The role of protein degradation in mitochondrial function and biogenesis

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REVIEW

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Abstract It has been known for a long time that mitochondria contain their own protein-degradation systems. Only recently, however, have genes for mitochondrial proteases been identified and the powerful techniques of molecular biology been applied to gain insight into the role of protein degradation in mitochondrial biogenesis. It is now clear that the mitochondrial proteases that are involved in the initial stages of degradation are similar to prokaryotic ATP-dependent proteases, and that a division of labour exists between soluble and membrane-bound systems. These systems are essential for the biogenesis of fully functional mitochondria. Their natural targets are currently being identified, and their co-operation with chaperones and possible dual functions as chaperones/proteases are being investigated.

Key words Mitochondrial proteases · Protein turnover · Proteolysis · Chaperones

Introduction

Biogenesis of mitochondria has been intensively studied for more than two decades. In particular, the mitochondrial system for gene expression, and the co-ordination of this system with the nucleo-cytoplasmic one, have received much attention (see Grivell 1995 for a review). While the bulk of mitochondrial research has concentrated on the synthesis of mitochondrial components, it has not gone unnoticed that protein turnover and degradation are also an important part of mitochondrial biogenesis (Luzikov 1985; Desautels 1986). In general, protein degradation may serve several distinct purposes: (1) protective, by removing polypeptides that are potentially harmful to the cell, (2) regulatory, by controlling the concentrations of enzymes or regulatory proteins, and (3) metabolic, by releasing amino acids to be used for other purposes. Like other supermolecular structures, the degradation of mitochondrial fragments is mediated by lysosomal or, in the case of yeast, vacuolar proteases via the process of autophagocytosis (Takeshige et al. 1992). This process falls into the third category. For the first two functions, however, mitochondrial proteases are responsible (Desautels 1986). This review summarizes what is known to-date on protein degradation in mitochondria, the proteases involved, and the significance of protein degradation for mitochondrial biogenesis.

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Targets for turnover

It is well established that half-lives are widely divergent among proteins of every mitochondrial compartment (Desautels 1986; Hare 1990). A major class of targets for proteolysis are subunits of enzymes that carry out electron transport and oxidative phosphorylation. These enzymes constitute about 50% of the protein content of the mitochondrial inner membrane (Hatefi 1985) and, generally speaking, each consists of a combination of subunits that are encoded by either the mitochondrial or the nuclear genome. The regulation of the rate of subunit synthesis through transcriptional, and perhaps translational, control may assure the production of roughly comparable amounts of subunits belonging to a particular complex; but it is clear that this is not (and perhaps cannot be) achieved with high precision, especially since two different genetic systems are involved. Turnover of subunits is therefore necessary to prevent accumulation of single subunits and sub-complexes in the mitochondrial inner membrane, which may disturb assembly processes or change the properties of the inner membrane (Manoil and Traxler 1995). Indeed, many subunits of mitochondrial inner membrane enzyme complexes are quickly degraded when assembly into a com-

Table 1 Sensitivity towards degradation of unassembled subunits of mitochondrial inner membrane complexes[a]. Hs: *Homo sapiens*; Nc: *Neurospora crassa* (all other data were obtained with *Saccharomyces cerevisiae*); FP: flavoprotein; IP: iron-sulfur protein; FeS: Rieske iron-sulfur protein; Co*b*: cytochrome *b*; Cytc_1: cytochrome c_1

