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Review

Processing peptidases in mitochondria and chloroplasts ☆

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ABSTRACT

Most of the mitochondrial and chloroplastic proteins are nuclear encoded and synthesized in the cytosol as precursor proteins with N-terminal extensions called targeting peptides. Targeting peptides function as organellar import signals, they are recognized by the import receptors and route precursors through the protein translocons across the organellar membranes. After the fulfilled function, targeting peptides are proteolytically cleaved off inside the organelles by different processing peptidases. The processing of mitochondrial precursors is catalyzed in the matrix by the Mitochondrial Processing Peptidase, MPP, the Mitochondrial Intermediate Peptidase, MIP (recently called Octapeptidyl aminopeptidase 1, Oct1) and the Intermediate cleaving peptidase of 55 kDa, Icp55. Furthermore, different inner membrane peptidases (Inner Membrane Proteases, IMPs, Atp23, rhomboids and AAA proteases) catalyze additional processing functions, resulting in intra-mitochondrial sorting of proteins, the targeting to the intermembrane space or in the assembly of proteins into inner membrane complexes. Chloroplast targeting peptides are cleaved off in the stroma by the Stromal Processing Peptidase, SPP. If the protein is further translocated to the thylakoid lumen, an additional thylakoid-transfer sequence is removed by the Thylakoidal Processing Peptidase, TPP. Proper function of the D1 protein of Photosystem II reaction center requires its C-terminal processing by Carboxy-terminal processing protease, CtpA. Both in mitochondria and in chloroplasts, the cleaved targeting peptides are finally degraded by the Presequence Protease, PreP. The organellar proteases involved in precursor processing and targeting peptide degradation constitute themselves a quality control system ensuring the correct maturation and localization of proteins as well as assembly of protein complexes, contributing to sustenance of organelle functions. Dysfunctions of several mitochondrial processing proteases have been shown to be associated with human diseases. This article is part of a Special Issue entitled: Protein Import and Quality Control in Mitochondria and Plastids.

1. Introduction

Despite the fact that both mitochondria and chloroplasts contain their own genomes and protein synthesizing machineries, an overwhelming majority of the proteins of these organelles are nuclear encoded, synthesized in the cytosol and imported into the respective organelle. There are approximately 1000 and 3000 proteins that have to be imported into mitochondria and to chloroplasts, respectively [1–5]. Most of the organellar proteins are synthesized as precursor proteins containing an N-terminal extension called targeting peptide. Traditionally, the mitochondrial targeting peptides are called presequences and the chloroplast ones, transit peptides. Both presequences and transit peptides are recognized by specific organellar receptors, enabling translocation of precursors across the organellar membranes and route proteins to correct intraorganellar compartments.

The mitochondrial precursors are translocated across the outer and the inner mitochondrial membrane via the Translocase of the Outer Membrane (TOM) and the Translocase of the Inner Membrane (TIM) machineries, respectively (for review see [6–9]). Transport across the outer membrane does not require energy, whereas transport across the inner membrane is an energy-dependent process that requires a membrane potential across the inner membrane (for review see [10]). Inside the mitochondrial matrix, the presequence of the imported precursor protein is cleaved off by the Mitochondrial Processing Peptidase (MPP) [11]. In yeast and mammals, MPP is localized to the matrix, whereas in plants, MPP is integrated into the cytochrome bc_1 complex of the respiratory chain as Core proteins of the complex facing the matrix [12,13]. Cleavage by MPP may be followed by additional trimming of some precursors in the mitochondrial matrix by the Mitochondrial Intermediate Peptidase (MIP, also designated Octapeptidyl aminopeptidase 1, Oct1) or the Intermediate cleaving peptidase of 55 kDa, Icp55 [14–16]. Some of the nuclear encoded proteins are first imported into the matrix and then exported to the intermembrane space (IMS), requiring an additional cleavage of a peptide following processing by MPP. This peptide, called hydrophobic sorting signal, is subsequently cleaved off

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by Inner Membrane Peptidase (IMP) [17,18]. Also other inner membrane proteases, such as rhomboid and AAA proteases are involved in processing of inner membrane and IMS proteins (for review see [19,20]) or, as Atp23, involved in processing required for assembly of a substrate protein into an oligomeric protein complex [21,22].

Chloroplastic precursor proteins destined to the stroma are translocated via the Translocon at the Outer envelope membrane of Chloroplasts (TOC) and further via the Translocase at the Inner envelope membrane of Chloroplasts (TIC) [23–26]. In the stroma, Stromal Processing Peptidase (SPP) cleaves off the transit peptide [27]. The thylakoid lumen proteins and some integral thylakoid membrane proteins require additional signals for proper intra-organellar sorting. These signals called thylakoid-transfer sequences are removed by Thylakoidal Processing Peptidase (TPP) on the luminal side [23,28,29]. C-terminal processing of the D1 protein of Photosystem II by C-terminal processing protease A, CtpA, has also been reported [30].

The free targeting peptides inside mitochondria and chloroplasts are potentially harmful for the integrity and function of the organelles as they can penetrate membranes and dissipate membrane potential. Therefore the free targeting peptides have to be degraded or removed from the organelles. This is achieved by the targeting peptide degrading peptidasome, called Presequence Protease, PreP [31–33].

In the present review we focus on proteolytic enzymes in mitochondria and chloroplasts involved in processing of organellar precursors and on turnover of the cleaved targeting peptides. All these processes are likely to have profound influence on organelle function and organism physiology and consequently, dysfunction of the mitochondrial processing proteases has been associated with several human diseases.

2. Mitochondrial and chloroplastic targeting peptides

Most of the mitochondrial and chloroplastic precursor proteins are synthesized with N-terminal targeting peptides that are removed upon import into the organelle. Protein import is usually organelle specific, but mitochondrial and chloroplastic targeting peptides show remarkable similarity with respect to amino acid composition. The overall composition of the mitochondrial and chloroplastic targeting peptides shows a high content of hydroxylated, hydrophobic and positively charged amino acid residues and a very low abundance of acidic amino acids. Furthermore, proline and glycine are well represented. The length of mitochondrial presequences varies from 6 to 122 amino acids in the yeast *Saccharomyces cerevisiae* [34], 19 to 109 in *Arabidopsis thaliana* and 18 to 117 in *Oryza sativa* (rice) [35]. On average, yeast and mammalian presequences have a length of about 30 amino acids [36], whereas *Arabidopsis* and rice presequences are longer with an average length of 50 and 45 amino acids, respectively. The length of chloroplast transit peptides can vary from 13 to 146 amino acids. They are usually longer than mitochondrial presequences; the most abundant lengths being around 50–70 amino acids with an average of 58 residues. In *Arabidopsis*, the most abundant residues in both mitochondrial and chloroplastic targeting peptides are serine, leucine, alanine and arginine [35,37,38]. There is no conservation in the primary sequence of *Arabidopsis* plastid transit peptides and the hierarchical clustering revealed that transit peptides can be further divided into at least seven subgroups, with each subgroup containing distinct sequence motifs [39].

