Protein degradation in mitochondria

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The biogenesis of mitochondria and the maintenance of mitochondrial functions depends on an autonomous proteolytic system in the organelle which is highly conserved throughout evolution. Components of this system include processing peptidases and ATP-dependent proteases, as well as molecular chaperone proteins and protein complexes with apparently regulatory functions. While processing peptidases mediate maturation of nuclear-encoded mitochondrial preproteins, quality control within various subcompartments of mitochondria is ensured by ATP-dependent proteases which selectively remove non-assembled or misfolded polypeptides. Moreover, these proteases appear to control the activity- or steady-state levels of specific regulatory proteins and thereby ensure mitochondrial genome integrity, gene expression and protein assembly.

Key words: AAA proteases / Lon proteases / mitochondria / prohibitins / proteolysis

Introduction

The selective degradation of proteins is essential for cellular homeostasis and allows its adaptation to altered environmental conditions. Similar to the turnover of cytosolic proteins, proteolysis of mitochondrial proteins can occur in the lysosomal compartment upon autophagy of the whole organelle.[1] While this process is predominant under starvation conditions and results in the non-selective removal of mitochondrial proteins, processing or proteolysis of specific mitochondrial proteins is

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mediated by peptidases within mitochondria themselves.[2, 3] In general, these peptidases are highly conserved and, at least in most cases, appear to be ubiquitously present in mitochondria of eukaryotic cells (Figure 1). Mitochondrial peptidases can be divided into three groups: processing peptidases, oligopeptidases and ATP-dependent proteases. We will focus in this review on ATP-dependent proteases in mitochondria and only briefly summarize the current understanding on other peptidases which is described in a comprehensive manner elsewhere.[4–9]

Mitochondrial processing peptidases

The vast majority of mitochondrial proteins is nuclear encoded. The notion, that targeting to mitochondria and intramitochondrial sorting is ensured by N-terminal presequences which are proteolytically cleaved off once proteins reach their final destination, provided first evidence for the existence of specific peptidases in mitochondria. Processing enzymes have been identified since then in various mitochondrial subcompartments (Table 1). Despite rather degenerate cleavage motifs, processing of mitochondrial preproteins occurs with high fidelity. It depends on structural information within the presequence and in regions adjacent to the cleavage site.