Subunit	Lower levels due to loss or mutation of:	References
Complex I		
All mitochondrially encoded subunits (Hs)	ND4	Hofhaus and Attardi 1993
21 and 21.3 of peripheral arm (Nc)	12.3 and 29.9 of the peripheral arm	Duarte et al. 1995
	51 of the peripheral arm	Fecke et al. 1994
Complex II		
FP	IP	Saghbini et al. 1994
Complex III		
Core I	Co*b*	Sen and Beattie 1985; Chevillotte-Brivet et al. 1987
Core II	Co*b*	Sen and Beattie 1985
	Core I	Gatti and Tzagoloff 1990
Co*b**	Core I, core II, Qcr7p, Qcr8p	De Haan et al. 1984; Berden et al. 1988; Crivellone et al. 1988; Schoppink et al. 1989
	Cbp3p**, Cbp4p**	Wu and Tzagoloff 1989; Crivellone 1994
Cytc_1	Qcr6p and Co*b* (simultaneously)	Schoppink et al. 1988
FeS	All complex III subunits except Qcr6p and Qcr10p	De Haan et al. 1984; Sen and Beattie 1985; Berden et al. 1988; Crivellone et al. 1988; Schoppink et al. 1989
	Bcs1p**	Nobrega et al. 1992
Qcr7p	Core I, core II, Qcr7p, Co*b*	De Haan et al. 1984; Berden et al. 1988; Crivellone et al. 1988; Schoppink et al. 1989
	Qcr6p	Yang and Trumpower 1994
	Cbp3p**, Cbp4p**	Wu and Tzagoloff 1989; Crivellone 1994
Qcr8p	Core I, core II, Qcr8p, Co*b*	De Haan et al. 1984; Berden et al. 1988; Crivellone et al. 1988; Schoppink et al. 1989
	Cbp3p**, Cbp4p**	Wu and Tzagoloff 1989; Crivellone 1994
Qcr9p	Qcr6p	Yang and Trumpower 1994
Complex IV		
CoxI*	CoxIV, CoxVa, CoxIX	McEwen et al. 1986
	CoxVII[b]	Calder and McEwen 1991
	Sco1p**	Krummeck and Rödel 1990
	Cytochrome *c***	Pearce and Sherman 1995a, b
CoxII*	CoxI, CoxIV, CoxVa, CoxIX	Dowhan et al. 1985; McEwen et al. 1986; Nakai et al. 1994
	Sco1p**	Schulze und Rödel 1989; Krummeck and Rödel 1990
	Cytochrome *c***	Pearce and Sherman 1995a
CoxIII*	CoxIV	Dowhan et al. 1985; Nakai et al. 1994
	Sco1p**	Krummeck and Rödel 1990
	Cytochrome *c***	Pearce and Sherman 1995a
CoxIV	CoxI, CoxII, Sco1p**	Krummeck and Rödel 1990
CoxV	CoxI	McEwen et al. 1986; Krummeck and Rödel 1990
	CoxII	Krummeck and Rödel 1990
	CoxIV	Dowhan et al. 1985
	Cytochrome *c***	Pearce and Sherman 1995a
CoxVI	CoxI, CoxII	Krummeck and Rödel 1990
CoxVII	CoxIX	McEwen et al. 1986
	CoxI, CoxII	Krummeck and Rödel 1990
CoxVIII	CoxIX	McEwen et al. 1986
Complex V		
Atp3p (γ)	Atp14p (δ) and Atp6,8,9p (simultaneously)	Giraud and Velours 1994
	Atp11p**, Atp12p**	Weber et al. 1996
Atp6p*	Atp4p (b)	Paul et al. 1989
	Atp7p (d)	Norais et al. 1991
	Atp8p, Atp9p	Jean-Francois et al. 1986a
Atp7p (d)	Atp4p (b)	Norais et al. 1994

[a] In most cases, sensitivity to degradation is inferred from low steady state amounts (see text). Data on the degradation of mitochondrial translation products caused by a general lack of cytoplasmically synthesized subunits (due to translation *in organello* or cycloheximide treatment of cells) are not included in this table

[b] The low level of CoxI in a strain disrupted for *COX7* could be due either to reduced synthesis or very fast (co-translational) turnover

* Mitochondrially encoded subunits

** Proteins not part of the complex

plex is made impossible by the lack of (an)other subunit(s) or of assembly-assisting proteins (Table 1, see also Hare 1990). Proteolytic systems responsible for the turnover of subunits in such artificial situations are likely to be also involved in maintaining the stoichiometry of subunits and the removal of non-functional proteins under natural conditions.

In addition to unassembled but otherwise normal membrane proteins, other polypeptides may threaten the integrity of the membrane. These include: (1) hydrophobic presequences, produced by proteolytic processing of cytochrome b_2, cytochrome c_1 and cytochrome *c* peroxidase (Kaput et al. 1982; Nunnari et al. 1993), (2) denatured matrix proteins that partition to the membrane due to the exposure of hydrophobic amino-acid sequences, as suggested for bacteria (Voellmy and Goldberg 1981), and (3) membrane proteins that have suffered oxygen damage. Substantial research has shown that, in diverse cell types, proteins damaged by oxygen radicals become sensitive to degradation (Davies et al. 1987; Pacifici and Davies 1990). Recently, membrane-bound proteases were shown to preferentially degrade oxidatively damaged erythrocyte membrane proteins. Inhibition studies point to the involvement of both serine- and metallo-proteases (Beppu et al. 1994). In rat liver mitochondria, conditions of enhanced radical flux indeed increased proteolysis by 20% (Dean and Pollak 1985). However, no such effect was seen in yeast mitochondria (Yasuhara et al. 1994).