Main differences in properties between the mitochondrial presequences and chloroplastic transit peptides are seen in the charge and structural properties of the N-terminal portions of targeting peptides [32,35]. In the N-terminal regions of mitochondrial presequences arginine is greatly overrepresented, whereas the N-terminal portions of chloroplast transit peptides have a significant excess of serine and proline [32]. Furthermore, the mitochondrial presequences have the propensity to form an amphiphilic α-helix, which has been shown to interact with the import receptor, Tom20 [40–43]. Chloroplastic transit peptides appear to be mostly unstructured in aqueous environment, but form helical structures in hydrophobic environments, although large portions still remain random coil [44–47].

There is also an ever-growing group of proteins containing ambiguous signal peptides targeting them to both mitochondria and chloroplasts, called dual targeting peptides. These peptides are intermediary in properties in comparison to the mitochondrial presequences and chloroplast transit peptides [48–50]. They have overlapping specificity, however a domain structure has also been reported [48,51–53].

There are several loosely defined scissile bond sites for processing of the mitochondrial and chloroplastic precursor proteins reported, which will be discussed in the sections following below.

3. Mitochondrial processing proteases

Mitochondrial processing proteases cleave off N-terminal cleavable presequences, which are usually found in precursor proteins destined for the matrix, and in the N-terminal of bipartite cleavable presequences found in proteins destined for the inner membrane or for the intermembrane space. There are several proteases in the mitochondrial matrix that cut off the presequence and trim the intermediate to generate the mature portion of the protein. The bipartite presequence is usually structured in two domains with a presequence containing the mitochondrial targeting information in the very N-terminal portion followed by a hydrophobic inner membrane sorting signal that is cleaved off by inner membrane located proteases. Processing of N-terminal presequences (and trimming of intermediate mature proteins) has also been shown to be important for the stability and correct localization of proteins and assembly of protein complexes. Fig. 1 shows an overview of the mitochondrial proteolytic systems involved in presequence processing and degradation.

3.1. Mitochondrial Processing Peptidase, MPP

MPP is a metalloendopeptidase that specifically cleaves off presequences from several hundred of mitochondrial precursor proteins (for reviews see [7,54]. MPP belongs to the pitrilysin family (MEROPS[1] subfamily M16B) of proteases as it contains an inverted Zn-binding motif, ($HXXEHX_{74-76}E$), in the catalytic site. MPP forms a heterodimer consisting of two structurally related subunits, α-MPP and β-MPP of ~50 kDa each, which co-operate in processing. The catalytic site is located on the β-MPP subunit. In yeast and mammals both MPP subunits are localized to the matrix [11,55]. In *Neurospora crassa*, 70% of β-MPP was found as a Core 1 protein of the cytochrome bc_1 complex of the respiratory chain [56], whereas in plants, both α-MPP and β-MPP subunits are completely integrated into the bc_1 complex of the respiratory chain as Core1 and Core 2 proteins, respectively [12,57–62]. The integration of MPP into the respiratory bc_1 complex in plants is a general feature for both higher and lower plants. Despite the fact that MPP/bc_1 complex in plants is bifunctional, the processing activity is not dependent on the electron transfer within the complex [58,61,62]. Furthermore, it is not clear whether presequences in plants are cleaved off while crossing the mitochondrial membrane by MPP/bc_1 localized in the membrane or if the processing occurs after translocation. One report indicated a connection between precursor import and processing catalyzed by MPP/bc_1 complex in plants [63], however other studies show that presequence removal occurs first after complete translocation of the precursor across the membrane [64].

[1] Throughout the text we refer whenever possible to the systematic classification of proteases in the MEROPS database (http://merops.sanger.ac.uk/index.shtml).

