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Metalloproteases of the Inner Mitochondrial Membrane

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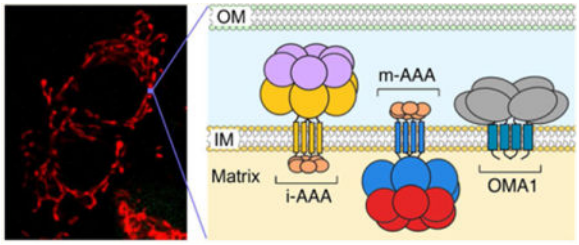
Abstract

The inner mitochondrial membrane (IM) is among the most protein-rich cellular compartments. The metastable IM subproteome where the concentration of proteins is approaching oversaturation creates a challenging protein folding environment with a high probability of protein malfunction or aggregation. Failure to maintain protein homeostasis in such a setting can impair the functional integrity of the mitochondria and drive clinical manifestations. The IM is equipped with a series of highly conserved, proteolytic complexes dedicated to the maintenance of normal protein homeostasis within this mitochondrial subcompartment. Particularly important is a group of membrane-anchored metallopeptidases commonly known as m-AAA and i-AAA proteases, and the ATP-independent Oma1 protease. Herein, we will summarize the current biochemical knowledge of these proteolytic machines and discuss recent advances in our understanding of mechanistic aspects of their functioning.

Graphical abstract



Mitochondria are complex organelles involved in a plethora of vital cellular functions, including energy conversion, cofactor and metabolite synthesis, maintenance of ion

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I.B. and R.M.L. analyzed the literature, designed the figures, and co-wrote the manuscript. O.K. conceived the idea, helped with the literature analysis, and co-wrote the manuscript. R.M.L. and I.B. contributed equally to this work.

Notes

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homeostasis, cell signaling, and death.[1] Two membranes with distinctive lipid compositions, known as the outer (OM) and inner (IM) membranes, segregate mitochondrial compartments from the rest of the cell and harbor a significant portion of proteins comprising the mitochondrial proteome. Particularly fascinating is the IM, which is estimated to house more than 800 integral and peripherally associated proteins originating from both nuclear and mitochondrial genomes and is one of the major sites of essential mitochondrial functions.[2] The extremely protein-rich environment of the IM subcompartment poses a major homeostatic challenge and necessitates tight control of its biogenesis and maintenance. Not surprisingly, the failure to properly fold, assemble, and maintain protein complexes within the IM is increasingly recognized as a root cause of a wide spectrum of pathological conditions.[3–5] Several interdependent mechanisms known as mitochondrial quality control (MQC) are involved in the maintenance of protein homeostasis within the IM. A major facet of MQC that operates in this mitochondrial subcompartment is represented by three evolutionarily conserved metallopeptidases: the intermembrane space (IMS)-oriented AAA (i-AAA) protease, the matrix-oriented AAA (m-AAA) protease, and the Oma1 protease. In this review, we will summarize current knowledge gained from the biochemical studies focusing on the mechanistic aspects of these proteolytic machines.

MITOCHONDRIAL AAA METALLOPROTEASES

The AAA+ (ATPases associated with diverse cellular activities) metalloproteases in mitochondria are direct descendants of the bacterial FtsH AAA+ protease[6] and share common domain and structure organization features with members of the so-called classic clade of AAA proteins.[7–9] They are a subgroup of AAA+ proteases classified as ring-shaped P-loop NTPases,[10] a diverse family of proteins responsible for unfolding misfolded or damaged polypeptides.[11] The proteins of the FtsH family form hexameric complexes that utilize the energy of coordinated ATP hydrolysis to propel target proteins through the central pore for unfolding[12] (Figure 1A). A typical AAA+ metalloprotease comprises an N-terminal domain, a AAA+ ATPase domain that contains the nucleotide binding Walker A and B (also known as NBD1 and NBD2) motifs, and the second region of homology (SRH) domain,[11] and the Zn^{2+} metalloprotease C-terminal domain.[7,11] The N-terminal portion is usually the least conserved part of AAA+ metalloproteases.[7] Metalloproteinase domains have either HExxH or variant HxxEH motifs and belong to the M41 protease domain family.[13] In this setup, histidines bind a Zn^{2+} ion and keep it in the correct orientation while glutamine takes part in the catalytic reaction as a proton donor[14] through an “oxyanion hole” mechanism that was described in detail for carboxypeptidase A.[15] While bacteria are usually equipped with only one FtsH-type metalloprotease,[16] the mitochondria of eukaryotic cells bear several different homologues, the homomeric i-AAA and heteromeric m-AAA, with their active sites facing the IMS and mitochondrial matrix, respectively.[11] The following sections describe these proteolytic machines in greater detail.