The turnover of matrix proteins has been studied mainly in mammalian mitochondria. These studies have shown that half-lives vary among proteins of this class (Nicoletti et al. 1977; Hare and Hodges 1982b). Several studies indicate that a high turnover rate of key enzymes in biosynthesis pathways may facilitate the control of mitochondrial function. Cholesterol side-chain desmolase and p450, two enzymes involved in steroid biosynthesis in adrenal mitochondria and whose levels are regulated by ACTH (adrenocorticotropic hormone), have relatively short half-lives (Kimura 1969; Purvis et al. 1973). In the case of p450, ACTH may inhibit the rate of its degradation (Purvis et al. 1973). Other inducible mitochondrial enzymes that are characterized by high turnover rates are δ-aminolevulinic acid synthetase, a key enzyme for heme synthesis (Marver et al. 1966), and alanine- and ornithine-aminotransferase (Swick et al. 1968).

Degradation of mitochondrial translation products

Much of the information on mitochondrial protein degradation has come from studies of the turnover of mitochondrial translation products. This section summarizes the main results of these studies. It should be remembered, however, that most of these proteins are highly hydrophobic. The mechanism(s) of turnover may therefore not be applicable to mitochondrial proteins in general.

The degradation of mitochondrial proteins synthesized within the organelle can be conveniently analysed either by blocking cytoplasmic translation with cycloheximide or by studying protein synthesis and turnover in isolated mitochondria. A high turnover of mitochondrial translation products (half-life approximately 60 min) in isolated yeast mitochondria was observed for the first time by Bakalkin et al. (1978). In 1979, Kalnov et al. reported that one-third to one-half of the proteins synthesized in isolated yeast mitochondria were degraded with a half-life of about 35 min (Kalnov et al. 1979b). This degradation occurred more efficiently in mitochondria isolated from cells growing in logarithmic phase than in stationary phase (Kalnov et al. 1979a) and is inhibited by the addition of glucose to stationary cells (Luzikov et al. 1983). These differences in the rate of proteolysis under different growth conditions were suggested to be caused by changes in the fluidity of the inner membrane (Luzikov et al. 1983, 1984, see also Luzikov 1986). Black-Schaefer et al. (1991) also observed two pools with different rates of turnover, one with a half-life of minutes, the other with a half-life of hours. Instability of (a subset of) mitochondrial translation products has also been observed in rat liver mitochondria (Wheeldon et al. 1974; Desautels and Goldberg 1982b), as well as in HeLa cells and rat hepatoma cells in the presence of cycloheximide (Constantino and Attardi 1977; Hall and Hare 1990). As assembly of mitochondrially encoded subunits of inner membrane complexes depends on the presence of imported subunits, and these imported subunits will become limiting at some point in isolated mitochondria or when cytoplasmic translation is inhibited, the rapidly degraded mitochondrial translation products probably represent unassembled subunits.

Incomplete translation products

Incomplete mitochondrial translation products produced by carrying out translation in the presence of puromycin are rapidly degraded in yeast (Kalnov et al. 1979b; Pajic et al. 1994) and rat liver mitochondria (Desautels and Goldberg 1982b). In rat heart mitochondria, degradation of 50–60% of newly made polypeptides was observed in the absence of the membrane potential. Most of the proteins subject to the observed degradation were of abnormal size, and were presumed to result from premature chain-termination (Cote et al. 1990). C-terminal truncation as a result of nonsense mutations can also result in instability. This has been reported for cytochrome *b* (di Rago et al. 1993) and implied for CoxII and CoxIII by the observation that mutations in the *COX2* and *COX3* genes (of which most are nonsense mutations) in the majority of cases result in the complete absence of the corresponding translation product (Weiss-Brummer et al. 1979; Baranowska et al. 1983). In several cases, however, truncated translation products of variable lengths were detected. Apparently, the recognition of truncated CoxII and CoxIII proteins by a proteolytic system depends on the exact C-terminus, which may influence the degree of folding and/or the accessibility of 'sensitive' sequences.

