presence of nucleotides but not ATP hydrolysis. No other component in addition to Yta10p and Yta12p has been detected in coimmunoprecipitation experiments with partially purified *m*-AAA protease, suggesting the presence of multiple copies of Yta10p and Yta12p in the complex [12].

m-AAA protease mutants are respiratory deficient

Deletion of either the *YTA10* or *YTA12* gene, or both, results in the absence of the high molecular mass proteolytic complex and causes a *petite* phenotype, i.e. impaired cellular growth on nonfermentable carbon sources [45–47]. In contrast to *pim1* mutant cells, the integrity of mtDNA is not affected in cells lacking Yta10p or Yta12p. Rather, the *petite* phenotype of *yta10* and *yta12* null mutants is attributed to impaired assembly of respiratory chain complexes [45–47, 54].

Replacement of the glutamate residue by glutamine in the consensus metal binding site HEXXH of either Yta10p or Yta12p subunits does not impair complex formation but prevents the proteolysis of all substrates of the *m*-AAA protease known so far [12, 53]. Thus, membrane protein degradation, a process which presumably requires multiple cleavage events, seems to depend on the proteolytic activity of both subunits of the protease. On the other hand, the activity of one subunit of the *m*-AAA protease apparently still allows the processing or the clipping of polypeptides: Yta10p was shown to mediate the cleavage of a model substrate if assembled with proteolytically inactive Yta12p [13]. Notably, the proteolytic activity of the *m*-AAA protease following inactivation of one subunit is sufficient to maintain respiratory competence of the cell [12, 53]. These observations suggest that the lack of specific processing steps rather than the deleterious effect of overaccumulating nonnative polypeptides is causing respiratory defects in the absence of the protease. Respiration, however, is impaired if point mutations are

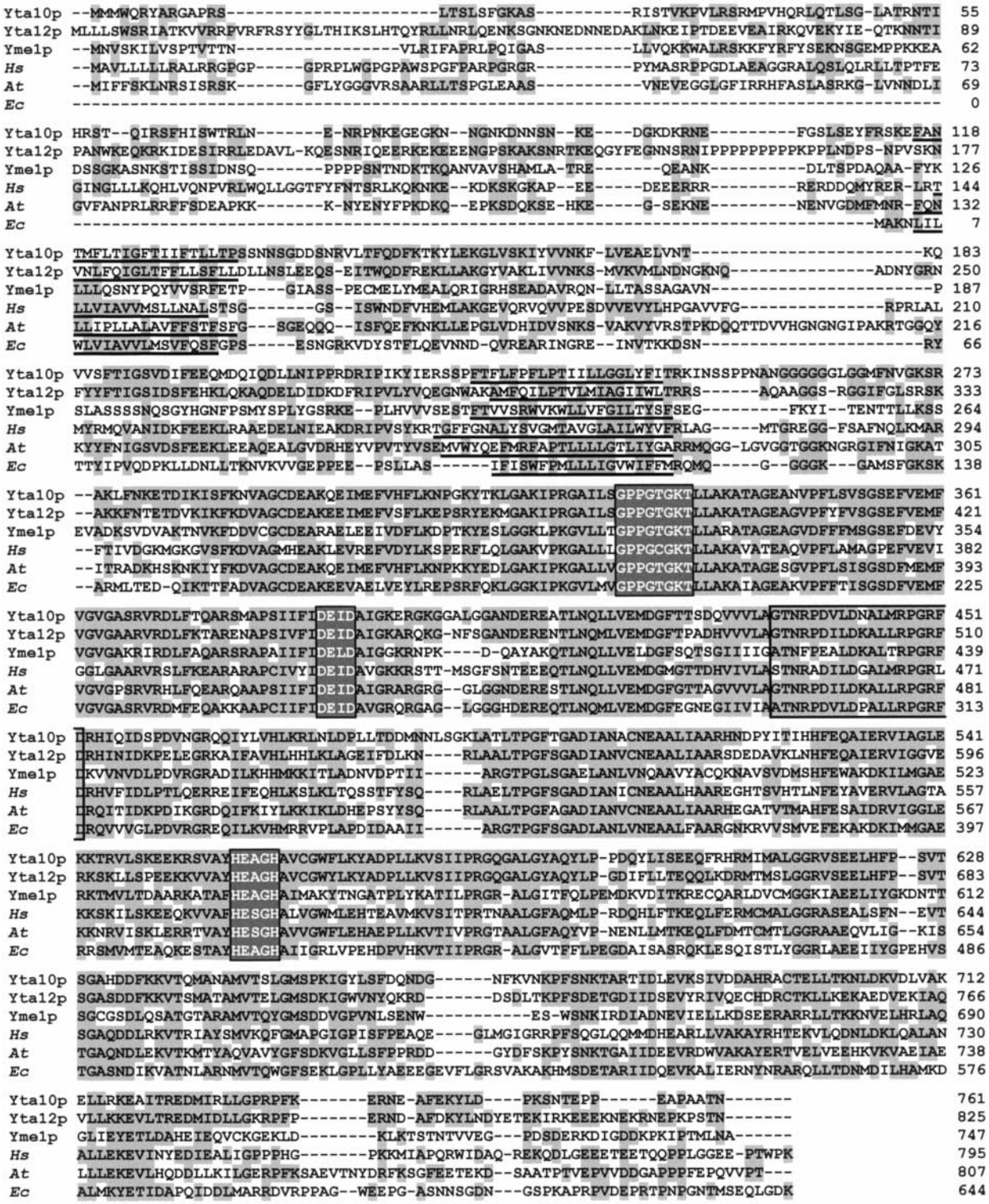

Figure 3. Amino acid sequence alignment of AAA proteases from eukaryotes and prokaryotes. The following sequences have been aligned using the CLUSTAL program: Yta10p/Afg3p from *S. cerevisiae* (Yta10p; accession number X81066); Yta12p/Rca1p from *S. cerevisiae* (Yta12p; accession number Z49259); Yme1p/Yta11p from *S. cerevisiae* (Yme1p; accession number Z49274); the Paraplegin protein from human (*Hs*; Y16610); a homologue from *A. thaliana* (*At*; AC005315); and the FtsH protease from *E. coli* (*Ec*; M83138). Identical residues in at least two sequences are indicated with a grey background. ATP-binding site Walker A and B motifs and metal-binding HEXXH motif are written in white and indicated with a dark background. The so-called 'second region of homology' present in all AAA proteins is boxed [136]. Predicted transmembrane regions are underlined. Only Yme1p contains a single membrane anchor.

introduced in the proteolytic center of both Yta10p and Yta12p, demonstrating that the proteolytic activity of the *m*-AAA protease is essential for mitochondrial function [10].