I-AAA PROTEASE

Six copies of the inner membrane-anchored, IMS-facing protein Yme1 (Yta11, also known as YME1L in mammals) form an active homohexameric complex commonly termed i-AAA protease.[17] These eukaryote-specific proteins are believed to have evolved from bacterial

FtsH peptidases by losing a transmembrane helix and substituting an $\alpha + \beta$ fold with a tetratricopeptide repeat (TPR) fold.[7,18] It is postulated that Yme1 proteins have further diverged in the animal kingdom by acquiring an additional domain, Yme1-NN (at present found only in the *Hydra* genus), which along with the TPR fold was replaced by the Yme1-NC domain following the evolutionary split between Cnidaria and Bilateria phylae.[7] In contrast, Nematoda and Biptera harbor yet another Yme1 variant, where Yme1-NN is replaced with other domains.[7] i-AAA function is highly conserved in eukaryotes.[19]

Most of the current knowledge of i-AAA was derived from studies in yeast. NMR analysis of the yeast enzyme showed that the N-terminal domain of Yme1 comprises a short mitochondrial targeting sequence, and a single, 19 kDa polypeptide chain, which in turn contains a core and loosely folded flanking regions. The core region harbors a fold with five helices, four of which are forming TPR hairpins. Such a fold structure is conserved in most eukaryotes, ranging from yeast to mammals, but has an additional extended loop in some fungi and plants.[7] The N-terminal domain of Yme1 is protein-specific and does not share any homology with the N-termini of the other AAA+ proteins. These domains mediate hexamerization of Yme1, thereby forming the central pore structure of the complex that leads a target protein to the complex's catalytic chamber[7] (Figure 1A,B). The remainder of the protein consists of the AAA+ ATPase domain, the conserved SRH motif, the catalytic (protease) domain, and the C-terminal calponin homology (CH) domain.[20]

The E541Q mutation in the HExxH domain of Yme1 disables the enzyme's catalytic site.[21] The SRC domain also contributes to enzymatic activity by co-localizing conservative arginine residues close to the catalytic center of the neighboring subunit, thereby boosting the catalytic efficiency of the enzyme.[22] An intact AAA+ domain structure with a hydrophobic amino acid at position 354 within the pore loop of Yme1 was postulated to be critical for substrate unfolding;[23] however, neither the catalytic domain nor the AAA+ module appears to play a role in determining substrate specificity. Two helical subdomains of the C-terminal CH domain appear to be responsible for substrate recognition,[21] even though the NH domain also seems to play some role in substrate binding.[21] The ATPase module of Yme1 can translocate proteins through the membrane into the IMS without engaging the cleavage by the catalytic domain[24] (Figure 1B). In line with this are the observations that i-AAA can perform chaperone-like functions both *in vitro*[25] and *in vivo*.[26,27] At present, Yme1 appears to be the only protein with the ATP-dependent chaperone function that operates in the IMS.[26]

The recent elegant *in vitro* studies using an engineered soluble version of mammalian Yme1 ortholog YME1L, stabilized via replacement of its native transmembrane segment with the *cc-hex* artificial peptide permitting the enzyme's assembly into an enzymatically active i-AAA complex in solution, showed that Yme1 recognizes certain specific motifs (degrons) in its substrate proteins, and the simple unfolding of a substrate is insufficient to initiate degradation.[28] While the molecular nature of i-AAA degrons remains to be elucidated, the study by the Glynn lab identified an F-*h*-*h*-F (where *h* is any hydrophobic residue) as a potential recognition signal for YME1L.[28] It is noteworthy that this motif or its variants are found in ~40 proteins comprising the IMS and IM subproteomes.[28] Further analyses determined that Yme1 exhibits specificity toward unfolded degrons but does not seem to

recognize them in a folded state.[29] Only when an unfolded degron is recognized can the i-AAA protease subsequently unfurl the rest of the protein and proteolyze it in a processive manner. This is achieved by active unfolding and feeding of the substrate into the enzyme's catalytic chamber through the complex's central pore using the energy of ATP hydrolysis.[28]

In line with the functional versatility of Yme1 are its multifaceted activities within the mitochondria; the i-AAA protease has been implicated in a variety of processes, including regulation of the mitochondrial fission/fusion mechanism by processing the IM GTPase OPA1[30] and metallopeptidase OMA1;[31] assembly, maintenance, and quality control of IM proteins and respiratory complexes;[32,33] IMS protein transport;[24] and the mitochondrial unfolded protein response (UPRmt).[34] While physiological roles of i-AAA remain to be clarified, the enzyme's functional importance is underscored by studies in rodents showing its crucial role in embryonic development and maintenance of normal mitochondrial populations.[35] In addition, a recent clinical study identified the homozygous R149W mutation in YME1L in patients with an infantile-onset mitochondriopathy.[36] This substitution affects the protein's maturation and impairs its accumulation within the mitochondria.[36]