Characterization of the proteolytic system

In all systems investigated thus far, degradation of mitochondrial translation products is dependent on ATP. Recently, several groups have reported that this process is also dependent on divalent metal-ions (Nakai et al. 1994; Pajic et al. 1994; Yasuhara et al. 1994). These reports differ, however, as to the ability of Zn^{2+} to restore proteolysis after treatment with the divalent metal-ion chelator o-phenantroline. Whereas Yasuhara et al. (1994) found inhibition by Zn^{2+}, Nakai et al. (1994) reported stimulation by the same ion. Both groups found reversal of inhibition by Mn^{2+} and Co^{2+}. Looking at the degradation of incomplete mitochondrial translation products induced by puromycin, Pajic et al. (1994) also report stimulation by Zn^{2+}, although Co^{2+}, Fe^{2+} and Mn^{2+} yield higher activities. Interestingly, only Co^{2+}, Fe^{2+} and Mn^{2+} can replace Zn^{2+} in the metalloprotease thermolysin to yield an active enzyme (Mn^{2+} restores only 10% of the activity), while a high amount of Zn^{2+} inhibits thermolysin activity (Holland et al. 1995). High concentrations of Zn^{2+} also inhibit mitochondrial-processing peptidases (Kalousek et al. 1992). A similar inhibitory effect of Zn^{2+} on other mitochondrial metalloproteases may explain the ambiguity concerning the effect of Zn^{2+} on mitochondrial protein degradation.

In addition to metal chelators, vanadate (an inhibitor of various ATPases) inhibits the degradation of mitochondrial translation products (Desautels and Goldberg 1982b). The same holds for chloramphenicol, an inhibitor of mitochondrial translation (Wheeldon et al. 1974; Kalnov et al. 1979b; Black-Schaefer et al. 1991), and hemin (Yasuhara et al. 1994). It is unclear at present why chloramphenicol should inhibit degradation, as other inhibitors of translation do not have this effect (Langer et al. 1995). Inhibition by hemin may reflect a positive regulation of heme on the production of respiratory chain components. However, such an interpretation is weakened by the fact that hemin also inhibits proteases from other sources (Tanaka et al. 1983; Waxman et al. 1985).

The inhibition by the protease inhibitors phenylmethylsulphonyl fluoride (PMSF), leupeptin, antipain and chymostatin as shown by Kalnov et al. (1979b) could not be reproduced in later studies, and may be related to the experimental set-up these authors used (discussed in Yasuhara et al. 1994).

Degradation of nuclear-encoded subunits of membrane complexes

In addition to mitochondrial translation products, many nuclear-encoded subunits of mitochondrial inner membrane complexes in yeast are known to be degraded rapidly when not assembled (Table 1). Increased turnover of imported subunits of complex IV was also observed in human and mouse cell lines in the absence of mitochondrial protein synthesis (Hayashi et al. 1990; Chrzanowska-Lightowlers et al. 1993; Nijtmans et al. 1995). It should be noted, however, that in most cases only steady state amounts of subunits were measured, high turnover being inferred from the assumption that synthesis and import were not affected. In only a few cases has subunit turnover actually been shown by pulse-chase experiments (De Haan et al. 1984; Nijtmans et al. 1995).

For some subunits, reports differ as to their stability in the absence of complex assembly in yeast. For example, in some studies core I and II were found to be stable in strains disrupted for other subunits of complex III (Berden et al. 1988; Crivellone et al. 1988), while other investigators have reported the instability of core I in *cob* mutants (Sen and Beattie 1985; Chevillotte-Brivet et al. 1987) and of core II in a core-I mutant (Gatti and Tzagoloff 1990). Nuclear-encoded subunits of complex IV accumulate in the absence of mitochondrially encoded subunits in yeast and *N. crassa*, even though they do not form stable sub-complexes (Sebald et al. 1972; Bertrand and Werner 1977; Cabral and Schatz 1978). However, strongly reduced levels of some of these subunits have been found in *cox1*, *cox4*, *cox9* and cytochrome *c* mutants (Dowhan et al. 1985; McEwen et al. 1986; Pearce and Sherman 1995a, see Table 1). The observed differences are possibly due to variations in experimental conditions and/or strain differences.