The *petite* phenotype of *yta10* or *yta12* mutants can be suppressed by overproduction of PIM1 protease [55]. Although the complementation is only partial as indicated by the slow growth of the transformants on nonfermentable carbon sources, this observation points to an overlapping substrate specificity of both ATP-dependent proteases. Indeed, bovine cytochrome P-450 expressed in yeast was found to be degraded by either PIM1 protease or the *m*-AAA protease depending on its localization within mitochondria [56]. Furthermore, as will be discussed later, the division of work between the proteases according to their location does not preclude cooperation and/or overlapping regulatory function between these proteolytic systems. The local concentration of PIM1 protease at the vicinity of the inner membrane may thus be sufficiently increased upon overexpression to substitute for the *m*-AAA protease functions required for respiration in a *yta10yta12* mutant.

Structure and biogenesis of *i*-AAA protease

The catalytic subunit of the *i*-AAA protease is encoded by the *YME1* (*YTA11*) gene [57, 58]. The deduced Yme1 polypeptide, which is 747 amino acids long, is highly homologous to Yta10p and Yta12p (32% identical residues in both cases) and belongs to the same subfamily of AAA proteins (fig. 3). Yme1p contains a predicted N-terminal mitochondrial sorting signal of 47 amino acid residues and the characteristic HEXXH zinc-binding motif of metalloproteases in its C-terminal domain. In contrast to Yta10p and Yta12p, Yme1p spans the inner mitochondrial membrane only once; a 20-kDa N-terminal domain is located in the matrix, whereas a 55-kDa catalytic domain is exposed to the intermembrane space (fig. 1) [13]. The *i*-AAA protease thus displays an inverted topology when compared with the *m*-AAA protease. A human Yme1p homologue present in the databases also contains only one predicted transmembrane region, suggesting a conserved topology of *i*-AAA proteases from yeast to humans.

Although Yme1p is the only subunit of the *i*-AAA protease identified so far, its overexpression did not result in a significant increase of *i*-AAA protease, suggesting that the amounts of another putative component(s) might be the limiting factor [57, 59]. The *i*-AAA protease forms a high molecular weight complex of about 1000 kDa similar to the *m*-AAA protease [13]. The stability of this complex, however, does not depend on the availability of nucleotides and is not affected by mutations in the ATP-binding motif of Yme1p. Deletion of *YTA10* or *YTA12* alters neither the assembly of Yme1p subunits nor their proteolytic activity, indicating that the *i*-AAA protease and the *m*-AAA protease form two independent complexes in the mitochondrial inner membrane.

Pleiotropic defects associated with *yme1* mutations

The *YME1* gene was originally isolated in a genetic screen as a factor which, upon mutation, increases the rate of mtDNA escape to the nucleus [60, 61]. The molecular basis of this phenotype is still obscure; however, the dependence of this process on a vacuolar peptidase suggests that mitochondria of *yme1* mutant cells are targeted for degradation by the vacuole during growth [62]. Pleiotropic phenotypes are associated to *yme1* null mutations [57]: reduced activity of the respiratory chain complexes at permissive temperature, respiratory growth defect at elevated temperature, cold-sensitive growth defect on fermentable carbon sources, and extremely slow growth or lethality when the mutation is expressed in a ρ^- mitochondrial background. Notably, swollen and nonreticulated mitochondria are present in *yme1* mutant cells, suggesting a role of Yme1p in maintaining the morphology of the mitochondrial compartment [62].

Phenotypes identical to those displayed by *yme1* knockout cells are observed for Yme1p variants with mutations impairing either the proteolytic or the ATPase activity [63]. This finding demonstrates the essential role of Yme1p-mediated proteolysis in mitochondria. It has been speculated that the various phenotypes of *yme1* mutations are due to alterations of the inner membrane composition and fluidity caused by the accumulation of detrimental polypeptides in the absence of the protease [62]. On the other hand, Yme1p displays genetic interactions with several factors which, upon mutation, lead to the suppression of one or more *yme1* mutant phenotypes or provide additional growth defects to *yme1* strains. The list of these factors includes Pep4p and Prc1p, two vacuolar proteases [62]; ATP3, the γ subunit of the ATP synthase [64]; Ynt20p, a putative mitochondrial 3′-5′ exonuclease [65]; Ynt1p/Rpt3p, a subunit of the 26S proteasome [66]; or the mitochondrial inner membrane protein Yme2p [67]. It is therefore conceivable that the various defects associated with *yme1* mutations reflect the requirement of the *i*-AAA protease for the turnover or the processing of different specific substrates regulating mitochondrial homeostasis.