The mitochondrial processing peptidase (MPP) cleaves off N-terminal mitochondrial targeting sequences of nuclear-encoded precursor proteins in the mitochondrial matrix space[5, 6, 8, 9] (Table 1). The heterodimeric Zn^{2+}-metallopeptidase consists of two subunits of about 50 kDa. Initial substrate recognition and binding is mediated by α-MPP which presents presequences to the proteolytically active β-subunit for cleavage.[10, 11] While many matrix and inner membrane proteins are released in their mature form from MPP, maturation of some matrix and intermembrane space proteins depends on a second processing step. The mitochondrial intermediate peptidase (MIP) cleaves off N-terminal octapeptides from some
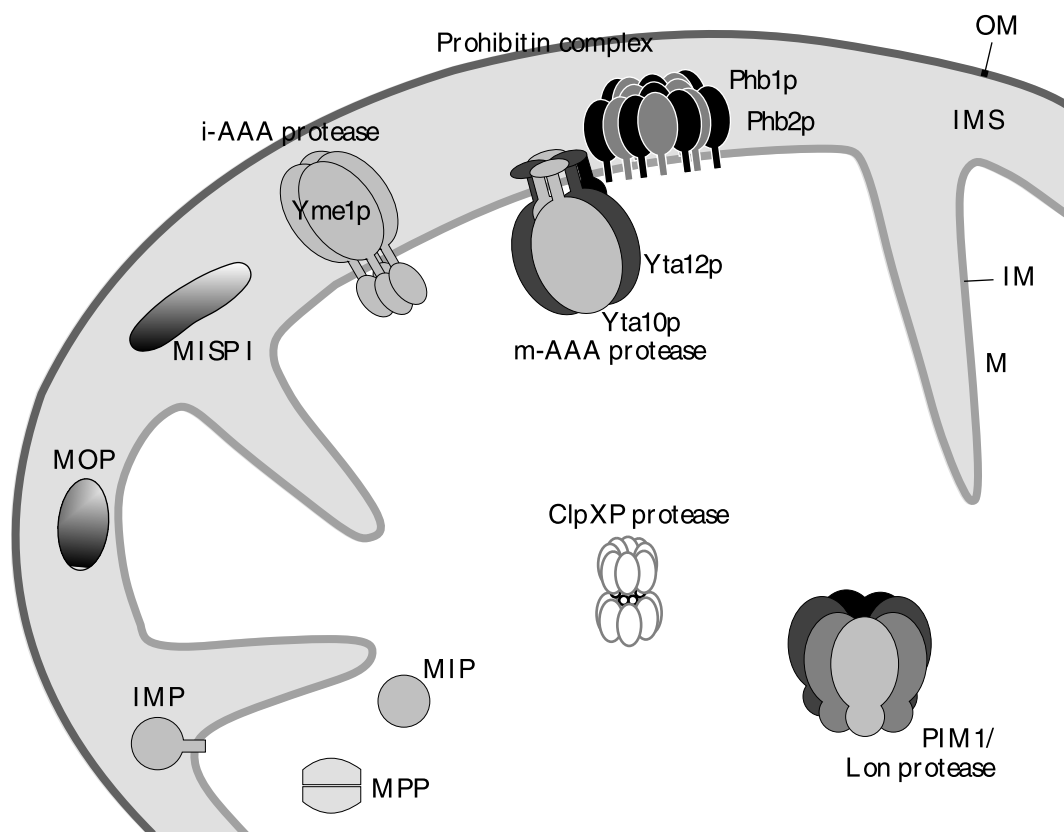
Figure 1. The proteolytic system of mitochondria. Maturation of nuclear-encoded preproteins is mediated by specific processing peptidases in various mitochondrial subcompartments: mitochondrial processing peptidase (MPP), mitochondrial intermediate peptidase (MIP), and inner membrane peptidase (IMP). ATP-dependent proteases degrade non-native polypeptides and exert crucial regulatory functions in mitochondrial biogenesis: PIM1/Lon protease, ClpXP protease (only identified in mammalian mitochondria), *m*-AAA protease and *i*-AAA protease. An additional ATP-dependent protease has been identified in the intermembrane space of mammalian mitochondria (MISP I; mitochondrial intermembrane space protease I).[88] The mitochondrial oligopeptidase MOP (termed yscD/Prd1p in yeast) in the intermembrane space represents the only identified oligopeptidase in mitochondria.[91, 92] The prohibitin complex does not exhibit proteolytic activity but modulates proteolysis by the *m*-AAA protease. See text for details. OM, mitochondrial outer membrane: IMS, mitochondrial intermembrane space; IM, mitochondrial inner membrane; M, mitochondrial matrix.

matrix-localized proteins including iron-utilizing proteins and components of the electron transport chain, the tricarboxic cycle and the mitochondrial genetic machinery.[12–15] The physiological function of processing by MIP, however, remains to be elucidated. Maturation of intermembrane space proteins with a bipartite presequence occurs by consecutive cleavage by MPP in the matrix and by the inner membrane protease (IMP) in the intermembrane space.[4, 16, 17] The latter protease is homologous to eubacterial and eukaryotic signal peptidases.[18] It is composed of two related subunits with non-overlapping substrate specificities, Imp1p and Imp2p, both of which are an integral part of the inner membrane and expose their catalytic sites to the intermembrane space.[19]

ATP-dependent proteases of mitochondria

In contrast to limited proteolytic events mediated by processing peptidases, ATP-dependent proteases mediate the complete degradation of dispensable mitochondrial proteins. Several ATP-dependent proteases have been identified in different subcompartments of mitochondria (Table 2). They are all derived from bacterial ancestors and comprise highly conserved protein families in eukaryotic cells.[2, 3, 20] Studies in the yeast *Saccharomyces cerevisiae* revealed a dual function of ATP-dependent proteases in mitochondria. On one hand, they constitute a quality control system and prevent the possibly deleterious

accumulation of non-assembled and misfolded polypeptides in the organelle. On the other hand, the selective proteolysis of some mitochondrial proteins by ATP-dependent proteases appears to be crucial for mitochondrial biogenesis. Increasing evidence suggests that a loss of the latter activity explains severe phenotypes associated with mutations in ATP-dependent proteases in various organisms including man.