Although cytochrome *c* is not part of any complex (it shuttles between complex III and IV transferring electrons down the respiratory chain), in terms of stability it does, under certain circumstances, behave like a subunit of these complexes. Certain variants are rapidly degraded when cytochromes aa_3 or c_1, its physiological partners, are absent (Pearce and Sherman 1995b). Conversely, cytochrome aa_3 is absent in *cyc1*/*cyc7* double mutants, lacking both isoforms of cytochrome *c* (Pearce and Sherman 1995a). In fact, these mutants had diminished amounts of both nuclear- and mitochondrially encoded subunits of complex IV, with the latter group being virtually undetectable by Western analysis.

Can fully assembled complexes also be targets for degradation?

Generally, intact enzyme complexes of the mitochondrial inner membrane are found to be quite stable in mammals (Hare 1990) and yeast (represented by the 'stable' fraction of mitochondrial translation products, see above). However, several reports mention either turnover of subunits that are already in a complex, or turnover of whole complexes. In rat hepatoma cells, all subunits of complex IV and V turn over very slowly (half-life >100 h). For complex III, however, four of eight subunits (core II, cytochrome *b* and two smaller subunits) turned over more rapidly (half-life 35–42 h) (Hare and Hodges 1982a). Presumably, the subunits with short half-lives are replaced by newly synthesized subunits after the complex has fallen apart. The question remains whether disassembly of the complex simply represents a random process or whether

(damaged?) complexes are actively disassembled by "quality control" factors.

Active turnover of complete complexes has received experimental support. Part of the cytochromes *b*, *c*, c_1 and aa_3 that accumulate in yeast cells during exponential growth on galactose disappears in the course of further culture growth without loss of respiratory rate (Galkin et al. 1975). This loss of cytochromes is inhibited by the protease inhibitors PMSF and pepstatin (Luzikov et al. 1976). Administration of these inhibitors also increases the accumulation of cytochromes in glucose-grown cells (Galkin et al. 1980). These changes in cytochrome content are not accompanied by changes in the activity of the respiratory chain complexes, indicating that the 'surplus' cytochromes accumulated during exponential growth are not functional (Galkin et al. 1979a, b, 1980). The nature of these non-functional cytochromes is unclear. One possibility is that they represent incomplete complexes, and are "edited out" by specific recognition/protease systems, as occurs with plasma-membrane complexes (Klausner 1989). This is not likely, however, since solubilization of mitochondria led to the apparent activation of the extra cytochromes (Galkin et al. 1979a,b, 1980). Moreover, surplus cytochrome *c* is made and degraded in a similar way as the complex-bound cytochromes. This suggests the presence of whole mitochondrial compartments which for some reason are non-functional and so may be degraded by autophagocytosis (discussed in Luzikov 1986).

Co-ordinated loss of all cytochromes is also observed when yeast cells are transferred from aerobic to anaerobic growth conditions. In this case, however, the loss is complete and appears not to be due to lysosomal degradation, as mutations that alter the stability of iso-1-cytochrome *c* in vitro and its steady state level in vivo also alter its half-life after the shift to anaerobic conditions (Pearce and Sherman 1995c). Another indication that complete complexes may be actively and selectively degraded *in mitochondria* is the loss of cytochrome *c* oxidase in cytochrome *c*-less yeast (Pearce and Sherman 1995a) and *N. crassa* (Bottorff et al. 1994). If cytochrome *c* has no role in the assembly of cytochrome *c* oxidase, one has to conclude that cytochrome *c* protects the complex from proteolytic breakdown. The initial attack by the responsible protease may then be on the 'unprotected' subunit 2, the cytochrome *c*-binding subunit (Hatefi 1985). If this is true, interpretations of gene disruption experiments designed to uncover the role of many 'accessory' subunits of mitochondrial respiratory chain complexes (see Table 1 for references) need to take into account the possibility that these subunits are not involved in complex assembly, but protect an already assembled complex from proteolytic attack (in addition to having possible functional roles).