Role of ATP-dependent proteases in mitochondrial gene expression

Mitochondria are composed of proteins of dual genetic origin: the vast majority of the proteins are encoded in the nucleus, whereas a handful of proteins are derived

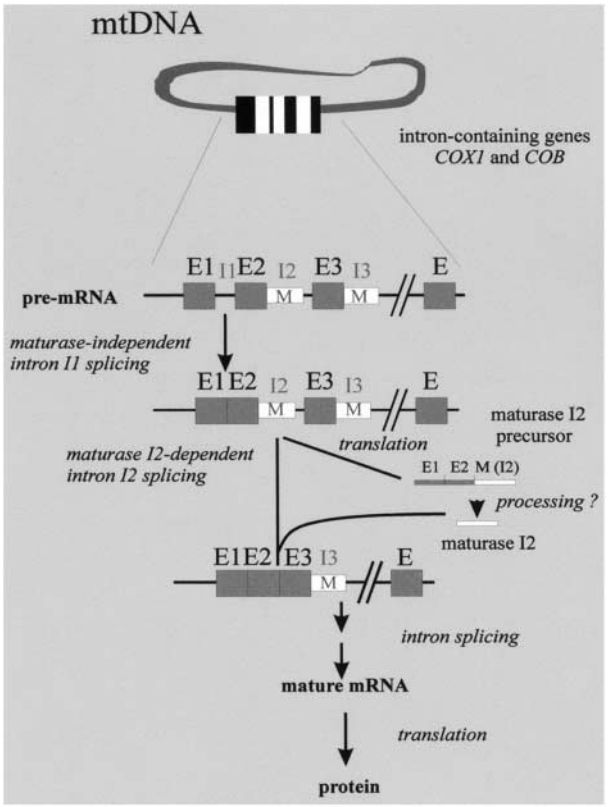

Figure 4. The maturase concept of intron splicing in yeast mitochondria. Polycistronic precursors are processed yielding single gene mRNA precursors (pre-mRNAs). Two respiratory chain subunits, CoxI and Cob, are encoded by split genes. Introns which do not encode ORFs (I1) are cotranscriptionally excised from the pre-mRNA. This mechanism involves nuclear-encoded factors. Introns I2 and I3 contain long ORFs in frame with the preceding exons. The excision of intron I1 creates an in-frame fusion between exons E1 and E2 and the ORF of intron I2. Translation of this pre-mRNA yields the synthesis of a fusion protein whose C-terminus is the product of the I2 ORF. The chimeric protein contains an mRNA maturase which, presumably after proteolytic processing, catalyses the removal of its encoding intron in cooperation with nuclear-encoded factors. Excision of I2 connects then the ORF of I3 with the fused exons E1, E2 and E3. In a similar process, the translation product of this precursor splices out the intron I3. This intron-splicing cascade results in the formation of mature mRNA which can be translated into protein. I, intron; E, exon; M, mRNA maturase.

from the mitochondrial genome. Energy-transducing enzymes are organized in five large, multisubunit complexes in the inner membrane of mitochondria (reviewed in [68, 69]): NADH:CoQ reductase (complex I; this complex is replaced by NADH-dehydrogenase in yeast); succinate:CoQ reductase (complex II); ubiquinol-cytochrome-*c*-reductase (complex III or bc_1 complex); cytochrome *c* oxidase (complex IV); and ATP synthase (complex V). These complexes are largely composed of nuclear-encoded subunits which are synthesized on cytosolic ribosomes and posttranslationally imported into the organelle [70, 71]. Some subunits, however, are encoded by the mitochondrial DNA (mtDNA) and synthesized in the organelle. The human mitochondrial genome, for instance, encodes 13 respiratory chain subunits [72]. From eight major translation products of the mtDNA in the yeast *S. cerevisiae*, seven are essential respiratory chain subunits [73–75]: cytochrome *b* (*COB*), three subunits of the cytochrome *c* oxidase (*COX1*, *COX2* and *COX3*) and three subunits of the ATP-synthase (*ATP6*, *ATP8* and *ATP9*). The mitochondrially encoded subunits assemble with nuclear-encoded subunits into functional multienzyme complexes and are essential for respiratory competence.

The analysis of mitochondrial translation products in *pim1* null mutants containing mtDNA and in cells lacking Yta10p or Yta12p provided the first evidence for a role of ATP-dependent proteases in gene expression [10, 11]. The synthesis of two respiratory chain subunits, CoxI and Cob, is under the proteolytic control of both PIM1 and *m*-AAA proteases. The role of these proteases in the expression of *COX1* and *COB*, two genes containing introns, will therefore be described in light of the mitochondrial gene expression system of *S. cerevisiae*.

Expression of mitochondrially encoded respiratory chain subunits

Gene expression in mitochondria of *S. cerevisiae* differs in many respects from nuclear gene expression, as it involves several peculiar RNA processing steps (reviewed in [76, 77]). First, many genes lack their own promoter and are transcribed as part of large transcriptional units. This is exemplified by the genes *COX1*, *ATP8* and *ATP6*, which are all transcribed as one polycistronic precursor RNA [78]. The primary transcripts must therefore be processed in order to generate messenger RNAs (mRNAs) and transfer RNAs (tRNAs). Second, in contrast to mammals, three mitochondrial genes of *S. cerevisiae* contain introns. The gene for the large subunit ribosomal RNA (LSU rRNA) contains one intron, *COB* up to five and *COX1* up to seven depending on the yeast strain (reviewed in [79]). The precursor transcripts of these three split genes must undergo a cleavage-ligation reaction—the RNA splicing—in order to generate active rRNA or translatable mRNAs.

In vivo splicing of introns is facilitated by proteins encoded by the nuclear genome and, in some cases, by the mitochondrial introns themselves (reviewed in [80]). Several yeast mitochondrial introns specify mRNA maturases which, according to the 'maturase concept' of intron splicing, are required for the splicing of the intron in which they are encoded (fig. 4) [81]. Such is the

case for the open reading frames (ORFs) present in introns bI2, bI3 and bI4 of the long *COB* gene [81–84] and introns aI1 and aI2 in *COX1* [85–87]. Maturase-encoding ORFs are in-frame with the preceding exon. Precursor forms of mRNA maturases are synthesized as fusion products with the preceding exon(s) and, presumably after proteolytic cleavage, active maturases are released [83, 88]. The 'maturase concept' of intron splicing involves several key features [81]: (i) Because splicing depends on the synthesis of the intron-encoded mRNA maturase, a block in translation will automatically result in splicing defects; (ii) the maturase removes its cognate intron from the precursor and thereby prevents its own synthesis; (iii) maturase synthesis and thus intron splicing require the excision of the preceding introns.