M-AAA PROTEASE

The matrix-facing m-AAA protease has been originally described in yeast as a heterohexameric 850 kDa complex consisting of paralogous Yta10 and Yta12 subunits (SPG7/ paraplegin and AFG3L2 in humans, respectively)[37,38] (Figure 1C), which are well-conserved across the Eukarya.[39] In addition to the usual AAA+ and M41 metallopeptidase domains, these proteins harbor a specific IMS domain (IMSD) and two transmembrane helices at their N-termini.[12] In humans, IMSD contains an $\alpha + \beta$ fold of two α helices and five β sheets.[39] Both catalytic ATPase domains of m-AAA subunits are exposed to the matrix[25,37] (Figure 1A). These modules not only are responsible for protein unfolding but also appear to be specifically driving formation of an active hexameric m-AAA complex. Swapping of catalytic domains between Yta10 and Yta12 prevents the complex from developing functionality, while switching other domains does not.[12] Interestingly, heterooligomeric assembly of the Yta10−Yta12 complex is dependent upon the proteolytic domains of both subunits; only two amino acid substitutions in this region can promote homooligomerization of Yta12. These mutations have been allocated to the interface between promoters in the m-AAA hexamer.[12] Coordinated ATP hydrolysis is ensured by a complex signaling network within the AAA ring with the primary role given to the arginine finger, followed by the intersubunit signaling motif (located at the end of helix $\alpha 7$) and the pore loop 2 region of Yta10.[40] Similar to the AAA+ domain of Yme1, the ATPase domains of Yta10 and Yta12 were shown to exert some chaperone-like properties.[25,37] The molecular organization and architecture of m-AAA appear to be similar between eukaryotes.[39,41] Human m-AAA complexes can rescue yeast cells with m-AAA deletions, which points to a highly conserved structure and function.[41,42]

Structural analysis of human SPG7 revealed that this domain comprises a large β sheet consisting of five parallel strands, surrounded by two α helices, and terminated with four antiparallel α helices at the C-terminus. The domain's ATP/ ADP binding site is situated

between these structures.[43] The Walker A and B motifs in SPG7 are highly conserved and are responsible for handling ADP and Mg^{2+} ions, respectively.[43] The activity of the catalytic domains of each subunit is boosted in the hexameric m-AAA complex because of the intersubunit influence of the Walker A motifs from neighboring subunits, and the resulting cooperative mechanism allowing simultaneous coordinated ATP hydrolysis by all subunits of the complex.[40] In addition to heteromeric SPG7/AFG3L2 m-AAA complexes, human mitochondria contain m-AAA variants formed by six copies of AFG3L2[41,42] that have overlapping functionality.[44] Interestingly, SPG7 does not appear to homo-oligomerize, likely because its own maturation requires AFG3L2.[45] Additionally, murine mitochondria contain yet another AFG3L2 paralog, Afg3L1, which has transformed into a pseudogene in humans.[45,46] The abundance of each variant complex appears to be determined merely by the abundance of a particular subunit.[42] Inceptive evidence of differential substrate preference and/or specificity was provided for both murine and human homo- versus hetero-oligomeric m-AAA complexes.[20] It is noteworthy that tissue-specific patterns of m-AAA subunit composition have been observed in rodents;[42] however, the functional significance of this observation remains to be clarified.

Numerous studies have catalogued information about mutations in the m-AAA complex. For example, mutations in SPG7's catalytic center or hydrophobic core, such as A510V, completely abrogate m-AAA complex activity.[43] Interestingly, however, a constitutively active R688Q substitution, which renders SPG7 insusceptible to AFG3L2-mediated maturation, has also been reported.[47] Several mutations affecting the function of AFG3L2's peptidase domain have also been described. Most of these substitutions occur in the region spanning amino acid residues 654−700.[48–50] Additionally, the E575Q, M625I, and K354A mutations disable the catalytic center of the enzyme,[51–53] while the Y616C substitution prevents hetero-oligomerization of AFG3L2 with SPG7;[54] the E408Q mutation ablates ATPase activity by disrupting the Walker B motif.[52] Given the detrimental effect of the AFG3L2 and paraplegin mutations mentioned above, their association with clinical manifestations is not surprising. Many of these substitutions manifest in neurological pathologies such as hereditary spastic paraplegia (HSP type 7), spinocerebellar ataxia type 28 (SCA28), and spastic ataxia-neuropathy syndrome (SPAX5). More detailed reviews of the roles of m-AAA protease in neuronal dysfunctions can be found elsewhere.[4]

The molecular architecture of m-AAA appears to be similar to that of the i-AAA protease.[7,39] It is therefore plausible that the mechanism of substrate recognition by m-AAA may be similar to that reported for YME1L. Available reports suggest that AAA proteases may be finding their substrates in the protein-rich IM by recognizing specific patterns that become accessible upon polypeptide's domain unfolding and/or its projection from the membrane face. Consistent with this idea, studies in yeast have determined that a certain length of the polypeptide's unfolded region is required for substrate retention and processing by m-AAA.[55] However, molecular determinants of substrate recognition by m-AAA, as well as the identity of potential degrons, remain to be investigated.