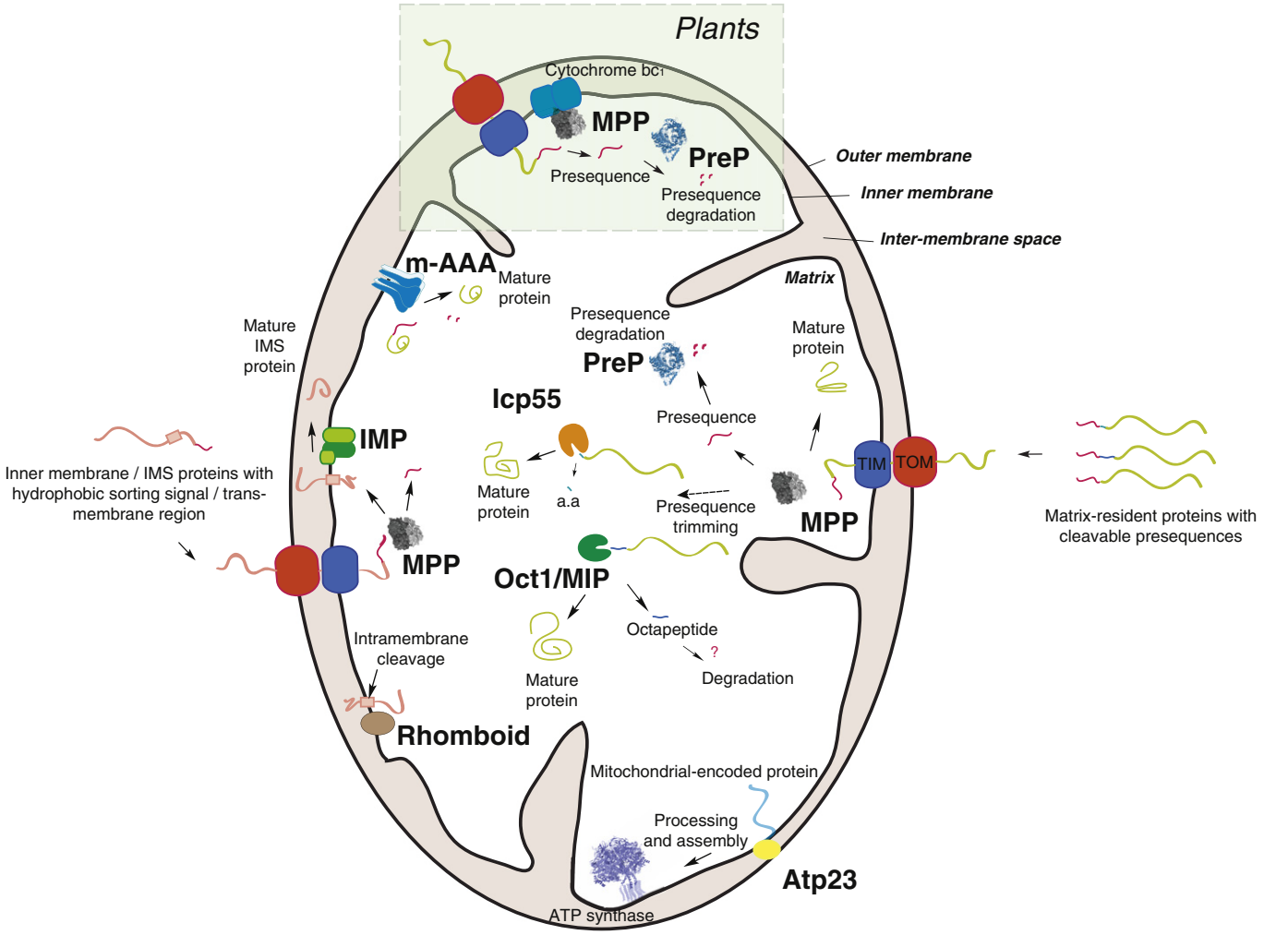

Fig. 1. Mitochondrial processing proteases. The mitochondrial precursors are translocated across the outer and the inner mitochondrial membrane via the Translocase of the Outer Membrane (TOM) and the Translocase of the Inner Membrane (TIM). Inside the mitochondrial matrix, the presequences are cleaved off by the Mitochondrial Processing Peptidase (MPP). In yeast and mammals, MPP is localized to the matrix, whereas in plants, MPP is integrated into the cytochrome bc_1 complex of the respiratory chain as Core proteins of the complex facing the matrix (see upper insert in the figure). Cleavage by MPP may be followed by additional trimming of some precursors in the mitochondrial matrix by the Mitochondrial Intermediate Peptidase (MIP/Oct1) and/or the Intermediate cleaving peptidase of 55 kDa, Icp55. Some intermembrane space (IMS) proteins are synthesized with a bipartite targeting signal containing sequence processed by MPP followed by a hydrophobic sorting signal that is subsequently cleaved by the Inner Membrane Peptidase (IMP). Also other inner membrane proteases, such as rhomboid and AAA proteases are involved in processing of inner membrane and IMS proteins or, as Atp23, involved in processing of Atp6 subunit required for assembly of a functional ATPsynthase. The free targeting peptides inside mitochondrial matrix are degraded by an organellar peptidasome, called Presequence Protease, PreP.

Mitochondrial presequences are processed by MPP at distinct sites although they vary greatly in size and do not share any sequence similarity. Early analysis of MPP processing sites grouped scissile bonds in four classes related to the position of an arginine residue upstream of the cleavage site [65]. Two major groups representing about 80% of presequences contain an arginine either at position −2 (−2R) or −3 (−3R) relative to the cleavage site and have a loosely conserved motif around the cleavage site, R-X↓X and R-X-F/Y/L↓A/S-X, respectively [14,36,66]. The third group includes presequences lacking a conserved arginine (no-R) close to the processing site [66,67], whereas the fourth group contains arginine in position −10 (−10R), R-X↓F/L/I-X_2-T/S/G-X_4↓X [14]. Studies in plants revealed a motif of (F/Y↓S/A) for no-R group of proteins [35]. The −2R, −3R and no-R motifs were found in presequences of all species, whereas −10R motif was not found in plants [35,66]. Global analysis of mature mitochondrial N-termini in plants and in yeast, using mass spectrometry, enabled identification of MPP cleavage sites in a much larger amount of precursor proteins than what was possible until then. From these proteomic studies 114 cleavage sites were determined in plants [35] and 279 in yeast [34]. Studies in plants found the −3R motif as the most frequent cleavage site, with phenylalanine in *Arabidopsis*, and phenylalanine, leucine or tyrosine in rice in position −1 in relation to the cleavage site [35]. Analysis in yeast revealed that −2R and −3R cleavage motifs were most predominant and that tyrosine, leucine and phenylalanine were the most frequent in the −1 position. The identification of N-terminal peptides differing by a single residue for several precursors led to revision of the −3R motif in yeast and resulted in the identification of a novel mitochondrial aminopeptidase, Icp55 that cleaves single phenylalanine, tyrosine or leucine residues at the N-terminus after initial processing by MPP [34] (see Section 3.2.). Also, the occurrence of the −10R motif is due to additional cleavage of an octapeptide by mitochondrial intermediate peptidase, Oct1/MIP following cleavage by MPP (see Section 3.2.).

Many mutational studies showed that the above described cleavage motifs were important, however they are not the only determinants for processing. Recognition by MPP was also dependent on the residues in the mature portion of the protein downstream to the cleavage site [68–70] and on structural elements in the presequence, such as e.g. presence of flexible linkers [71]. The 3D structure of the recombinant yeast MPP in complex with synthetic targeting peptides revealed

binding properties of the targeting sequences to MPP [72] and the recognition sites for the −2R and the +1 aromatic residue were observed. In their work, Deisenhofer and coworkers suggested that the presequences adopt context-dependent conformations throughout mitochondrial import and processing, helical for recognition by mitochondrial import machinery and extended for cleavage by MPP [72].

3.2. Presequence trimming by Oct1/MIP and Icp55

It is well established that several mitochondrial proteins can undergo a second proteolytic event following MPP cleavage, even though the usefulness and necessity for this secondary cleavage was not clear [14]. Currently, there are two known matrix proteases that exclusively cleave mitochondrial presequences after an initial cleavage by MPP. One of these proteases (MIP) has been known for about 20 years [14,73–79], and shown to cleave an octapeptide from the MPP generated N-terminus in the substrate proteins, reason that motivated the current designation of Octapeptidyl aminopeptidase 1 (Oct1). Oct1 (MEROPS M3 family) is a soluble mitochondrial matrix protein containing a conserved Zn-binding motif (HEXXH) required for activity [76,77]. Purified Oct1/MIP from rat liver mitochondria is a monomer of ~75 kDa and shows a broad optimum pH for activity (6.6 to 8.9) [76]. The processing activity was stimulated by divalent cations (Mg^{2+}, Mn^{2+} and Ca^{2+}) and inhibited by EDTA (metal chelator) and NEM (reagent blocking sulfhydryl groups).

Following an analysis of determined N-termini from both yeast and mammalian mitochondrial proteins, most substrates of Oct1 were shown to contain R in the −10 position from the mature protein (MPP recognition site), a bulky hydrophobic residue (F, L or I) at the −8 position and S/T at positions −5, −6 and −7 [74,79,80]. An interesting exception to this consensus sequence is the uncharacterized yeast protein Imo32, which contains a −10C residue, implying that in this case a -10R is not required for MPP cleavage [79]. An Oct1 homolog was found in plants through sequence analysis [81], even though it is presently unknown if it localizes in mitochondria or in chloroplasts.