PIM1 protease is required for the translation of COX1 transcripts

The synthesis of CoxI and Cob is impaired in the absence of PIM1 protease [11]. Because the corresponding genes both contain introns, PIM1 could affect *COX1* and *COB* pre-mRNA stability and/or processing, and mRNA translation. A possible role of the protease in translation was, therefore, studied in strains where *COX1* and *COB* contain no introns. Cob is normally synthesized in a *pim1* deletion mutant containing wild-type mtDNA devoid of introns [11]. In contrast, CoxI protein is not synthesized in these cells despite overaccumulation of *COX1* mRNA. CoxI synthesis is not restored upon expression of a proteolytically inactive PIM1 variant, demonstrating the requirement of PIM1-mediated proteolysis for *COX1* mRNA translation.

Most of the proteins known to be involved in the translation of mRNAs in mitochondria of *S. cerevisiae* are specific for one of these mRNAs (reviewed in [89–91]). Similarly, only the synthesis of CoxI is defective in *pim1* mutants [11]. PIM1 protease may regulate the activity of specific RNA-binding proteins and, thereby, modulate the translation of *COX1* transcripts. Yeast mitochondrial translational activators involved in the expression of respiratory chain subunits mediate direct interactions between specific regions of the mRNA 5′ leaders and the mitochondrial ribosomes [92–94]. Possible targets of PIM1 protease thus include translation activator(s) or ribosomal protein(s) which might have to be processed or degraded to allow translation of *COX1* mRNA. Alternatively, translation repression has also been documented in yeast mitochondria [95, 96]. Synthesis of CoxI may thus depend on the processing and/or degradation of translation repressor(s) of *COX1* mRNA by PIM1 protease.

PIM1 protease affects the stability of pre-mRNAs containing multiple introns

Northern blot analysis of RNA derived from mitochondria lacking PIM1 protease revealed a role for ATP-dependent proteolysis in RNA metabolism. No precursor or mature forms of *COX1* and *COB* RNAs can be detected in *pim1* null mutant strains containing wild-type mtDNA due to the overproduction of Sss1p, although the genes are transcribed normally in these cells [11].

Several proteins are known to be required for the stability of *COX1* and/or *COB* pre-mRNAs [97–100]. Stability defects resulting from the inactivation of these proteins are often linked to a high number of introns in the genes. The role of PIM1 protease in *COX1* and *COB* RNA metabolism is also connected to the presence of multiple introns in these genes (seven introns in *COX1* and five introns in *COB*). In *pim1* mutant strains devoid of mitochondrial introns, *COX1* and *COB* mRNAs accumulate at normal levels [11]. Furthermore, if mitochondria harbor a short *COB* gene containing only intron bI1 and bI2, PIM1 protease is not required for the stability of *COB* RNAs (fig. 5b). However, only reduced amounts of *COB* mRNA can be detected, indicating the involvement of the protease in intron splicing (discussed below).

PIM1 protease is thus necessary for the stability of *COX1* and *COB* pre-mRNAs containing multiple introns. Not much is known about the mechanism regulating mtRNA turnover in yeast mitochondria [101]. The pre-mRNA stability defects observed in some mutants may be an indirect effect resulting from impaired gene expression or polycistronic precursor processing, as incorrectly processed RNAs are often unstable and rapidly degraded. Splicing defects, however, usually lead to accumulation of precursors and not to their degradation. Therefore, specific roles of ATP-dependent proteases in mtRNA stability could be envisioned.

PIM1 protease is required for maturase-dependent intron splicing

Mutations in the *PIM1* gene affect *COX1* and *COB* splicing processes through defects in CoxI translation and pre-mRNA stability. Complementation studies of *pim1* deletion by the *E. coli* Lon protease also suggested that the protease functions in intron splicing [11]. To unambiguously establish a direct role of PIM1 protease in RNA splicing, we recently constructed a *pim1* mutant strain maintaining wild-type mtDNA due to the overexpression of Sss1p and containing a short *COB* gene with introns bI1 and bI2 only (fig. 5a). Intron bI1 does not encode an mRNA maturase and is processed regardless of the presence of PIM1 protease [11], whereas intron bI2 encodes an mRNA maturase [81]. Northern blot

analysis using a *COB* exon-specific probe revealed that, when compared with wild-type cells, *pim1* mutant cells accumulate high amounts of a stable *COB* precursor RNA containing intron bI2 and only minute amounts of mature *COB* mRNA (fig. 5b). These results demonstrate that PIM1 protease is required for efficient splicing of at least one intron encoding an mRNA maturase. Possible ways by which PIM1 protease affects intron splicing are discussed in the next paragraph. It should be noted that, despite the drastic reduction of *COB* mRNA, the synthesis of Cob protein is not affected in this strain (data not shown). This observation is not unique, as normal synthesis of CoxI has been observed in a mutant having a 20-fold decrease in the level of *COX1* mRNA [102].