Lon-like proteases in the matrix

Lon-like proteases build up a conserved protein family with members in eubacteria, archaebacteria and eukaryotic cells where they appear to be exclusively localized to the matrix space of mitochondria.[21–24] Functional conservation between various members of this family has been demonstrated by complementation studies in yeast.[25, 26] Lon-like proteases form homo-oligomeric complexes. While the stoichiometry of *Escherichia coli* Lon protease is still a matter of debate, a heptameric stoichiometry has recently been described for the yeast homologue[27] which is also termed PIM1 protease.[21, 22] The analysis of mitochondrial extracts provided first evidence for the existence of an even larger high molecular mass assembly *in vivo.*[28]

Several domains can be distinguished in subunits of Lon-like proteases. They harbour an ATPase domain characteristic of Walker-type P-loop ATPases which exhibits a tertiary fold similar to other ATP-dependent proteases.[29, 30] ATP hydrolysis is indispensable for proteolysis whereas ATP binding was found to be required for oligomerization of yeast PIM1 protease.[28, 31] A proteolytic domain containing the catalytically active serine residue is present at the C-terminus of the protease subunits. Mitochondrial Lon-like proteases contain an additional N-terminal domain of unknown function which is absent in eubacterial homologues. As shown for PIM1 protease in yeast, sorting to mitochondria is ensured by a targeting sequence and a pro-region at the N-terminus.[28] While the targeting sequence is cleaved off by MPP in the matrix, the pro-region is autocatalytically removed upon assembly of PIM1 subunits.

Substrates of Lon-like proteases in mitochondria have only been identified in yeast and include various non-assembled polypeptides, such as β-MPP, subunits α, β and γ of the F_1F_0-ATP synthase and ribosomal proteins,[20] as well as missorted and misfolded model proteins.[32] PIM1 protease is thus part of a quality control system in the matrix preventing the accumulation of non-native polypeptides. Consistently,

inclusion bodies containing most likely aggregated mitochondrial proteins have been observed in yeast mitochondria lacking PIM1 protease.[22] It is conceivable that the ATPase domain of Lon proteases exerts chaperone-like activity, promotes substrate unfolding and ensures the specificity of proteolysis, as such a role has been demonstrated for structurally related ATPase domains of other ATP-dependent proteases.[33] This activity, however, is apparently not sufficient to prevent the aggregation of substrate polypeptides, a prerequisite for their degradation by Lon-like proteases. This is achieved by the mitochondrial Hsp70 system which was found to cooperate with PIM1 protease in the degradation of misfolded polypeptides in the matrix of mitochondria.[32] The Hsp70 system also promotes folding of newly imported proteins in the matrix and thus represents a checkpoint between folding and degradation of mitochondrial proteins. The fate of a polypeptide is thought to be determined by the kinetics of partitioning between an association with PIM1 protease for proteolysis and binding to the Hsp70 system for folding.[3, 32]