Another protease performing secondary presequence trimming was recently identified and termed Intermediate Cleaving Peptidase of 55 kDa (Icp55) [16,34]. Icp55 is a peripheral membrane protein [34] of ~55 kDa, attached to the mitochondrial inner membrane from the matrix side, classified in the aminopeptidase P family (MEROPS M24B subfamily). Icp55 was found to cleave one amino acid from an MPP-generated intermediate, specifically from substrates containing the consensus Y/L/F↓S/A. Interestingly, this is in contrast to *Escherichia coli* aminopeptidase P, that requires the presence of a proline residue in the P1′ position [82]. Presently, Icp55 has been characterized only in yeast even though homologs exist in plants [81] and mammalian organisms [83]. Icp55 has not yet been purified and therefore the mechanism of substrate recognition and catalysis is presently unknown. Interestingly, Icp55 was reported to be dual localized to both mitochondria and the nucleus using α-complementation assays [16].

The identification of Icp55 substrates is of particular importance as it resolved the issue of −2R vs. −3R MPP recognition motifs in yeast, showing that the −3R motif is in fact a −2R, followed by a one amino acid trimming by Icp55 [34]. In the case of 38 substrates, Icp55 was shown to cleave a single amino acid from the MPP-generated N-termini. In contrast, Icp55 cleaved 3 amino acid residues from the N-terminus of Nsf1 [16]. However, due to the possible dual localization (mitochondria/nucleus) of both Icp55 and Nfs1, it is presently unclear whether this processing even occurs in mitochondria or in the nucleus [16].

Cleavage of the MPP generated intermediates by Icp55 or Oct1 gives rise to new N-termini on substrate proteins, with the interesting feature of converting an N-terminal primary destabilizing amino acid into a stabilizing one, in accordance to the N-end rule [34,79]. The N-end rule pathway was proposed in 1986 by Varshavsky and coworkers based on elegant experiments showing that the stability of an engineered protein was dramatically dependent on the identity of the N-terminal amino acid residue [84,85]. Since these pioneering experiments, the N-end rule pathway was found in both prokaryotes and eukaryotes and its components elucidated at the molecular level [85–87]. Considering the observed cleavage specificity of Oct1 and Icp55 derived from the mitochondrial protein N-terminome data, Meisinger and co-workers proposed these two proteases as effectors in a mitochondrial N-end rule pathway [34,79]. All Icp55 identified substrates and most of the currently known Oct1 substrates in yeast (13 out of 14) share as a common feature the presence of an N-terminal primary destabilizing amino acid according to the N-end rule. The mature proteins generated by Oct1 or Icp55 cleavage were found to have a more stabilizing residue at the N-terminus. In accordance with this, a decreased half-life of the substrates was observed in yeast strains lacking the respective processing protease [34,79].

Functionally, Oct1 can be grouped with Icp55 as secondary processing peptidases that influence protein stability as part of the mitochondrial N-end rule pathway [15]. It is presently unknown how are the unstable intermediates degraded if they fail to be processed by Oct1/Icp55, if this degradation is performed by a specific protease or just a shared functional characteristic among different ones (LON, ClpXP, etc.). Additionally, it is also possible that these unstable intermediates are degraded by the ubiquitin–proteasome system as the connection between mitochondria and the ubiquitin system has been described for a few mitochondrial proteins [88,89].

Other interesting and still unexplored point is the cellular fate of the octapeptides removed by Oct1 from the unstable MPP-generated intermediates. The octapeptides produced were shown to inhibit Oct1 activity [75] and therefore must be removed. In analogy with the presequence peptides, these octapeptides can either be exported from mitochondria for degradation in the cytosol or possibly degraded within the mitochondrial matrix by the Presequence Protease PreP [90] or in the IMS by other oligopeptidases such as Prd1 in yeast [91,92] or neurolysin in mammals [93].

3.3. Additional mitochondrial proteases with processing activity

3.3.1. Inner membrane protease

The mitochondrial Inner Membrane Protease (IMP) is, in yeast, a hetero-oligomer composed of 3 subunits [18,94]: the catalytic components Imp1 and Imp2 and the non-catalytic subunit Som1. The catalytic subunits Imp 1/2 are integral membrane proteins, with the active site facing the inter-membrane space [18]. In this way, IMP cleaves off the hydrophobic sorting signal from proteins that face the IMS. Imp1 and Imp2 possess a conserved Ser-Leu dyad [95] and are classified in the MEROPS family S26 together with the bacterial signal peptidase I and the Thylakoid Processing Peptidase (see Section 4.2.). Interestingly, Imp1 and Imp2 have non-overlapping cleavage specificity to the known (relatively few) substrates [14,18]. Up to now only 4 substrates were identified for Imp1 (Cox2, Mcr1, Gut2 and Cyb2) and 1 for Imp2 (Cyc1) [17,18,96,97]. In terms of substrate cleavage specificity, Imp1 seems to have a requirement for an acidic amino acid (E or D) at the P1′ position and hydrophobic non-aromatic residues at position P3. In the case of the only identified substrate of Imp2 (Cyc1) a strict requirement for alanine at positions P1 and P3 was demonstrated [98]. Up to now, the information about Imp proteins resulted from studies performed in yeast. Sequence analysis revealed that *Arabidopsis* genome contains six putative homologs of IMP, even though none of them has been characterized [81]. Mammalian IMPs have been studied only by expression in yeast and not the in native organism. In this context, a possible substrate for mammalian IMP is the apoptosis regulator smac/DIABLO [99]. Using yeast as a host organism, DIABLO was found to contain a bipartite presequence, being processed by Imp1, possibly upon initial cleavage by MPP. Even though these studies were only performed in yeast and not in the mammalian system, they suggest

that IMP may have a role in the apoptotic process, as it cleaves and thereby activates the pro-apoptotic protein DIABLO [99].

3.3.2. Atp23

The metalloprotease Atp23 (MEROPS family M76) was identified in the inter-membrane space of yeast mitochondria and deletion of the *atp23* gene results in impaired growth in non-fermentative carbon sources [21,22]. Atp23 was found to cleave the 10 amino acid presequence of Atp6, a component of the F_OF_1-ATPsynthase encoded in the mitochondrial genome, translated on mitochondrial ribosomes and processed before assembly. However, two independent studies concluded that the proteolytic activity of Atp23 is not an essential function, as a substitution in the conserved glutamate residue for glutamine in the putative Zn-binding motif abolishes proteolysis but still supports respiratory growth of yeast [21,22]. Additionally, it was found that the function of Atp23 goes beyond proteolytic processing of Atp6 and may in fact work as a chaperone in the assembly of functional ATP synthase complexes [21,22]. It is presently unknown if the function of Atp23 is conserved in other organisms, as the activity of mammalian and plant homologs was not yet characterized. In fact, if mammalian Atp23 has proteolytic activity it will act on different substrates than Atp6 as the mammalian Atp6 homolog is synthesized without the N-terminal extension observed in the yeast Atp6 [21,22]. Recently, also the yeast Ups1 protein was identified as new possible substrate for Atp23 [100]. Ups1 is a protein involved in regulating the accumulation of phosphatidylethanolamine and cardiolipin in mitochondria and was found by Langer and co-workers to be degraded by Atp23 [100]. This represents the third activity of Atp23, protein degradation and turnover in addition to presequence processing and chaperone activities.