The *m*-AAA protease controls COX1 and COB pre-mRNA stability and splicing

Similar to PIM1, the *m*-AAA protease is required for the expression of *COXI* and *COB* [10]. In Δ*yta10* and Δ*yta12* strains devoid of mitochondrial introns, however, synthesis of CoxI and Cob as well as of the other mitochondrially encoded proteins is normal, indicating that the *m*-AAA protease is not required for translation but rather for RNA metabolism. Indeed, no mature forms and only reduced amounts of *COX1* and *COB* pre-mRNAs containing multiple introns are observed in mitochondria lacking Yta10p and/or Yta12p [10]. Though to a different extent, PIM1 and *m*-AAA proteases are thus both necessary for the stability of *COX1* and *COB* pre-mRNAs containing multiple in-

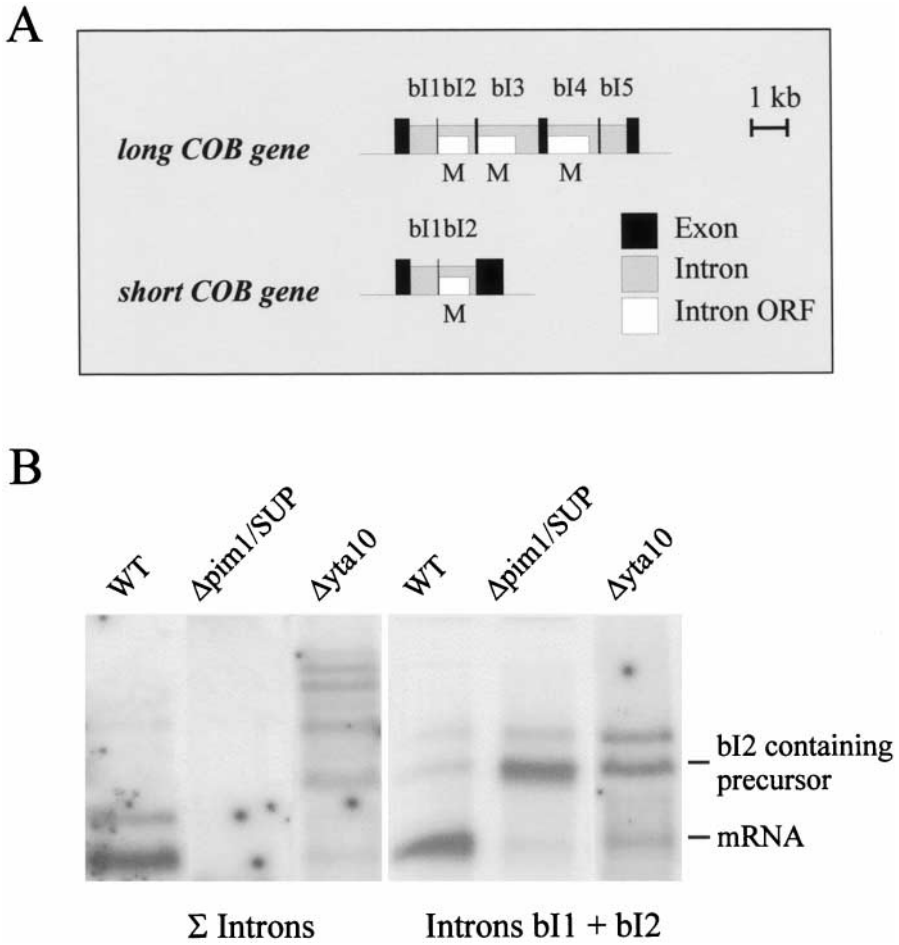

Figure 5. Expression of long and short *COB* genes in mitochondria devoid of PIM1 or *m*-AAA protease. (*A*) Structure of the long *COB* gene with the five introns and of a short variant of *COB* containing only intron bI1 and bI2, which encodes an mRNA maturase (adapted from [79]). M; mRNA maturase. (*B*) *COB* pre-mRNA processing in *pim1* and *yta10* deletion mutants. Three micrograms of mtRNA isolated from wild-type cells (WT), *pim1* cells containing mtDNA due to the expression of the multicopy suppressor gene *SSS1* (Δpim1/SUP), and *yta10* cells (Δyta10) were analysed with a *COB* probe specific for exons B1 as described in [11]. See text for further details. Σ Introns, strains containing the long *COB* gene with five introns; Introns bI1 + bI2, strains containing the short version of *COB* with intron bI1 and intron bI2 which encodes an mRNA maturase.

trons. The *m*-AAA protease is also involved in the maturase-dependent intron splicing of *COX1*- and *COB*-pre-mRNAs [10]. For instance, splicing of the *COB* intron bI2 is affected, though to a lesser extent than in *pim1* mutants, in cells lacking *m*-AAA protease. Reduced amounts of mature *COB* mRNA as well as accumulation of a precursor containing intron bI2 are observed in *yta10* null mutant cells harboring a short *COB* gene with introns bI1 and bI2 ([10] and fig. 5b). These results do not only establish a role of the *m*-AAA-protease for the splicing of a maturase-encoding intron, but also demonstrate that the maturase-dependent splicing of at least one intron is under the control of two ATP-dependent proteases in the mitochondrial matrix space.

A possible function of ATP-dependent proteases in maturase-dependent intron splicing

How PIM1 protease and the *m*-AAA protease affect pre-mRNA splicing is still a matter of speculation. Mutations in the proteolytic site of either Yta10p or Yta12p prevent degradation of all known substrates of the *m*-AAA protease [12]. Such mutations, however, do not affect stability and splicing of *COX1* and *COB* transcripts [10]. In contrast, cells with mutations in the proteolytic center of both Yta10p and Yta12p display the same phenotype as *yta10* and *yta12* deletion mutants [10]. Thus, the proteolytic activity of the *m*-AAA protease is required for pre-mRNA stability and processing, but the activity of only one subunit of the complex is sufficient for the expression of CoxI and Cob. Similarly, the proteolytically inactive variant $Pim1p^{S1015A}$ cannot promote mitochondrial gene expression in *pim1* mutants containing mtDNA [11]. *COX1* and *COB* pre-mRNA stability and splicing thus require the proteolytic activity of both PIM1 and *m*-AAA proteases.