Inactivation of PIM1 protease in yeast causes severe phenotypes which appear to reflect specific regulatory functions of the protease during mitochondrial biogenesis rather than the deleterious effect of non-native polypeptides accumulating in the absence of the protease. PIM1 protease affects the expression of mitochondrially encoded respiratory chain subunits at multiple steps and is therefore required for cell growth on non-fermentable carbon sources (Figure 2) (see Reference 34 for a comprehensive review). Cells lacking PIM1 protease accumulate extensive mutations in the mitochondrial DNA (mtDNA).[21, 22] The molecular basis of this phenotype is presently unclear, but the peculiar property of bacterial and human Lon proteases to bind single-stranded DNA in a site-specific manner suggests a direct role of Lon-like proteases in the mtDNA metabolism.[35, 36] Moreover, PIM1 protease controls the expression of two mitochondrial mosaic genes, *COX1* and *COB*, which encode the essential respiratory chain subunits Cox1p (subunit 1 of cytochrome *c* oxidase) and Cob (cytochrome *b* of the cytochrome bc_1-complex).[37] PIM1-mediated proteolysis is required for the splicing of introns in both genes which code for RNA maturases. These enzymes are synthesized as fusion proteins with preceeding exons and activated by proteolytic removal of the exon-encoded moiety.[38, 39] It is an attractive possibility that PIM1 protease mediates this cleavage reaction. Alternatively, the protease may control the activity of

Table 1. Processing peptidases of mitochondria

Peptidase	Localization	Subunits	Proteolytic activity	Substrates
MPP (mitochondrial processing peptidase)	matrix	α-MPP β-MPP	Zn^{2+}-metallopeptidase	• soluble matrix proteins • IM proteins • IMS proteins with bipartite presequences
MIP (mitochondrial intermediate peptidase)	matrix		metallopeptidase	• iron-utilizing proteins • respiratory chain subunits • tricarboxic cycle enzymes • components of the mitochondrial genetic machinery
IMP (inner membrane peptidase	IM, facing the IMS	Imp1p Imp2p	serine-peptidase	• IM and IMS proteins with bipartite presequences

regulatory proteins directly involved in pre-mRNA splicing. After transcript maturation, translation of *COX1* mRNA also depends on PIM1 protease[37] which thus exerts multiple functions in mitochondrial gene expression essential for the maintenance of the respiratory competence of the cell.

Clp-like proteases in the matrix

Proteases homologous to eubacterial Clp proteases have been identified in the matrix of mammalian mitochondria but are absent in lower eukaryotes such as yeast.[37, 40, 41] Next to nothing, however, is known about their physiological function. Clp-like proteases form hetero-oligomeric complexes with an interior chamber for proteolysis and are built up by ATPase and proteolytic subunits.[33, 42] The ATPase subunits belong to the Hsp100/Clp family,[43, 44] members of which function both as chaperones and as subunits of bacterial Clp proteases. They unfold misfolded polypeptides allowing either their refolding by other chaperone systems or, if associated with proteolytic subunits, their degradation. Notably, though lacking proteolytic subunits, homologues of the ATPase subunits are present in the matrix of yeast mitochondria. Yeast Hsp78, a member of the ClpB subfamily of chaperones in the matrix,[45] has apparently no proteolytic function but is required for mitochondrial thermotolerance.[46] Homologues of *E. coli* ClpX have been identified in mammals and yeast. While forming the ATPase subunit of a Clp-like protease in mammalian mitochondria,[41] it might act as a chaperone on its own in yeast lacking an apparent proteolytic partner.[47]

AAA proteases in the mitochondrial inner membrane

A large number of mitochondrial proteins are located in the inner membrane which is characterized by an extremely high protein content.[48] Quality control of inner membrane proteins is ensured by two ATP-dependent proteases, termed AAA proteases, which are an integral part of this membrane and exert a key function in the maintenance of its integrity.[49] They expose their catalytic sites to opposite membrane surfaces, the *m*atrix and the *i*ntermembrane space side, and are accordingly termed *m*- and *i*-AAA protease. Orthologues of both proteases are seemingly present in mitochondria of all eukaryotic cells but are best studied in the yeast *S. cerevisiae*.