Mitochondrial proteases

Early attempts to characterize mitochondrial proteases in mammalian cells led to the identification of soluble (Alberti and Bartley 1969; Subramanian et al. 1975) and membrane-bound (Aoki 1978; Haas and Heinrich 1978; Hare 1978) proteases. However, it has been difficult to exclude the contamination of mitochondrial preparations with proteases from lysosomes or mast cell granules (see, for instance, Rubio and Grisolia 1977; Duque-Magalhães 1979; Haas et al. 1979). A major breakthrough was the demonstration of an ATP-dependent protease with a similarity to *E. coli* Lon (or La) in the matrix of mitochondria from rat liver (Desautels and Goldberg 1982a, 1985), counterparts of which were later found in bovine adrenal cortex (Watabe and Kimura 1985a,b) and yeast (Kutejová et al. 1993). In addition, non-ATP-dependent metalloproteases involved in the processing of precursor proteins were identified in mitochondria from rat liver (Conboy et al. 1982; Miura et al. 1982), bovine adrenal cortex (Kumamoto et al. 1986), yeast (McAda and Douglas 1982; Böhni et al. 1983), and *N. crassa* (Hawlitchek et al. 1988). Only in the last few years have genes that encode these and other (putative) mitochondrial proteases been isolated from *S. cerevisiae* and other organisms (Table 2).

As shown in Table 2, mitochondrial proteases can be divided into two groups: proteases involved in processing precursor proteins and proteases involved in degradation. The first group falls outside the scope of this review and will not be considered further. To the second group belong Pim1p(Lon), a matrix-localized protease of which the human gene has also been cloned (Wang et al. 1993; Amerik et al. 1994), and the related proteins Yme1p(Yta11p), Rca1p(Yta12p) and Afg3p(Yta10p) that belong to a subgroup of the AAA family of ATPases (Confalonieri and Duguet 1995) and are associated with the inner membrane (see Table 2 for references). Pim1p is related to *E. coli* Lon/La (Charette et al. 1981; Chung and Goldberg 1981), while Afg3p, Rca1p and Yme1p are all related to *E. coli* FtsH (Tomoyasu et al. 1993a, b).

The members of this group are (putative) ATP-dependent proteases. Such proteases are involved in catalyzing the first step of degradation: cleaving the substrate into peptides that are subsequently degraded by non-ATP-dependent proteases. As ATP hydrolysis is only required for the degradation of large proteins, the energy from ATP hydrolysis probably allows the protease to function in a processive manner (Goldberg 1992; Gottesman and Maurizi 1992) and/or to unfold proteins so as to allow entry of the polypeptide chain into the proteolytic 'core' of the protease (see below).

Are all mitochondrial ATP-dependent proteases multimers?

The formation of multimers is common among ATP-dependent proteases (Rechsteiner et al. 1993). Bacterial Lon is a homotetramer (Chung and Goldberg 1981), while its homologues in mammalian and yeast mitochondria appear to be hexamers (Watabe and Kimura 1985a; Kutejová et al. 1993). Homo-multimerization of FtsH has been shown by Akiyama et al. (1995). There have been several indica-

Table 2 Mitochondrial proteases of *S. cerevisiae* for which the genes have been cloned

Item	Type	*E. coli* homologue	Location	Composition/structure	Proteolytic function	References[a]
Processing peptidases						
MPP	Metallo		Matrix	Heterodimer of protease and protease enhancer	Cleavage of mitochondrial targeting sequence	Jensen and Yaffe 1988; Pollock et al. 1988
MIP	Metallo		Matrix	Monomer	Cleavage of octapeptide of bipartite presequence	Isaya et al. 1994
IMP	Serine	Lep	Inner membrane, facing IMS	Dimer of two homologous subunits with different substrate specificities	Cleavage of N-terminal (sorting) sequence of some inner membrane and IMS proteins	Behrens et al. 1991; Nunnari et al. 1993
ATP-dependent proteases						
Pim1p/Lon	Serine	Lon(La)	Matrix	Homohexamer	Degrades (unfolded) matrix proteins	Van Dyck et al. 1994; Suzuki et al. 1994
Afg3p/Yta10p	Metallo	FtsH	Inner membrane, facing matrix	In a complex with Rca1p/Yta12p	Degrades incomplete and mature mitochondrial translation products	Schnall et al. 1994; Guélin et al. 1994; Tauer et al. 1994
Rca1p/Yta12p	Metallo	FtsH	Inner membrane, facing matrix	In a complex with Afg3p/Yta10p	Degrades incomplete and mature mitochondrial translation products	Schnall et al. 1994; Tzagoloff et al. 1994
Yme1p/Yta11p	Metallo	FtsH	Inner membrane	In a complex	Degrades CoxII	Thorsness et al. 1993; Nakai et al. 1995