3.3.3. m-AAA (mitochondrial matrix ATPase Associated with cellular Activities)

The m-AAA proteases (MEROPS family M41) are organized as hexameric complexes (consisting of the Yta12 and Yta10 subunits in yeast) with the active sites containing a metal-binding motif and facing the mitochondrial matrix [20]. The two transmembrane regions of each m-AAA monomer are inserted into the mitochondrial inner membrane and the matrix facing regions contain the conserved P-loop ATPase domain (characteristic of AAA + proteins) and the metallopeptidase domain with a metal binding motif HEXXH [20].

m-AAA proteins were originally identified for their role in protein degradation / turnover of misfolded or unassembled proteins, playing a role in the general quality control of mitochondrial inner membrane proteins [101,102]. Additionally, m-AAA proteases participate in the proteolytic maturation of some proteins (particularly the ribosomal protein MrpL2) [103], even though it is still poorly understood how they discriminate between substrates, shifting between the degradation and processing activities [20]. Removal of the N-terminal presequence of MrpL32 by m-AAA protease is essential for ribosome assembly and mitochondrial translation, and it is a process conserved between yeast and mammals [103]. In fact, impaired maturation of MrpL32 is the cause of the respiratory growth phenotype observed in yeast strains lacking m-AAA proteins [103]. Recent work by Langer and co-workers suggested a mechanism for MrpL32 presequence removal by m-AAA protease. It was shown that m-AAA does not recognize a specific scissile sequence, but rather starts degrading from the unstructured N-terminal presequence until reaching the tightly folded mature portion of MrpL32 [104].

The mammalian m-AAA proteases are involved in the processing of newly imported m-AAA subunits and also in the processing of the dynamin-like GTPase OPA1, a protein important for the mitochondrial fusion process [105–107]. In plants, the processing activity of m-AAA proteases has not been characterized, even though the plant homologs AtFtsH3 and AtFtsH10 can complement the growth phenotype of the yeast cells lacking m-AAA proteins [81,108].

3.3.4. Rhomboid proteases

Rhomboids are integral membrane proteins that form an unusual family of serine proteases, catalyzing proteolysis of their substrate within the lipid bilayer environment [19,109]. Rhomboids contain a conserved Ser-His dyad (instead of the Ser-His-Asp triad characteristic of serine proteases) and cleave substrate proteins within or at the boundaries of their transmembrane domains. Detailed structural and mechanistic analysis has been performed for the bacterial Rhomboid GlpG [19,109]. Members of this protein family were shown to localize to mitochondria in yeast, plants and in mammals [110,111]. It is presently unclear what are the requirements for cleavage by the mitochondrial Rhomboids and a specific cleavage motif has not yet been identified [19]. In yeast the mitochondrial Rhomboid protease Pcp1 (MEROPS family S54) has two known substrates: Ccp1 and Mgm1, both cleaved within the moderately hydrophobic sequence of their transmembrane region [110,112,113]. The cytochrome c peroxidase Ccp1 is first integrated into the inner membrane as a precursor, requiring then the action of the m-AAA protease (though its ATPase activity) for the correct positioning within the membrane, prior to cleavage by Pcp1 and release of the mature portion to the IMS [21,112]. In the case of Mgm1, there is a first cleavage of the presequence by MPP before processing by Pcp1 [113,114]. In plants, the mitochondrial Rhomboid AtRbl12 was shown to have different specificity than Pcp1, but its substrate repertoire is presently unknown [115]. In mammals, the mitochondrial Rhomboid PARL is involved in the control of mitochondrial morphology, in the response to stress and in the apoptotic process [116].

4. Chloroplast processing proteases

Most chloroplast proteins are synthesized in the cytosol as precursor proteins and are imported into the organelle post-translationally. The precursors associate in the cytosol with molecular chaperones HSP70, HSP90, and 14-3-3 proteins that prevent protein aggregation and assists in maintaining an import competent conformation [26,117]. The precursor proteins destined either to the chloroplast stroma or thylakoids are typically equipped with an N-terminal extension, a transit peptide. Chloroplast transit peptides have been shown to be phosphorylated by cytosolic kinases and then dephosphorylated before binding to chloroplastic receptors in a process regulating protein import [118–120]. Inside the chloroplasts, transit peptides are proteolytically removed by processing peptidases, the Stromal Processing Peptidase, SPP, a soluble enzyme operating in stroma and by the membrane bound Thylakoidal Processing Peptidase, TPP, if the protein is further translocated from the stroma across the thylakoid membrane to the thylakoid lumen. Interestingly, the D1 protein of PSII reaction center has been shown to be proteolytically processed at its C-terminus by a thylakoid lumen peptidase, called Carboxy-terminal processing protease, CtpA. Fig. 2 depicts an overview of the known proteases involved in transit peptide processing and degradation within the chloroplast.

4.1. Stromal Processing Peptidase, SPP

SPP has been originally identified as a chloroplast processing enzyme, a 124 kDa soluble protein in the chloroplast stroma, that cleaves the precursor for the major light-harvesting chlorophyll binding protein (preLHCP) [121] and later on recognized as a general Stromal Processing Peptidase, SPP, in plastids in both photosynthetic and non-photosynthetic tissues [122,123]. SPP contains an inverted Zn-binding motif, HXXEH, and it belongs to the MEROPS metallopeptidase family M16, which also includes *E. coli* protease III, insulin-degrading enzyme, MPP and PreP [27,123]. SPP recognizes and binds the transit peptides of a wide variety of substrates and it cleaves them off in one endoproteolytic step. After cleavage, the mature protein is released, but the transit peptide remains bound to SPP. SPP can then cleave the transit peptide a second time turning it into a sub-fragment

without its original C-terminus [124,125]. The sub-fragment can be then further degraded in the stroma by PreP [126,127]. Examination of binding and processing characteristics of several chloroplastic precursor proteins was shown to be dependent on specific interactions of SPP with the region consisting of the 10–15 C-terminal residues of the transit peptides, independently on its primary sequence and length [125]. Further analysis of processing determinants identified a loosely conserved sequence consensus for SPP cleavage, (V/I)-X-(A/C)↓A, in which the −3V and the −1A seem to be most conserved [35,38,65]. However, this motif is not as conserved as the cleavage motifs for MPP [66]. Investigation of determinants for processing of a dually targeted protein to mitochondria and chloroplasts, pea glutathione reductase, revealed that the 60 amino acids long N-terminal targeting peptide was recognized by separate information patterns by MPP in mitochondria and by SPP in chloroplasts [69]. Whereas numerous single substitutions of amino acid residues in proximity of the cleavage site did not affect processing by SPP, replacing two amino acid residues on either side of the processing site had inhibitory effect on processing by MPP, showing that processing by SPP has a lower level of sensitivity to single mutations on targeting peptide than MPP. That led to the conclusion that recognition of precursors by SPP is likely to involve recognition of the physicochemical properties of the sequence in the vicinity of cleavage rather than a requirement for specific amino acid residues [69].