Several nuclear-encoded factors have been shown to be involved in intron splicing in mitochondria of *S. cerevisiae* (reviewed in [77]). The list of these factors comprises proteins specifically involved in splicing, bifunctional enzymes like tRNA synthetases which affect RNA conformations, and proteins required for the synthesis of mRNA maturases, including proteins involved in mitochondrial translation or RNA helicases. Proteolysis and/or processing of any of these factors by ATP-dependent proteases could be required for efficient splicing of *COX1* and *COB* precursors. It should be kept in mind, however, that both PIM1 and *m*-AAA proteases affect specifically maturase-dependent intron splicing. The proteases could thus be directly involved in the biogenesis of mRNA maturases. Maturases are synthesized as fusion products of the intron-encoded ORF with the preceding exon(s) (reviewed in [80]; fig. 4), and the precursor polypeptides have to be processed in order to release the active maturase [88]. It has been suggested that this processing step could be accomplished by ATP-dependent proteases [103]. Both PIM1 and *m*-AAA proteases are likely candidates for this function. In this respect, it is interesting to note that neither *pim1* mutations nor *yta10* or *yta12* mutations lead to a total block in *COB* intron bI2 splicing (fig. 5b). These results could easily be explained if overlapping substrates and functions are considered for PIM1 protease and the *m*-AAA protease.

Proteolysis-dependent assembly of respiratory chain complexes

The requirement of both PIM1 and *m*-AAA proteases for the expression of the mitochondrially encoded respiratory chain subunits CoxI and Cob as well as the dependence of mtDNA stability on Pim1p provide an explanation for the *petite* phenotype observed in cells carrying mutations in any of the subunits of these proteases. The analysis of a *yta10* null mutant strain harboring an intronless mitochondrial genome, however, revealed an additional role of the *m*-AAA protease in post-translational complex assembly. Although CoxI and Cob synthesis is not affected in these cells, subunits of complex III and IV of the respiratory chain do not assemble into functional complexes [10].

To examine the dependence of respiratory chain complex assembly on the proteolytic activity of the *m*-AAA protease, a strain devoid of mitochondrial introns which expresses proteolytically inactive Yta10p and Yta12p subunits has been constructed [10]. The synthesis of all mitochondrially encoded polypeptides is normal in these cells; however, assembly of cytochrome *c* oxidase is impaired [10]. This observation indicates that the proteolytic activity of the protease is required for the assembly of this complex. Similarly, null mutants of *YTA10* or *YTA12* fail to assemble the F_1 subunit of the ATP synthase [54], and this phenotype was also observed in strains harboring proteolytically inactive Yta10p and Yta12p subunits [H. Arlt and T. Langer, unpublished observation]. How the *m*-AAA protease affects complex formation posttranslationally is still unknown. In any case, the *petite* phenotype displayed by *yta10* and *yta12* null mutants results from pleiotropic effects of the *m*-AAA protease on the biogenesis of respiratory chain complexes.

Protein substrates of mitochondrial ATP-dependent proteases

Substrate proteins of mitochondrial ATP-dependent proteases with regulatory functions, which would rationalize defects observed in protease-deficient cells, remain to be identified. All known authentic substrates are nonnative polypeptides and therefore reflect the quality control function of ATP-dependent proteases in mitochondria. PIM1 protease is required for the turnover of nonassembled α-, β- and γ-subunits of the F_1-ATPase, the β-subunit of the matrix processing peptidase (Mas1p) and several mitochondrial ribosomal proteins [9], and mediates the proteolytic breakdown of newly imported, misfolded model substrates in the matrix space of mitochondria [104]. Several nonassembled mitochondrially encoded inner membrane proteins, namely cytochrome *c* oxidase subunits I and III, cytochrome *b* and ATP-synthase subunits 6, 8 and 9, are substrates of the *m*-AAA protease [12, 53, 105]. The *i*-AAA protease has only been shown to be involved in the degradation of the cytochrome *c* oxidase subunit II [63, 106, 107].

Degradation of some other respiratory chain components located in the intermembrane space like cytochrome *c* or CoxV is not affected by mutations in the *i*-AAA protease [107, 108]. Therefore, additional proteolytic pathways which mediate the degradation of intermembrane space or inner membrane bound proteins must exist. Some polypeptides may diffuse from the intermembrane space to the cytosol where they are degraded by the 26S proteasome. Evidence for the existence of such a pathway was recently provided by the analysis of the proteolytic degradation of apo-cytochrome *c* [109].

Chaperone-like properties of ATP-dependent proteases

Substrate recognition and binding

The specificity of substrate recognition by ATP-dependent proteases is crucial to prevent cell damage. Very little is known about the determinants which signal the specific turnover of mitochondrial proteins; however, it appears likely that several mechanisms are involved in the recognition of different substrates. Targets of ATP-dependent proteases include mitochondrial proteins with apparently regulatory functions in the biogenesis of the organelle as well as nonassembled or misfolded polypeptides. It is therefore conceivable that specific sequence motifs trigger the proteolysis of some proteins, whereas others are recognized due to their nonnative conformation. Evidence for the importance of the folding state of mitochondrial proteins for proteolysis was provided by studies on the stability of hybrid proteins containing dihydrofolate reductase (DHFR). Destabilization of the DHFR domain at high temperature or by point mutations results in turnover of the hybrid proteins. This holds true for the proteolytic breakdown of soluble proteins by PIM1 protease in the matrix [104, 110] as well as for the turnover of integral membrane proteins, which expose an unfolded DHFR domain to the intermembrane space by the *i*-AAA-protease [111].

The specific recognition of unfolded polypeptides by the mitochondrial proteolytic system is reminiscent of molecular chaperone proteins, which stabilize nonnative polypeptides against aggregation and thereby ensure their folding to the native state (reviewed in [112–114]). Notably, the mitochondrial Hsp70 system is required for the degradation of misfolded proteins by the PIM1 protease and, at least for some model proteins, for the turnover of inner membrane proteins by the *m*-AAA protease [56, 104]. The fate of an unfolded polypeptide chain associated with Hsp70 is apparently determined by kinetic partitioning. Newly imported preproteins, which are still in a folding-competent state, attain their native conformation upon cycles of binding to and release from Hsp70. Misfolded polypeptides, however, remain associated with the chaperone protein for a prolonged time, thus allowing for their degradation.