Just like i-AAA, the m-AAA protease can perform both proteolytic and chaperoning functions. The enzyme is responsible for maturation, maintenance, and quality control of membrane proteins on the matrix side of the IM[56,57] as well as certain proteins in the

matrix[58] (Figure 1C), most notably the key ribosomal protein MRPL32.[56] The m-AAA protease is also known to regulate mitochondrial fusion by influencing the cleavage of the OPA1 GTPase, albeit likely in an indirect manner.[52] Interestingly, both m-AAA and i-AAA complexes can degrade IM-anchored polypeptides with at least a short tail exposed to the matrix or IMS by ATP-dependent unfolding and translocation of the proteins across the membrane for degradation.[59] An interesting example of protein translocation that is uncoupled from degradation has been reported in yeast, wherein m-AAA dislocates fungus-specific protein cytochrome *c* peroxidase (Ccp1) from its initial position in the IM and correctly positions it for subsequent cleavage by rhomboid protease Pcp1.[60,61] The intact central pore loop and the insertion of Ccp1 into the protease's central channel are critical requirements of this process.[61]

OMA1 PROTEASE

Initially postulated to be a backup protease for the m-AAA proteolytic module, Oma1 belongs to the M48C family of conserved metalloproteases[62] with homologues found in both prokaryotes and eukaryotes.[63] Some notable exceptions include Nematoda and Trematoda worms and certain flies (Drosophilidae), which appear to have lost OMA1 homologues throughout their evolution. The enzyme comprises a eukaryote-specific N-terminal region[63] and a M48 metallopeptidase domain.[13] Unlike i-AAA and m-AAA proteases, Oma1 lacks the ATPase domain and thus is ATP-independent (Figure 2A,B). Amino acid sequence analysis predicts Oma1 to contain two hydrophobic transmembrane segments (residues 63−83 and 220−240 in *S. cerevisiae*)[62] that anchor OMA1 to the IM and orient the enzyme's active site to the IMS[64] (Figure 2A). However, the experimental evidence rather suggests that the enzyme contains a single transmembrane segment.[65] Mutations of catalytic glutamate and histidine residues within the protease's HExxH catalytic domain ablate its enzymatic activity.[63,66]

Oma1 is assumed to exist as a homo-oligomeric complex.[66,67] The protease appears to be largely dormant under basal conditions but becomes rapidly activated by various homeostatic challenges such as loss of mitochondrial membrane potential, oxidative stress, and heat stress[65,66,68] (Figure 2C). With no available structural data about Oma1, the mechanism of its stress activation remains largely obscure. However, several insights have emerged from studies of the yeast and mammalian enzyme. Probing yeast mitochondrion-derived Oma1 oligomers by limited proteolysis revealed that conditions of mitochondrial stress alter the conformation and/or stability of the protease complex, while constituent Oma1 subunits remain stable.[66] In contrast, mammalian OMA1 was reported to undergo autocleavage upon its activation.[64,65,68] OMA1 is presumed to self-cleave at C-terminal residues 443−452 upon mitochondrial depolarization.[68] It is noteworthy that the C-terminal moiety of metazoan Oma1 is significantly extended as compared to the yeast enzyme. However, a more recent study by Rainbolt et al. has challenged the OMA1 self-cleavage model by demonstrating that degradation of activated OMA1 occurs via YME1L-mediated proteolysis.[69] Mammalian OMA1 harbors another metazoan-specific element, a positively charged N-terminal region spanning amino acid residues 144−163. This region appears to be important for OMA1 activation; mutations in the residues constituting the segment impair the enzyme's stress activation and processing.[65]

Heterozygous mutations in conserved His-69, Glu-272, and Asp-365 residues of OMA1 have been reported in several patients afflicted with familial and sporadic forms of amyotrophic lateral sclerosis.[72] While molecular effects of these mutations remain to be tested, these substitutions are likely to exert negative effects due to the likely homooligomeric nature of the OMA1 complex.

FUNCTIONAL INTERACTIONS OF THE IM METALLOPEPTIDASES

Functional interactions between the IM proteases can manifest in various forms, including (1) specific condition-defined reciprocal proteolysis of the enzymes, (2) sequential processing or degradation of shared substrates, and/or (3) formation of supramolecular complexes to facilitate processing of certain substrates or modulate enzymes' activity. These functional scenarios are not mutually exclusive, as exemplified, for instance, by versatile functional interactions between Oma1 and i-AAA proteases. We will discuss some of these activities.

The study by Rainbolt et al.[69] reported that mammalian YME1L and OMA1 can reciprocally regulate each other's steady state levels in response to membrane depolarization and ATP levels, respectively (Figure 3A). This work suggested that the i-AAA protease is responsible for degradation of depolarization-activated OMA1, whereas ATP-independent OMA1 peptidase may be involved in proteolytic processing of YME1L when mitochondrial ATP pools are critically small. At present, it is unclear whether this modus operandi is specific to mammalian enzymes; the stress-activated Oma1 appears to remain stable in yeast.[65,66] On the other hand, combined deletion of fungal Oma1 and Yme1 leads to a severe synthetic genetic effect.[66] Such synergy, however, may be related to functional cooperation between the enzymes toward certain substrates. For example, a recent study by Ogunbona et al. demonstrated in yeast that Oma1 and Yme1 are responsible for the processing of temperature-sensitive mutant phosphatidylserine decarboxylase Psd1.[70] Psd1 is a mitochondrial protein comprised of noncovalently associated α (required for enzymatic activity) and β subunits, which are separated as a result of autocatalysis. The i-AAA protease alone can degrade postautocatalysis-aggregated β-subunits. However, when the latter process is compromised, Oma1 is required to cleave the misfolded temperature-sensitive Psd1 precursor, which is subsequently degraded by Yme1.[70] It is noteworthy that similar cooperation has been reported for the Oma1 and m-AAA proteases during proteolytic removal of the temperature-sensitive L240S mutant of the IM insertase Oxa1.[63]