Mitochondrial AAA proteases belong to a highly conserved protein family with homologues also present in chloroplasts and eubacteria.[49, 50] They build up large complexes with a native molecular mass of approximately 1 MDa in the mitochondrial inner membrane which are composed of identical or closely related subunits of 70–80 kDa.[51, 52] All subunits contain an ATPase domain, which is characteristic of the AAA superfamily of ATPases (for ATPases associated with a variety of cellular activities)[30, 53, 54] and which has chaperone-like properties.[55] A proteolytic domain with metallopeptidase activity is present at their C-terminus. AAA proteases degrade, in contrast to soluble ATP-dependent proteases, membrane-embedded polypeptides if they are non-assembled or misfolded. Inactivation of AAA proteases causes severe defects in various organisms including neurodegeneration in humans, most likely reflecting regulatory functions of these proteases crucial for the biogenesis and homeostasis of mitochondria.
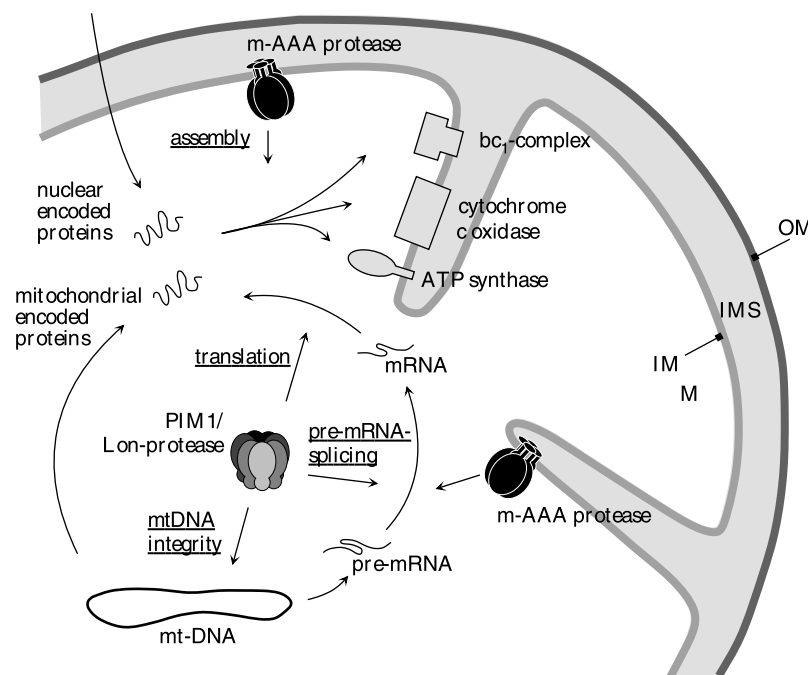
Figure 2. Roles of ATP-dependent proteases in mitochondrial gene expression and protein assembly in *S. cerevisiae*. Processes under the proteolytic control of ATP-dependent proteases are underlined. See text for details. OM, mitochondrial outer membrane; IMS, mitochondrial intermembrane space; IM, mitochondrial inner membrane; M, mitochondrial matrix.

i-AAA protease

The *i*-AAA protease in yeast appears to represent a homo-oligomeric complex composed of Yme1p subunits.[52] Yme1p contains one transmembrane segment. An N-terminal domain of approximately 170 amino acid residues is present in the matrix space while a large C-terminal domain with the catalytic sites is exposed to the intermembrane space.[52] Point mutations in the proteolytic center of Yme1p or a deletion of the complete *YME1* gene both result in identical pleiotropic defects in *S. cerevisiae*.[56–58] Cells lose their respiratory competence at elevated temperature and accumulate mitochondria with a punctate, non-reticulated morphology. The latter phenotype has been suggested to result in an increased turnover of mitochondria in the vacuolar compartment.[59] This scenario could provide an explanation for the increased rate of mEDNA escape which has originally lead to the identification of the YME1 gene.[60] The molecular basis of various phenotypes associated with *yme1* mutations is presently not understood, but it appears likely that multiple proteolytic substrates of Yme1p exist. Consistently, each of the various phenotypes can be suppressed individually by different extragenic mutations.[58, 59, 61, 62] The only

reported substrate of the *i*-AAA protease, however, is non-assembled subunit 2 of cytochrome *c* oxidase (Cox2p),[57, 63, 64] illustrating the quality control function of the protease in the inner membrane.