Studies of the role of SPP in vivo demonstrated that SPP is essential for chloroplast biogenesis and plant survival [128]. When SPP cDNA antisense constructs were introduced into *Arabidopsis*, a strong mutant phenotype was produced, with most plants dying as seedling lethals. Surviving plants exhibited slower growth, aberrant leaf morphology, abnormal pigmentation and not fully developed chloroplasts with accumulated starch granules. Also protein import into plastids was affected in antisense plants. This may reflect the fact that most components of the TOC-TIC import machinery are synthesized with transit peptides that have to be removed or it may also indicate that SPP cleavage is required for progression of the protein import pathway [128]. Analysis of a rice mutant with a glutamate deletion in the highly conserved C-terminus of SPP showed that the mutant rice plants exhibited chlorosis associated with small, underdeveloped chloroplasts as well as defective root development [129]. Recent knockout studies of SPP in *Arabidopsis* lines with T-DNA insertions in the SPP gene demonstrated that SPP is essential for embryo development. No homozygous mutant plants could be detected and the siliques of *spp* mutant plants contained a quarter of aborted seeds, suggesting embryo lethality. The mutant embryos exhibited delayed development, with cell divisions not terminating properly after the 16-cell stage [130].

4.2. Thylakoid Processing Peptidase, TPP

TPP removes the thylakoid-transfer domain from proteins translocated into the thylakoid lumen [131,132] using either the Sec or Tat (or ΔpH) pathways [133,134]. These proteins include plastocyanins, PsbO, PsbP and PsbQ subunits of the oxygen-evolving-complex (OEC), some other photosystem components, lumen located proteases and several other enzymes [135]. TPP belongs to a group of membrane bound serine proteases called the type I signal peptidase (SPase I) family (MEROPS S26 family). Members of the SPase I family are found in both prokaryotes and eukaryotes. In prokaryotes, SPases I are often called leader peptidases, which exist in the plasma membrane [136,137]. In eukaryotes, there are two distinct SPases I in addition to TPP. One is present in the endoplasmic reticulum (ER) cleaving the signal peptides [137,138]. Another activity is the mitochondrial inner membrane peptidase (Imp) responsible for removal the intramitochondrial sorting

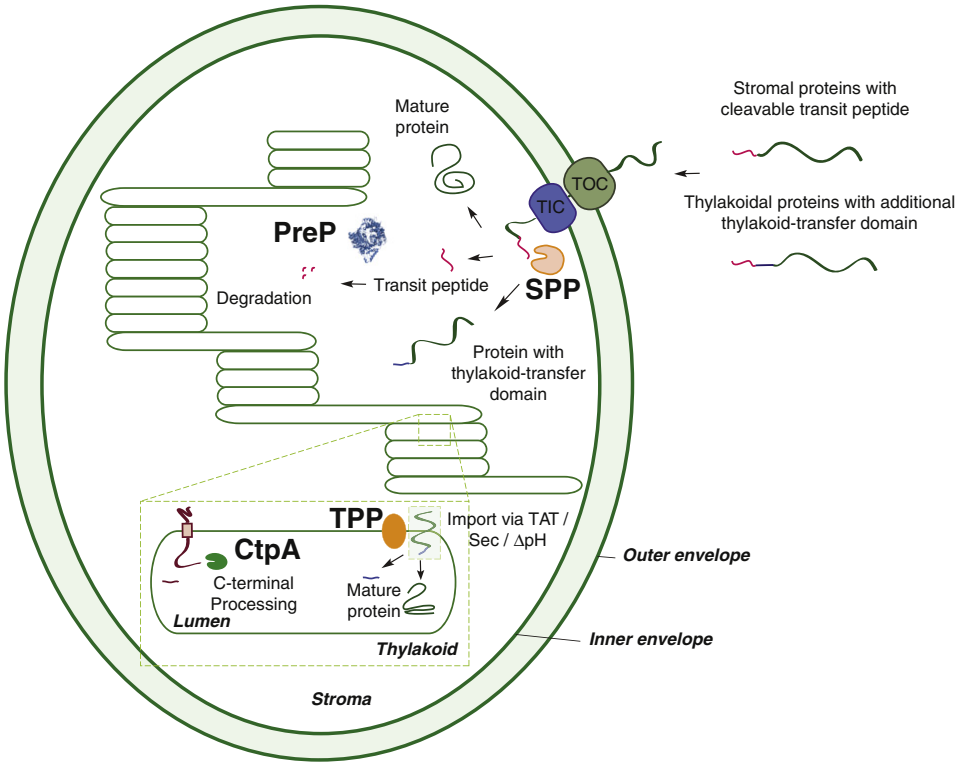

Fig. 2. Chloroplastic processing peptidases. The chloroplastic precursor proteins destined to the stroma are translocated via the Translocon of the Outer envelope membrane of Chloroplasts (TOC) and via the Translocase of the Inner envelope membrane of Chloroplasts (TIC). In the stroma, Stromal Processing Peptidase (SPP) cleaves off the transit peptide. The thylakoid lumen proteins and some integral thylakoid membrane proteins require an additional thylakoid-transfer sequence that is removed by membrane bound Thylakoidal Processing Peptidase (TPP) on the luminal side of the thylakoid membrane. C-terminal processing of the D1 protein of Photosystem II by C-terminal processing protease A, CtpA, has also been reported. The free targeting peptides inside chloroplast stroma are degraded by the organellar peptidasome, called Presequence Protease, PreP.

signals from a subset of proteins in the intermembrane space of mitochondria (see Section 3.3.1.) [17,18]. The conservation of SPases can be explained from an evolutionary point of view, as the translocation machinery of proteins across the thylakoid membrane resembles the secretion machinery [136] and export of mitochondrial proteins from matrix to intermembrane space (see Section 3.3.1).