Another well-characterized shared substrate of the IM metallopeptidases is mammalian dynamin-related GTPase OPA1.[65,73] This protein, which has emerged as a key mediator of IM fusion, is present in multiple variants in various tissues because of alternative splicing and processing resulting in six different isoforms in mice[74] and eight in humans.[75] Long OPA1 isoforms (L-OPA1) are produced because of alternative splicing; these polypeptides contain two physically separate cleavage sites, designated S1 and S2, which are recognized by OMA1 and YME1L, respectively (Figure 3B). Cleavage of L-OPA by either protease yields short forms of OPA1, known as S-OPA1 (reviewed in refs [76] and [77]). However, in this case, OMA1 and YME1L seem to engage under different physiological conditions. The i-AAA protease appears to be the key enzyme mediating L-OPA1 processing under nonstress

conditions; its activity yields roughly equimolar amounts of long and short OPA1 isoforms, the state required for an optimal fusion/fission balance of the mitochondrial network. Additionally, YME1L's activity toward L-OPA1 has been proposed to further stimulate IM fusion under conditions that demand increased bioenergetics capacity.[78] In contrast, OMA1 exhibits little or no involvement in L-OPA1 processing under basal conditions. However, it becomes activated in response to various homeostatic insults (Figure 2C) and mediates rapid conversion of all L-OPA1 isoforms into the short variant, thereby triggering IM fission and subsequent partitioning of the mitochondrial network[30,64,65,73] (Figure 3C). The balance between proteolytic activities of i-AAA and OMA1 appears to be crucial for normal cell functioning. For example, cardiac-specific inactivation of YME1L in mice triggers OMA1 activation and subsequent mitochondrial fragmentation, and metabolic alterations in cardiac tissue. These events further lead to the development of dilated cardiomyopathy and ultimately heart failure. Remarkably, these pathological conditions can be effectively alleviated by the additional deletion of OMA1, leading to L-OPA1 stabilization and consequent restoration of the mitochondrial network.[35]

It is noteworthy that the yeast OPA1 ortholog Mgm1 is not a substrate of either i-AAA or Oma1. The fungal GTPase is processed by the rhomboid serine protease Pcp1 (PARL in mammals), which also yields equimolar amounts of L-Mgm1 and its single short isoform (reviewed in ref [79]).

Both i-AAA and m-AAA proteases can form supermolecular structures with other proteins that are not directly required for their proteolytic activity. In some cases, such assemblies have been reported to display regulatory features, associate with specific substrate recruitment activities, or facilitate protease− substrate interactions. In yeast, nematodes, and mammals, the prohibitins Phb1 and Phb2 form a large IM-anchored ring-shaped complex of 1.2 MDa (PHB) (reviewed in ref [80]). The PHB complex assembles together with independently formed m-AAA protease;[80,81] however, the functional significance of this interaction remains unclear. Osman et al.[80] speculated that such assembly may be assisting recruitment of the m-AAA complex to specific membrane sites where lipid composition could activate the protease and/or modulate its activity. An additional component of the PHB −m-AAA complex has been reported recently.[81] The previously uncharacterized protein c2orf47 (now named MAIP1, for m-AAA protease interacting protein 1) is the matrix protein peripherally attached to the IM. MAIP1 is a binding partner but not a substrate of the m-AAA. It facilitates maturation of the EMRE subunit of the mitochondrial Ca^{2+} uniporter (MCU) by protecting it from degradation by YME1L. Interestingly, non-assembled EMRE also appears to be the substrate of m-AAA; however, its cleavage seems to be independent of its association with MAIP1.[81]

The i-AAA protease is also known to be a part of supramolecular assemblies regulating its proteolytic activity. The study by Dunn et al.[82] revealed that yeast i-AAA can associate into a structure comprising proteins Mgr1 and Mgr3. While this assembly is not essential for proteolysis per se, it appears to improve the protease's binding to model substrates, thereby suggesting an adaptor role for the Mgr1−Mgr3 subcomplex.[82] Another report studying i-AAA-mediated turnover of disease allele-mimicking mutant forms of phospholipid transacylase Taz1 demonstrated that degradation of these proteins is impaired but not ablated

in the absence of Mgr1 and Mgr3, further indicating their role in facilitating Yme1 function.[83]

Interestingly, in the absence of the adaptor subcomplex, Yme1 may function as a chaperone.[84] Similarly, the human YME1L protease also exists in several macromolecular complexes ranging from 600 to 2000 kDa in size.[32,85] At present, however, no functional homologues of the Mgr1− Mgr3 subcomplex have been identified outside of fungi. Instead, the recent study described a novel supramolecular complex consisting of the YME1L, membrane scaffold stomatin-like protein SLP2, and the rhomboid protease PARL.[85] This ~2 MDa complex, named SPY, appears to facilitate the proteolytic activity of i-AAA under conditions of high substrate load; in this role, it resembles the PHB−m-AAA supercomplex and may exert a modulatory effect on i-AAA actvity toward specific substrates.[85]