m-AAA protease

In *S. cerevisiae*, the *m*-AAA protease is composed of multiple copies of two homologous subunits, Yta10p (Afg3p)[65, 66] and Yta12p (Rca1p),[67, 68] which are closely related to each other and to the *i*-AAA protease subunit Yme1p.[51] In contrast to Yme1p, Yta10p and Yta12p span the inner membrane twice. A small N-terminal and a large C-terminal domain harbouring the catalytic sites are exposed to the matrix.[51] Mutational analysis of both proteins in yeast revealed first evidence for an overlapping but non-identical substrate specificity of Yta10p and Yta12p.[52, 69] A variety of substrate polypeptides has been identified including non-assembled subunits of respiratory chain complexes and of the F_1F_0-ATP synthase.[51, 70] All of these polypeptides are an integral part of the inner membrane but it is likely that the *m*-AAA protease is also capable of degrading proteins peripherally associated with the inner membrane.

The pivotal role of the *m*-AAA protease of mitochondrial biogenesis is illustrated by strong phenotypes associated with mutations in Yta10p and Yta12p in yeast. The *m*-AAA protease is essential for the maintenance of oxidative phosphorylation.[65, 66, 68, 69] The expression of the mitochondrially encoded respiratory chain subunits Cox1p and Cob is under the proteolytic control of the *m*-AAA protease.[69] Impaired splicing of *COX1* and *COB* introns encoding RNA maturases was observed in the cells lacking *m*-AAA protease (Figure 2). Similar to the matrix-localized PIM1 protease, the *m*-AAA protease might be involved in the proteolytic activation of RNA maturases (see Reference 34 for a comprehensive review). In any case, the activity of two ATP-dependent proteases, the PIM1 and the *m*-AAA protease, is required to ensure the expression of two mitochondrial mosaic genes coding for essential respiratory chain subunits. In addition to its role in mitochondrial gene expression, the *m*-AAA protease affects also the post-translational assembly of respiratory chain complexes and the F_1F_0-ATP synthase.[69, 71] While these results establish crucial functions of the *m*-AAA protease in mitochondrial biogenesis, a detailed understanding of these processes awaits the identification of the target proteins of the protease.

Two orthologues of yeast *m*-AAA protease subunits have been identified in humans.[72, 73] Mutations in one of them, paraplegin, cause an autosomal recessive form of hereditary spastic paraplegia.[72] Deficiencies in mitochondrial oxidative phosphorylation were observed in these cells, reminiscent of defects in yeast cells lacking Yta10p and Yta12p. These findings point to conserved functions in mitochondrial biogenesis of *m*-AAA proteases in yeast and mammals.

Regulation of m-AAA protease activity by prohibitins

The analysis of mitochondrial extracts by sizing chromatography in yeast revealed that the *m*-AAA protease is present in a supercomplex in the inner membrane which has a native molecular mass larger than 2 MDa.[74] It associates with another membrane protein complex containing the prohibitin homologues Phb1p and Phb2p. While also an integral part of the inner membrane, Phb1p and Phb2p are largely exposed to the intermembrane space, i.e. to the opposite membrane surface as the *m*-AAA protease.[74] The prohibitins do not represent novel subunits of the *m*-AAA protease as they are dispensable for its proteolytic activity. Rather, they appear to fulfill regulatory functions during proteolysis. An increased turnover of non-assembled inner membrane proteins

by the *m*-AAA protease was observed in mitochondria lacking prohibitins suggesting a negative regulatory effect.[74] Affecting the conformation of the *m*-AAA protease, the prohibitin complex may modulate its specific proteolytic activity. Alternatively, prohibitins may directly interact with substrate polypeptides and regulate their binding to the *m*-AAA protease. A similar function has been described for the *E. coli* proteins HflK and HflC which show sequence similarities to eukaryotic prohibitins and modulate the proteolytic activity of the *E. coli* AAA protease FtsH.[75–77] Thus, regulation of AAA proteases appears to be conserved and derived from an earlier common ancestor.