TPP is a 30 kDa protein in the thylakoid membrane having a Ser-Leu catalytic dyad. The thylakoid transfer domain of luminal proteins have features in common with signal peptides directing proteins to ER, such as a hydrophobic "core" region and the presence of short-chain residues at the −3 and −1 positions, relative to the processing site [139]. TPP specificity follows this well-known −1, −3 rule [139,140] for SPase I. Sequence analysis of chloroplast thylakoid preproteins with bipartite signal sequences shows that alanine is most frequently found at the −1 and −3 positions. Extensive mutagenesis studies confirmed that these residues play a critical role in defining the TPP's cleavage site, and that TPP has a stringent requirement at these positions [131]. However, despite the fact that the pre-33 K protein has a marked preference for an alanine at the −1 position TPP tolerates also a −3 valine instead of alanine for efficient processing [131,140].

Three TPP isoforms are encoded by the nuclear genome of *Arabidopsis*. Plastidic type I signal peptidase 1 (Plsp1) is necessary for proper thylakoid assembly processing of three thylakoidal proteins [141,142]. Interestingly, Plsp1 is also present in the outer chloroplast envelope and is responsible for maturation of Toc75 protein [142]. The lack of Plsp1 results in seedling lethality, apparently due to disruption of proper thylakoid development [143]. Phylogenetic analysis revealed that TPP may have originated before the endosymbiotic event; one group includes Plsp1 and another comprises Plsp2A and Plsp2B. The two PLSP2 genes were co-expressed in both photosynthetic tissues and roots, whereas the PLSP1 transcript accumulated predominantly in photosynthetic tissues. The seedling-lethal phenotype of the *plsp1*-null mutant could be rescued by a constitutive expression of Plsp1 but not by that of Plsp2A or Plsp2B indicating that Plsp1 and Plsp2 evolved to function differently, and that neither of the Plsp2 isoforms is necessary for proper thylakoid development in photosynthetic tissues [143].

4.3. Carboxy-terminal processing protease, CtpA

Another interesting type of proteolytic processing in thylakoids is processing of the carboxy-terminal extension of the D1 protein of PSII reaction center that is essential for biogenesis of functional PSII [144,145]. This cleavage event is catalyzed by a thylakoid lumen peptidase, called Carboxy-terminal processing protease, CtpA (MEROPS family S41), classified as a novel type of serine protease with a Ser-Leu catalytic dyad, a homologue of the bacterial Tail-specific protease (Tsp) [144,145]. In *E. coli*, Tsp is a periplasmic protease, either processing certain substrates by a single proteolytic cleavage, or totally degrading other ones [146]. The homologs of Ctps are found in a broad range of organisms, such as eubacteria, archaea, algae, plants and animals, but their physiological functions are poorly understood [30]. The structure of CtpA in *Scenedesmus obliquus* at 1.8 Å resolution indicated that the middle domain, topologically homologous to known PDZ motifs, is the site at which the substrate C-terminus binds [147]. Overexpressed spinach CtpA showed high pH dependency suggesting the presence of a specific interaction of CtpA with factors that modulate the proteolytic action in response to physiological conditions [148]. Three putative CtpA homologs have been identified in *Arabidopsis*. In an *Arabidopsis* mutant of one of the genes (*At3g57680*) accumulation of the D1 precursor was not detected suggesting that there may be functional redundancy between the three CTP homologs and that additional components are required to facilitate the processing of the D1 precursor by CtpA in *Arabidopsis* [148]. Furthermore, it has to be investigated whether the luminal Ctps are exclusively dedicated to pre-D1 processing, or if it is involved in total degradation of other substrates.

5. Turnover of mitochondrial presequences/chloroplast transit peptides

The targeting and import processes are, for most proteins, determined by the N-terminal targeting peptides [149]. Proteomic analysis performed in yeast mitochondria showed that about 70% of the proteins have a cleavable presequence. After import of the precursor proteins, the targeting peptides are removed by processing peptidases, resulting in the production of mature proteins and free targeting peptides. Several studies performed by different research groups have analyzed the effect of targeting peptides on the integrity of membranes and mitochondrial function. Due to their amphiphilic character, these peptides are thought to form pores in membranes, resulting in perturbation of the mitochondrial membrane, respiration uncoupling and dissipation of membrane potential [150–153]. Considering the threat posed by presequence peptides, mitochondria contain several important quality control mechanisms that ensure either their export or degradation. It was shown that a subset of presequence peptides could be exported from mitochondria through ABC-type transporters (Mdl1 in yeast and ABCB10 in humans) possibly for degradation by cytosolic oligopeptidases [154,155].

Presequence peptides are also degraded within mitochondria by a matrix resident peptidasome, the Presequence protease (PreP) [90,156]. PreP was initially identified in *Arabidopsis* [33], and later in yeast [92] (known as Cym1 or Mop112) and in humans [31,157] (known as hPreP or MP1). PreP (MEROPS family M16C) is a Zn-metallopeptidase containing the conserved inverted Zn-binding motif (HXXEH) [31–33].

In *Arabidopsis* there are two isoforms of PreP (AtPreP1 and AtPreP2) displaying 86% sequence identity [33,126,158]. Interestingly, both AtPreP1 and AtPreP2 are dually localized to both chloroplasts and mitochondria, with the targeting information contained in the ambiguous targeting peptides [51]. From the enzymatic point of view, both AtPreP1 and AtPreP2 were found to degrade peptides in the range 10–65 amino acid residues, corresponding to the average size of mitochondrial presequences and chloroplastic transit peptides [126,158]. Additionally, the structure of AtPreP1 at 2.1 Å resolution revealed four homologous domains, organized in two halves forming a peptidolytic chamber with an internal volume of 10000 $Å^3$, compatible with the degradation of targeting peptides [32]. Analysis of *Arabidopsis* double *atprep1 atprep2* knockout lines revealed strong phenotypes, related to both mitochondria and chloroplasts. Double knockout plants are characterized by a slower growth rate, reduced biomass and chlorosis in young leaves, when compared to wild type *Arabidopsis*. Additionally, *atprep1 atprep2* plants show partial loss of mitochondrial membrane integrity, reduced respiratory rate and altered morphology of both mitochondria and chloroplasts [127].

In *S. cerevisiae*, the PreP homolog Cym1/Mop112 is also located in the mitochondrial matrix [159]. Knockout of *cym1* results in altered mitochondrial morphology and a strong growth phenotype on non-fermentative carbon sources, when compared to wild type yeast [92,159]. In contrast, the knockout of *mdl1* had no effect on yeast respiratory growth [155], suggesting that the pathway for degradation of presequence peptides is more important than the export pathway.

The human homolog of PreP (hPreP) is also located in the mitochondrial matrix as analyzed in human brain samples and also in several cell lines ([31]; Teixeira et al., unpublished). Furthermore, recent proteomic analysis revealed that hPreP is part of the human "central proteome," a set of proteins that is common to seven different cells lines, and considered to be an approximation to the proteins ubiquitously expressed among different human cells [160]. In addition to the degradation of presequence peptides, hPreP was also found to degrade the amyloid-β peptide, one of the main factors involved in the progression of Alzheimer's disease ([31] see also Section 5).