ATP-dependent proteases do not only cooperate with molecular chaperone proteins during proteolysis but have chaperone-like properties themselves. This has been demonstrated for Yme1p, a subunit of the *i*-AAA protease [111]. Yme1p senses the folding state of solvent-exposed domains and specifically binds and degrades unfolded inner membrane proteins. Substrate recognition and binding is mediated by the conserved AAA domain of Yme1p which, when expressed and purified from *E. coli*, exerts chaperone-like properties in vitro and prevents the aggregation of various model substrates [111]. ATP-dependent conformational changes of Yme1p triggered by the AAA domain may induce the unfolding of associated substrate polypeptides and regulate the accessibility of the proteolytic sites. The *i*-AAA protease subunit Yme1p thus displays a modular structure: an ATPase domain with chaperone-like properties cooperating with a proteolytic domain. In view of the high sequence identity between Yme1p and the subunits of the *m*-AAA protease, Yta10p and Yta12p, a similar mode of action of both proteases is likely. Moreover, these findings are in agreement with studies on hetero-oligomeric Clp proteases in prokaryotes (reviewed in [26, 27, 115]). The regulatory subunits of these proteases exert ATPase and chaperone activity in the absence of a proteolytic subunit [116–118]. When complexed with ClpP, they present substrate proteins to the peptidase for degradation. Similar tertiary structures of the ATPase domains of AAA proteins have been predicted on the basis of sequence comparisons for Lon-like and Clp proteins

[30, 31]; therefore, a conserved mechanism for ATP-dependent proteolysis can be envisioned.

Chaperone function and proteolytic activity

The chaperone-like properties of mitochondrial ATP-dependent proteases raised the possibility that they also promote protein complex assembly, independent of their proteolytic activity [9]. Indeed, several findings have initially suggested nonproteolytic functions of ATP-dependent proteases in mitochondrial biogenesis: First, cells expressing either Yta10p or Yta12p with point mutations in the proteolytic site are respiratory-competent and contain normally assembled respiratory chain complexes, but are deficient in turnover of inner membrane proteins by the *m*-AAA protease [12, 53]. Second, the respiratory deficiency of *yta10yta12* null mutants was restored by overexpression of proteolytically inactive PIM1 protease carrying a point mutation in its proteolytic center [40]. In contrast, suppression is prevented by mutation of the ATP-binding site of Pim1p [40]. Third, Mba1p and Oxa1p, two proteins lacking proteolytic activity, were identified as multicopy suppressors of *yta10* and *yta12* null mutants [55]. Mba1p is a membrane-bound, mitochondrial matrix protein involved in the assembly of respiratory chain complexes [119], whereas the polytopic inner membrane protein Oxa1p is a component of a translocase which mediates export of nuclear- and mitochondrially encoded proteins from the matrix across the inner membrane [120–123].

A detailed examination of proteolytic site mutants of the mitochondrial ATP-dependent proteases, however, establishes that all functions documented so far can be attributed to their proteolytic activity. Cells containing proteolytically inactive variants of the proteases exhibit phenotypes identical to those of the respective null mutant cells. For instance, mutation of the proteolytic sites of both Yta10p and Yta12p impairs respiratory competence and the assembly of respiratory chain complexes to the same extent as manifested in the absence of either protein, demonstrating that the *m*-AAA protease exerts some proteolytic activity after inactivation of either Yta10p or Yta12p [10]. Similarly, overexpression of proteolytically inactive Pim1p was performed in *yta10yta12* null mutant cells containing the endogenous wild-type *PIM1* gene [40]. Wild-type and proteolytically inactive Pim1p subunits, however, have been demonstrated to form mixed oligomeric complexes which still exert sufficient proteolytic activity to stabilize mtDNA in these cells [34]. On the other hand, oligomerization of Pim1p subunits occurs in an ATP-dependent manner, and consequently ATPase-mutant subunits of Pim1p cannot assemble with wild-type subunits [34]. These observations provide a rationale for the different suppressive effects of point mutations in the proteolytic and ATP binding sites of PIM1 protease in *yta10yta12* null mutant cells.

ATP-dependent proteases in organelles of higher eukaryotes

Early reports suggested the occurrence of ATP-dependent degradation in mammalian mitochondria [124–127]; however, until recently, little was known about ATP-dependent proteolysis in organelles of higher eukaryotes. ATP-dependent proteases of the Lon-, AAA- and Clp families have now been found in almost all eukaryotic classes, and these enzymes are likely to be active in mitochondria or chloroplasts [17, 19, 20, 33, 128]. The existence of conserved physiological pathways and a conserved mode of action of these enzymes within their families is conceivable, though differences in activities between the enzymes of yeast and of higher eukaryotes cannot presently be excluded. Similar to the situation in yeast, mitochondrial ATP-dependent proteases of higher eukaryotes appear to fulfill important roles for organellar homeostasis. For instance, a regulatory role in mitochondrial biogenesis has been suggested for the rat Lon protease on the basis of expression studies [129], and a plant Lon protease has been shown to regulate the turnover of a peptide associated with cytoplasmic male sterility [130]. Most important, an ATP-dependent protease has recently been implicated in a mitochondrial pathology. The human Paraplegin gene encodes an AAA-protease subunit located in the inner membrane of mitochondria and highly homologous to yeast AAA-proteases [128]. Deletion and frameshift mutations in the Paraplegin gene have been found to cause a neurodegenerative disorder, hereditary spastic paraplegia (HSP) [128, 131, 132]. Patients suffering from HSP display typical signs of impaired oxidative phosphorylation, consistent with the role of homologous proteins in yeast.