Prohibitin was originally identified in mammals due to its decreased expression in tumor cells and its ability to negatively regulate cell proliferation.[78, 79] Highly conserved homologues appear to be ubiquitously present in all eukaryotic cells[80] and have been implicated in diverse processes, such as the regulation of the cellular life span[81] and the maintenance of mitochondrial morphology.[82] It remains to be determined whether the various effects of prohibitins reflect their role in proteolysis or whether additional functions have to be envisioned. The solvent-exposed domain of prohibitins exhibit significant sequence similarity to stomatin-like proteins and to the caveolae-associated flotillins, raising the intriguing possibility that these proteins are also components of membrane-associated proteolytic complexes.[83]

Quality control of mitochondrial proteins by ATP-dependent proteases

The fidelity of proteolysis, i.e. the specificity of substrate recognition by mitochondrial ATP-dependent proteases is crucial to prevent cell damage. In the eukaryotic cytosol, polyubiquitination of proteolytic substrates ensures their targeting to the 26S proteasome for proteolysis.[84] There is, however, no evidence for the existence of a similar system within mitochondria nor have sequence motifs been identified which trigger the degradation of specific mitochondrial proteins. Rather, identified proteolytic substrates appear to be solely recognized due to their non-native 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

Table 2. ATP-dependent proteases in mitochondria of *S. cerevisiae*. See text for details. Proteolytic substrates identified represent exclusively non-assembled membrane proteins illustrating the quality control function of the proteases within mitochondria

ATP-dependent protease	Localization	Subunits	Function	Substrates
PIM1/Lon protease	matrix	Pim1p	• mtDNA integrity • *COX1*- and *COB*-pre-mRNA splicing • *COX1* translation	• Mas1p • α-β-γ-subunit of the F_1F_0-ATP synthase • ribosomal proteins
m-AAA protease	IM, facing the matrix	Yta10p (Afg3p) Yta12p Rca1p	• *COX1*- and *COB*-pre-mRNA splicing • assembly of bc_1-, cytochrome *c* oxidase, ATP synthase complexes	• Cox1p • Cox3p • Cyt b_2 • subunits 6, 8, 9 of the F_1F_0-ATP synthase
i-AAA protease	IM, facing the IMS	Yme1p	• maintenance of respiratory competence at high temperature • mitochondrial morphology	• Cox2p

of soluble proteins by PIM1 protease in the matrix[26] 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.[55]

ATP-dependent proteases by themselves are capable of sensing the folding state of their substrates. The analysis of substrate binding to truncated versions of the *i*-AAA protease subunit Yme1p revealed a crucial function of its AAA domain, in particular of its N-terminal part, for substrate binding.[55] When expressed and purified from *E. coli*, the AAA-domain of Yme1p exerts chaperone-like properties: it binds specifically to non-native polypeptides and suppresses their aggregation.[55] ATP-dependent conformational changes may result in unfolding of associated substrate polypeptides facilitating their subsequent degradation at the proteolytic site. Notably, all known ATP-dependent proteases are thought to have a conserved fold of ATPase domains suggesting mechanistic similarities.[29, 30] Indeed, a chaperone-like activity has been established for the ATPase subunits of both Clp proteases and the 26S proteasome.[85, 86] *E. coli* ClpA was found to completely unfold a model substrate *in vitro*.[87] Unfolding of misfolded substrate polypeptides may therefore be a common function of the ATPase domain of ATP-dependent proteases.

Perspectives

Although recent years have seen rapid progress in the understanding of the proteolytic system of mitochon-

dria, many questions remain to be addressed. The mechanism of ATP-dependent proteolysis, in particular of membrane proteins, as well as the identification of authentic proteolytic substrates with regulatory functions in mitochondrial biogenesis will be a major focus of future studies. Moreover, additional proteolytic pathways may be established which, for instance, ensure the turnover of mitochondrial proteins in the outer membrane or intermembrane space. The existence of an ATP-dependent proteolytic activity in the mitochondrial intermembrane space in mammals has been reported.[88] Increasing evidence links the cytosolic 26S-ubiquitin-proteasome system to mitochondria[58, 89, 90] but the molecular basis of these observations is still elusive. It appears that the mitochondrial proteolytic system still keeps a lot of its secrets.

Acknowledgements

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