Taken together, the phenotypic analysis of PreP knockouts in both *Arabidopsis* and yeast strongly suggests that the degradation of

targeting peptides within the mitochondrial matrix (and also in the chloroplast stroma) is a very important process and that PreP exerts a fundamental role within the organellar quality control systems [92,127]. It is presently unknown whether the accumulation of peptides (in the absence of PreP) has solely a general effect on the organelle integrity (membrane perturbation by amphiphilic peptides) or more selective effects, for instance on signaling events or in the assembly of protein complexes. It is interesting that presequence peptides were shown to inhibit MPP activity [161] (and octapeptides shown to inhibit Oct1 activity [75]) and therefore, the perturbation of PreP activity can have unforeseen secondary effects on the maturation of mitochondrial proteins and general mitochondrial physiology.

6. Mitochondrial processing peptidases and human disease

Mitochondria have diverse cellular functions that go well beyond ATP production. Perturbation of the different aspects of mitochondrial functions can have severe cellular consequences, resulting in the development of many pathologies, including neurodegenerative disorders, some forms of cancer and metabolic syndromes [162]. It is clear that proper mitochondrial function requires not only the precise and timed production and targeting of many hundreds of proteins but also their correct sub-mitochondrial location, proper folding and often the correct assembly into multimeric complexes [9]. Considering all these aspects, it is likely that altered activity of the mitochondrial processing peptidases is essential to ensure the correct maturation of mitochondrial proteins and that altered activity of these proteases will have dramatic effects in the activity, stability and assembly of mitochondrial proteins. Even though the relation between processing peptidases and human disease has not been addressed systematically there are several examples pointing to the involvement of processing peptidases in several pathologies. Both MPP and Oct1/MIP are involved in the proteolytic maturation of Frataxin, a protein responsible for iron homeostasis and shown to be involved in Friedreich ataxia, an autossomic recessive neurodegenerative disorder [14,163–165]. Additionally, the human IMP homolog was proposed as a candidate protein involved in the Gilles de la Tourette syndrome (a neuropsychiatric disorder) [166]; the human mitochondrial rhomboid PARL is intimately connected to Parkinson's disease (a degenerative disease of the central nervous system) [167]; the human homolog of Icp55 (XPNPEP3) was recently suggested to be implicated in nephronophthisis-like nephropathy-1 (an autosomal recessive cystic kidney disease) [83]; perturbation of m-AAA protease activity and deficient maturation of MrpL32 are hallmarks of human hereditary spastic paraplegia (a disease causing progressive stiffness and contraction in the lower limbs) [103].

In the last few years it also became clear that the mitochondrial Presequence protease hPreP, responsible for the degradation of presequence peptides, might be involved in the progression of Alzheimer's disease (AD) [31,156,168]. In addition to the degradation of mitochondrial presequences, hPreP can also degrade the amyloid-β peptide, whose accumulation in the form of oligomers/fibrils causes AD. It is likely that hPreP is involved in the clearance of the mitochondrial amyloid-β pool. Recently, work from our laboratory showed that the activity of hPreP is severely reduced in hypothalamic mitochondrial samples of AD patients, when compared to age-matched controls. Also, this decrease in hPreP activity is recapitulated in AD mouse models [168]. Given the recent evidence for amyloid-β localization in mitochondria and in general the importance of this organelle in the disease progression, it is possible that hPreP plays an important role in Alzheimer's disease [156,169–171].

Even though all these examples substantiate a connection between the processes of mitochondrial presequence cleavage, trimming and turnover and the progression of several human diseases this may only be the tip of the iceberg. New technologies in proteomics, integrative genomics and large-scale gene expression analysis combined with a systems biology approach to study mitochondrial functions will certainly provide in the future a more substantial picture of the involvement of all these proteins in human pathologies.

7. Future perspectives

Biogenesis of both mitochondria and chloroplasts is strictly dependent on the posttranslational import and processing of nuclear-encoded proteins. Cleavage of the N-terminal cleavable targeting peptides inside the organelle and their degradation is essential for proper organellar function and organism development. Many proteases have been shown to be involved in these processes. Up to date, nine such proteases have been found in mitochondria (in yeast), and three proteases in chloroplasts. Most of them have been identified in the 1970s–1990s, but recent discoveries of PreP and Icp55 added new aspects to our understanding of the fate of targeting peptides and quality control of newly processed mature proteins. Nonetheless, there are still several intriguing questions that remain unanswered. Some of these questions concern the fate of targeting peptides in organellar compartments where no degrading proteases have been characterized so far, such as the IMS of mitochondria and the thylakoid lumen of chloroplasts. Furthermore, there are indications on occurrence of additional proteases participating in turnover of targeting peptides in the chloroplast stroma and inner membrane. Other questions concern quality control of the processed precursor proteins. So far, it has been shown only for MPP that its cleavage may result in the generation of destabilizing N-termini that require additional processing by Oct1 or Icp55. It would be of great interest to investigate if the trimming according to the N-end rule also applies to other processed intermediates and if this type of quality control system also exists in mitochondria of other organisms and in chloroplasts. Another intriguing question relates to the evolution of processing and degrading proteases, especially those belonging to the pitrilysin family (such as MPP, SPP and PreP). All three proteases appear to have evolved from non-photosynthetic bacteria, but while the evolution of MPP has been studied (this enzyme is soluble in yeast and mammals and integrated into the cytochrome bc_1 complex in plants) detailed information on the ancestry of SPP and PreP is still missing. Moreover, the 3D structure of MPP and PreP revealed a similar fold despite essentially no sequence conservation and the different functions of these proteins. In this context, it would be of great importance to solve the 3D structure of SPP. Comparison of the 3D structures of these proteases would allow us to understand the molecular basis of their functional differences, i.e. processing of precursors by MPP in comparison to processing of precursors and trimming of targeting peptides catalyzed by SPP and complete degradation of targeting peptides by PreP.

Furthermore, as already described in Section 6, due to the importance of precursor processing and removal of targeting peptides, it is likely that altered activity of the proteases involved in these processes will lead to dramatic effects on the activity, stability and assembly of organellar proteins and on the sustenance of organellar functions. Lack of correct processing of precursors may not only affect activity and stability of the protein itself, but it may lead to incorrect interactions with other proteins, altered folding and accumulation of misfolded proteins. Lack of targeting peptide degradation may result in their accumulation and interaction with other organellar proteins affecting their activity, folding and assembly status. Moreover, free targeting peptides cause uncoupling of biological membranes and a consequence of it can be organism pathology. It will be of great interest to understand the effects of protease dysfunctions or complete deletion on organellar activities and investigate therapeutical possibilities based on protease re-activation.

In summary, further studies on organellar proteases will not only advance our understanding of organelle biogenesis and evolution, but

may also contribute to our knowledge of medical aspects associated with mitochondrial dysfunctions.

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