CONCLUDING REMARKS

A significant body of experimental data pertaining to the IM metalloproteases has accumulated since the identification and initial characterization of the i-AAA enzyme more than two decades ago.[17,86,87] Nevertheless, despite intensive studies by numerous laboratories around the world, many outstanding questions remain to be addressed, for example, how the IM proteases, which appear to be predominantly localized to the inner boundary membrane region of the IM,[88,89] survey and access substrate proteins residing in the cristae compartments. Further challenges for the future include identification of substrate repertoires for each IM metallopeptidase, understanding structural bases for selective substrate recognition, and either modulation or activation of enzymes' proteolytic function. The physiological roles of the IM proteases under basal and homeostasis-perturbing conditions represent another exciting issue. While multiple lines of evidence indicate functional overlap and an apparent case of condition-specific cooperation of the IM metalloproteases in processing various substrates, the prospective therapeutic significance of enzymes like Oma1 has begun to emerge. These versatile enzymes are no longer viewed as mere "custodians" of the IM involved in simple removal of damaged or surplus proteins. The fine-tuned, multifaceted roles of the IM metalloproteases in mitochondrial and cellular physiology are being increasingly recognized and await further investigation.

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ABBREVIATIONS

IM	inner mitochondrial membrane
OM	outer mitochondrial membrane

IMS	intermembrane space
ATPase	adenosine triphosphate hydrolyzing enzyme
AAA+	ATPase associated with diverse cellular activities
i-AAA	IMS-oriented AAA+ protease
m-AAA	matrix-oriented AAA+ protease
MQC	mitochondrial quality control
NBD	nucleotide binding domain
SRH	second region of homology
TPR	tetratricopeptide repeat
NMR	nuclear magnetic resonance
UPRmt	mitochondrial unfolded protein response
GTPase	guanosine triphosphate hydrolyzing enzyme
IMSD	IMS domain
HSP	hereditary spastic paraplegia
SCA	spinocerebellar ataxia
SPAX	spastic ataxia-neuropathy syndrome
MCU	mitochondrial calcium uniporter
PHB	prohibitin complex

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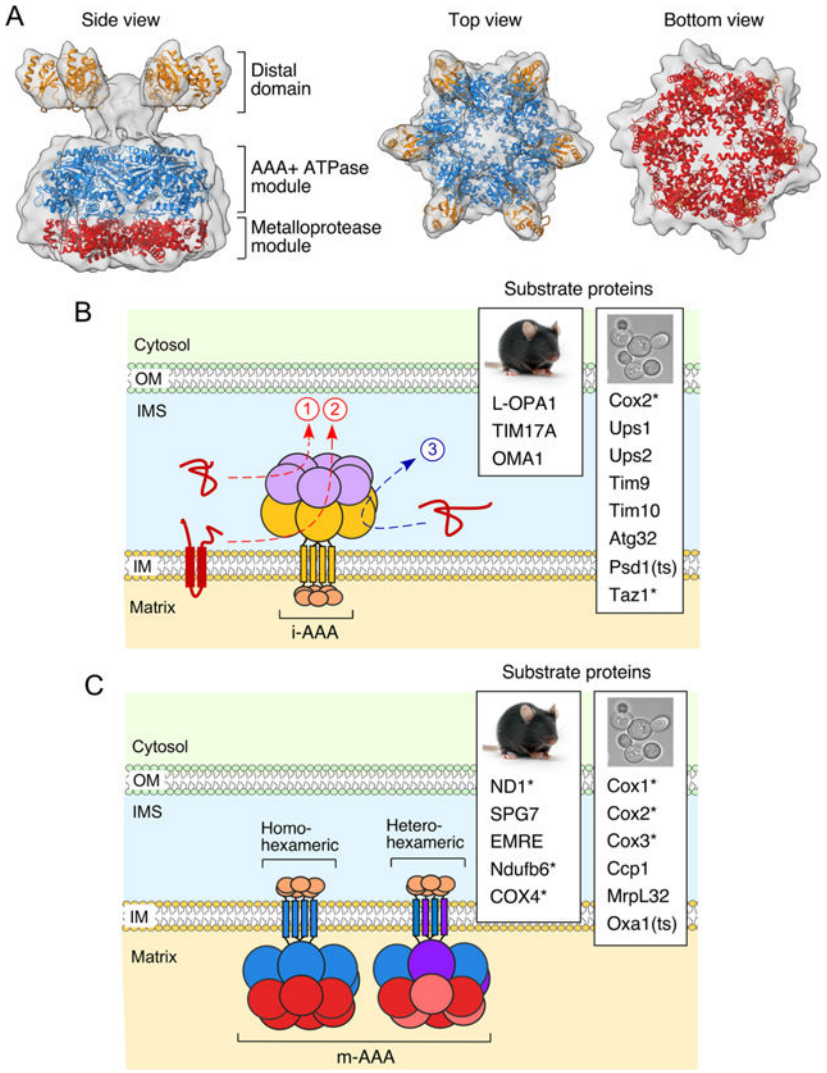

Figure 1.