[a] Reference is made to the cloning of *S. cerevisiae* genes; see text for references on biochemical characterization of the proteases from yeast and other organisms.
MPP: mitochondrial processing peptidase; MIP: mitochondrial intermediate peptidase; IMP: inner membrane protease; IMS: intermembrane space

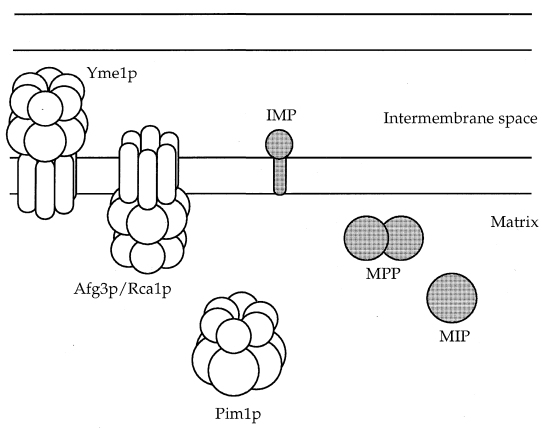

Fig. 1 Schematic picture of proteases identified in yeast mitochondria. Drawn approximately to scale, ATP-dependent proteases involved in protein degradation are in *white* and processing peptidases required for maturation of imported proteins and/or proteins translocated into the intermembrane space are in *grey*. The bipartite structure of the (extra-membrane part of) ATP-dependent proteases reflects the presence in the primary sequence of ATPase and protease domains. The hexameric nature of the Afg3p/Rca1p and Yme1p proteases in the picture is solely to indicate their oligomeric structure; the exact number of subunits present in the complexes is not known. The orientation of the Yme1p complex is controversial. The picture shows an orientation towards the inter-membrane space (Thomas Langer, personal communication). Weber et al. (1996), however, reported an orientation towards the matrix side. See Table 2 and text for details on the different proteases

tions that mitochondrial membrane-bound proteases are also present as homo- or hetero-multimers. Over-expression of *YME1* does not increase the amount of Yme1p protein, presumably because Yme1p which is not associated with other proteins in a complex is unstable (Thorsness et al. 1993). Candidates for Yme1p-associated proteins from genetic screens are the products of *YME2* (Thorsness and Fox 1993), *OSD2* and *OSD3* (Nakai et al. 1995). In addition, homo-multimerization of Yme1p was suggested by intragenic complementation between two *yme1* alleles (Thorsness and Fox 1993). Recently, a high-molecular-weight complex containing Yme1p has been identified, as well as a complex involving both Afg3p and Rca1p (Arlt et al. 1996; Thomas Langer, personal communication). Formation of homo-multimers is also seen with other members of the AAA family (Peters et al. 1990, 1993; Whiteheart et al. 1994; Fröhlich et al. 1995) and may be a common feature of these proteins.

Figure 1 shows the location and (putative) quaternary structure of the mitochondrial proteases the yeast genes of which have been cloned. In this picture, non-ATP-dependent proteases responsible for breaking down oligopeptides to amino acids are strikingly absent. Such proteases may be among proteases reported for the mitochondrial matrix (Beer et al. 1982; Yasuhara and Ohashi 1987; Yasuhara et al. 1994) and inner membrane (Novikova et al. 1981; Zubatov et al. 1984). However, their physiological functions remain to be established. Identification of the

corresponding genes will be an important step towards that end.

Division of labour

The division in the group of ATP-dependent proteases into matrix-localized (Pim1p) and membrane-localized (Afg3p, Rca1p, Yme1p) members parallels a division in substrate proteins. Until now, Pim1p has been implicated in (1) the degradation of imported proteins that fail to fold into a native structure (Wagner et al. 1994) and (2) the turnover of MPPβ and $F_1\beta$, two matrix-localized proteins (Suzuki et al. 1994). Furthermore, the *PIM1* gene is induced four-fold by heat-shock (Van Dyck et al. 1994) and electron-dense material, presumably consisting of protein-aggregates, accumulates in the mitochondrial matrix of *lon*$^-$ (*pim1*) mutants (Suzuki et al. 1994). Targets of the Pim1p counterpart in bovine adrenal cortex include SP-22, a matrix-localized oxygen-radical scavenger (Watabe et al. 1994, 1995).