Direct evidence for a functional conservation of organellar ATP-dependent proteases has been provided for Lon-like proteases by complementation studies in yeast. When expressed in yeast cells, Lon1p, one of the two Lon homologues identified in the plant *Zea mays*, is imported into yeast mitochondria and can stabilize wild-type mtDNA in the absence of PIM1 protease [133]. It does not, however, provide respiratory competence to *pim1* mutant cells. Analysis of the cytochrome content of *LON1*-complemented *pim1* mutant cells indicates the presence of cytochrome *b* but not of cytochrome *c* oxidase. As no data are available on the number of *COX1* and *COB* introns present in the yeast strain used in this study, a possible activity of the plant Lon1p in intron splicing and Cox1 translation in yeast

cannot be assessed. Similarly, *E. coli* Lon protease partially substitutes for Pim1p and allows respiratory growth at 30 °C but not at 37 °C [110]. Thus, Lon-like proteases have a conserved mode of action and can substitute for some, but not all, functions of their homologous proteins when expressed in heterologous systems. Failure to perform some activities may be related to the specificity of the different proteases.

Concluding remarks and perspectives

During the last few years there has been an accumulation of information on the biogenesis and role of membrane-bound and soluble ATP-dependent proteases within mitochondria. These proteases perform both regulatory and housekeeping functions and as a result are essential for mitochondrial homeostasis and cellular respiration (summarized in fig. 6).

The molecular mechanisms of regulated proteolysis in mitochondria, however, are still far from being elucidated. How substrates are selected and bound by the proteases is among the major questions to address. Similarly, the generation of proteolytic products, presumably peptides, by ATP-dependent proteases and their subsequent breakdown by non-ATP-dependent peptidases has not yet been analysed. Further studies should also focus on the regulation of mitochondrial proteases. A first step in this direction is the identification of Phb1p and Phb2p, members of the prohibitin family in yeast, as negative regulators of the *m*-AAA protease activity [134]. The fact that prohibitins are associated with senescence and tumor suppression in mammalian cells [135] may connect these processes to

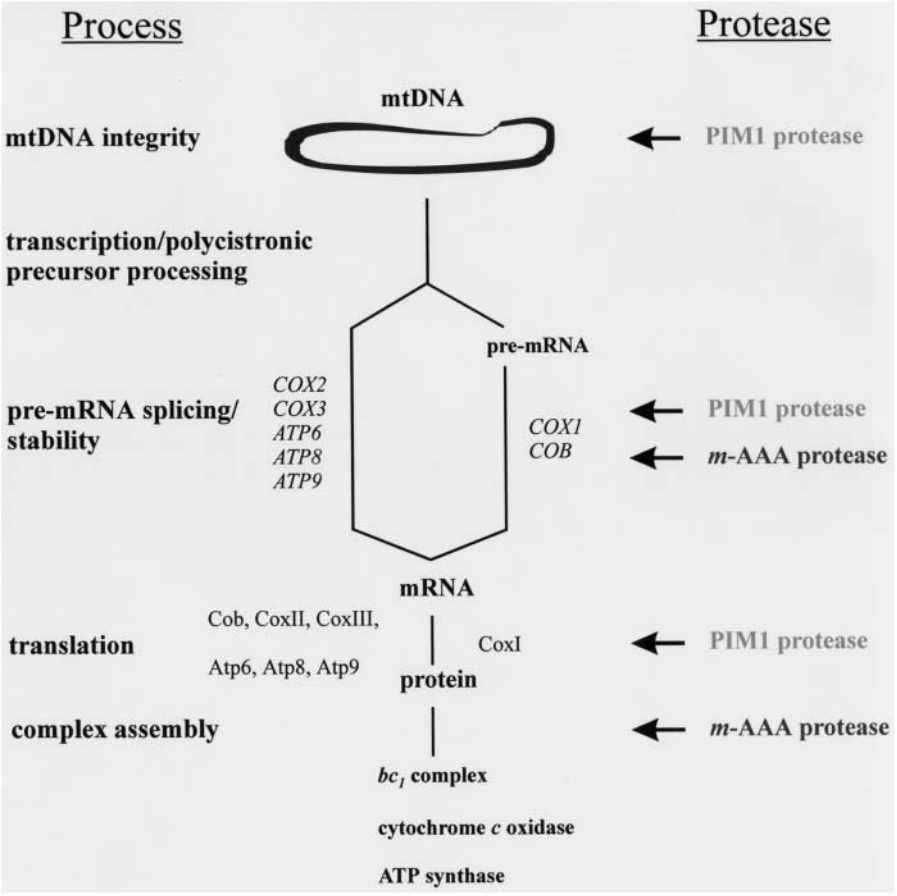

Figure 6. Role of ATP-dependent proteases in the biogenesis of respiratory complexes. The mitochondrial genome encodes seven respiratory chain subunits. PIM1-mediated proteolysis is required for mtDNA integrity. PIM1 and *m*-AAA proteases are involved in the expression of intron-containing *COX1* and *COB* genes: some maturase-encoding intron splicing steps as well as the stability of precursor RNAs containing multiple introns are affected in the absence of either protease. In addition, PIM1 protease is required for the translation of CoxI. Newly synthesized, mitochondrially encoded respiratory chain subunits assemble into functional complexes with nuclear-encoded proteins. *m*-AAA proteolytic activity is required posttranslationally for complex assembly.

regulated proteolysis in mitochondria. Thus, if some of the physiological pathways in which mitochondrial ATP-dependent proteases are involved are well documented, the identification of many others functions will be forthcoming.

Mitochondrial ATP-dependent proteases are highly conserved throughout all kingdoms. Based on studies in the yeast *S. cerevisiae*, a crucial role of homologous enzymes can also be expected in mitochondria of higher eukaryotes. The recent identification of a human mitochondrial AAA protease involved in a neurodegenerative mitochondrial disorder substantiates this hypothesis and suggests that, in the near future, more mitochondria-linked pathologies may be attributed to defects in ATP-dependent proteolysis.

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