Structural and functional organization of the AAA+ IM metalloproteases. (A) A structural model of prototypal IM AAA+ protease is presented as a hexameric structure with a distinct metalloprotease domain (red) and an AAA+ domain (blue) modeled by fitting the crystal structure of the FtsH protease from *Thermogota maritima* (3KDS) into the cryoEM envelope from *Saccharomyces cerevisiae* m-AAA enzyme (EMD-1712). The IMSD domain (orange) in the distal part is presented by the solution structure of a portion of human AFG3L2 (2LNA). Note that the AAA+ and metalloprotease modules are not color-highlighted in the top and bottom view projections, respectively, for the sake of clarity. The model was generated using ChimeraX. (B) i-AAA protease forms a homohexameric complex with the catalytic M41 zinc metalloprotease's domain (purple) and the AAA+ ATPase domain (yellow) facing the IMS, which are then followed by the transmembrane spans and the Yme1-specific N-terminus (orange). The enzyme can (1) proteolytically process soluble, IMS-residing substrates, presumably without engaging its AAA+ module, (2) extract and degrade IM-anchored proteins through the concerted action of the ATPase and proteolytic domains, or (3) merely unfold and/or refold them via its AAA+ module, thus functioning as

a chaperone. Various substrates have been identified for both mammalian and yeast i-AAA proteases, which, however, in many cases are not orthologous. The insets in panels B and C list proteins that have been experimentally confirmed as bona fide substrates of each respective protease. The asterisks or ts (temperature-sensitive) acronyms denote proteins that are perceived as substrates under a condition under which they are damaged, unassembled, and/or misfolded. Abbreviations: OM, outer mitochondrial membrane; IM, inner mitochondrial membrane; IMS, intermembrane space. (C) m-AAA proteases can form either homo- or hetero-oligomeric complexes with their proteolytic (red and pink) and ATPase (blue and dark purple) domains exposed to the matrix. Similar to the i-AAA enzyme, the m-AAA proteases demonstrate remarkable versatility in their substrate processing modes. Likewise, the m-AAA substrates appear to differ between mammalian and yeast systems. More details can be found in the text.

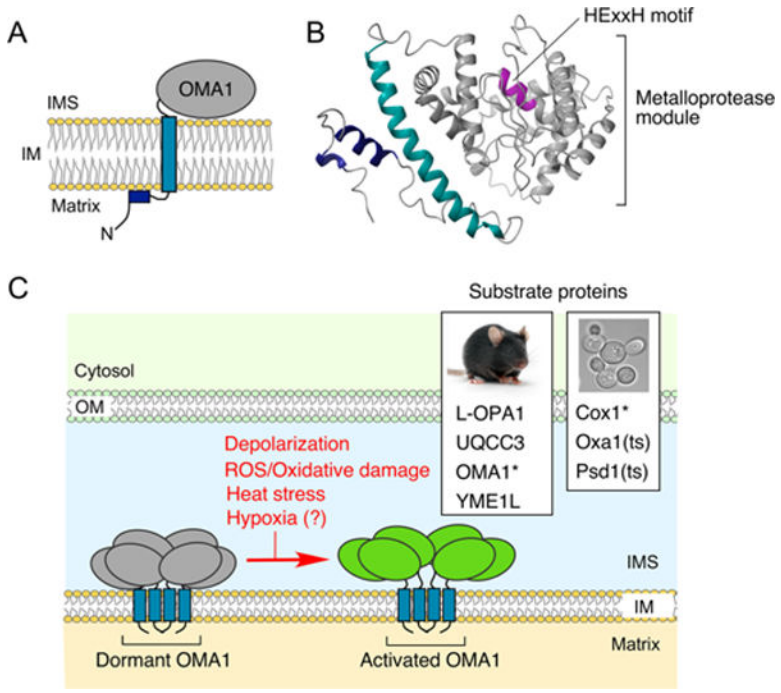

Figure 2.

Structural and functional organization of OMA1 protease. (A) Predicted molecular organization of mammalian OMA1. The enzyme comprises the IMS-facing proteolytic domain (gray; the position of the HExxH catalytic motif is colored purple), the transmembrane segment (teal), and a shorter N-terminal α helix that likely helps to further stabilize the enzyme's association with the IM (navy). The N-terminal moiety of vertebrate Oma1 orthologs also contains a stretch of charged amino acid residues that has been proposed to participate in stress-induced activation of the enzyme. (B) Predicted structural model of the yeast Oma1 protease. The model was produced using the iTASSER prediction software and further processed using ChimeraX. (C) OMA1 exists as a largely dormant, homo-oligomeric complex that is activated upon indicated homeostatic insults. While the exact mechanism behind this process remains to be clarified, changes in the conformation of the Oma1 oligomer were proposed to trigger the enzyme's activation. The inset shows currently known substrates of Oma1. The yeast Cox1 protein (marked with an asterisk) is degraded by Oma1 in a subset of cytochrome oxidase assembly mutants. Similarly, Oma1 appears to specifically degrade temperature-sensitive (ts), misfolding-prone variants of the IM translocase Oxa1 and phosphatidylserine decarboxylase Psd1. More details are available in the text. The current Oma1 substrate repertoire includes the misfolded and/or unassembled translocase Oxa1,[63] respiratory complex IV subunit Cox1,[67] and phosphatidylserine decarboxylase Psd1 in yeast,[70] and the GTPase OPA1[52,64,65] and complex III assembly factor UQCC3[71] in mammals (panel C). The mechanisms by which Oma1 selects and recognizes its substrates remain to be determined.

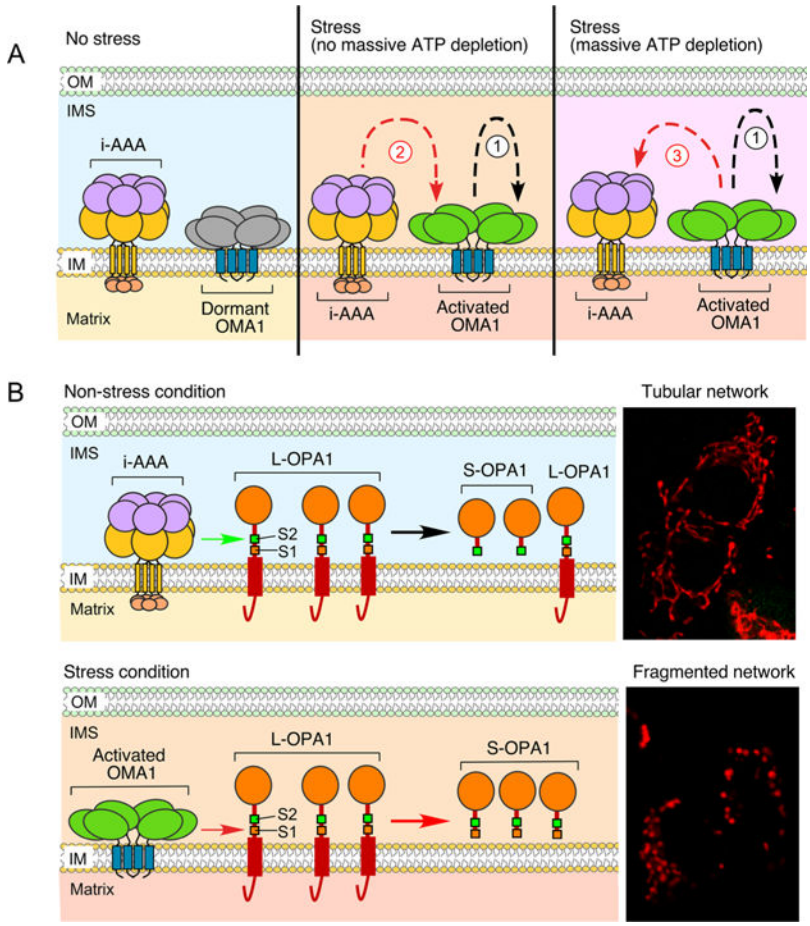

Figure 3.

Functional cooperation between the i-AAA and OMA1 proteases. (A) OMA1 is activated by conditions of mitochondrial stress. Original reports suggested that the activated protease undergoes autoproteolysis (1), thereby eliminating the over-reactive enzyme when the stressed condition no longer exists. However, a recent report proposed an alternative model in which the i-AAA protease is responsible for degradation of stress-activated OMA1 (2). Reciprocally, OMA1 may be involved in proteolytic cleavage of YME1L when mitochondrial ATP levels are attenuated (3). (B) Metalloproteases YME1L and OMA1 cooperate in proteolytic processing of long forms of GTPase OPA1 (L-OPA1). This variant is important for mitochondrial fusion and normal cristae ultrastructure. L-OPA1 forms harbor two physically separate cleavage sites, S1 and S2, which are recognized by OMA1 and YME1L, respectively. Cleavage of L-OPA by either protease yields short forms of the enzyme, S-OPA1, which facilitates partitioning of the mitochondrial network. The i-AAA protease appears to be the key enzyme mediating L-OPA1 processing under nonstress conditions, yielding nearly equimolar amounts of L-OPA1 and S-OPA1, and thus balances fusion and fission of the mitochondrial network. OMA1 exhibits little or no involvement in L-OPA1 processing under nonstress conditions. Homeostatic challenges or mitochondrial dysfunction trigger stress activation of OMA1, which mediates rapid processing of the entire L-OPA1 pool into S-OPA1 forms, thereby stimulating mitochondrial fission and massive fragmentation of the mitochondrial network. Further details can be found in the text.

Representative confocal fluorescent images show the MitoTracker Red-stained mitochondrial network in cultured SH-SY5Y neurobalstoma cells that have been challenged with an uncoupler CCCP or not challenged.