That degradation of matrix proteins is essential for mitochondrial biogenesis in yeast is apparent from the phenotype lacking the matrix protease. Such strains are respiratory deficient and quickly accumulate deletions in mtDNA ("rho$^-$ induction") (Suzuki et al. 1994; Van Dyck et al. 1994). In *pim1* cells, rho$^-$ induction may be related to over-accumulation of a particular protein(s) or perhaps to a disturbing effect of aggregated mitochondrial proteins. In contrast to Pim1p, Afg3p(Yta10p) is not involved in the degradation of misfolded proteins in the matrix but is necessary for the degradation of incomplete, membrane-bound mitochondrial translation products (Pajic et al. 1994). Recently, it has been shown that Afg3p is also involved in the degradation of most mature mitochondrial translation products destined for the inner membrane, with the notable exception of CoxII (Guélin et al. 1996). The latter appears to be degraded by Yme1p. Mutation of *YME1* has been found to attenuate degradation of CoxII in the absence of CoxIV (Nakai et al. 1995; Weber et al. 1996), and a stabilizing effect of *YME1*-inactivation on both CoxII and CoxIII has been observed in a strain lacking cytochrome *c* (Pearce and Sherman 1995a). The presence of ATP- and zinc-binding motifs typical of metalloproteases in Yme1p, Rca1p and Afg3p supports the notion that these three proteins are indeed responsible for the degradation of mitochondrial translation products destined for the inner membrane, which, as described above, is both ATP- and divalent metal ion-dependent.

The division of labour between soluble and membrane-bound proteases does not completely coincide with the division between soluble and membrane-bound polypeptides. Bovine p450scc and adrenodoxin reductase, both present on the matrix side of the inner membrane, can be degraded by the matrix ATP-dependent protease (Watabe et al. 1993). Possibly, the mitochondrial membrane-bound system is specific for integral membrane proteins. However, although no soluble targets for Afg3p, Rca1p or Yme1p have yet been identified, their existence should not be excluded at this point. The homologue of these proteins in *E. coli*, FtsH, governs the degradation of the soluble proteins λ-cII (Herman et al. 1993), λ-cIII and σ^{32} (Herman et al. 1995; Tomoyasu et al. 1995) in addition to being involved in degrading the integral membrane protein SecY (Kihara et al. 1995). Significantly, in vitro degradation of σ^{32} by purified FtsH is the first direct proof of ATP- and divalent metal-dependent protease activity of a member of the FtsH-subfamily (Tomoyasu et al. 1995).

Yet another protease seems to be responsible for the degradation of the intermembrane space (IMS)-proteins, as neither of the above mentioned ATP-dependent proteases is responsible for degradation of cytochrome *c* (Pearce and Sherman 1995b). Putative candidates are cytochrome *c* hydrolyzing proteases associated with sub-mitochondrial particles (Novikova et al. 1981; Zubatov et al. 1984), an ATP-dependent proteolytic activity in the inter-membrane space (Sitte et al. 1995) and, in mammalian cells, an ATP-dependent protease activity that requires ATP outside the inner membrane (Rapoport et al. 1982). Furthermore, the presence of an additional protease associated with the inner membrane is suggested by the fact that degradation of CoxV in a cytochrome *c*-less mutant is not suppressed by disrupting any of the genes for the currently known ATP-dependent proteases (Pearce and Sherman 1995a). In fact, it is not known at present which protease(s) is(are) responsible for the turnover of nuclear-encoded subunits of mitochondrial inner membrane complexes (except for $F_1\beta$, see above).

Dual roles of ATP-dependent proteases in mitochondrial biogenesis?

As described in the introductory sections, an important function of membrane-bound ATP-dependent proteases is the removal of polypeptides that may adversely influence the structure or function of the membrane. However, the defects arising in mitochondria or bacteria as a result of loss of an ATP-dependent protease may not (only) be caused by the loss of protease activity. Indeed, as will be discussed below, phenotypes associated with mutations of membrane-bound ATP-dependent proteases are sometimes more readily explained by assuming a dual function for these proteins. Independent of the protease activity, the ATPase domain of these proteins may have a chaperone-like activity.

Afg3p and Rca1p

Afg3p/Yta10p and Rca1p/Yta12p were both found to be essential for respiratory growth (Tauer et al. 1994; Tzagoloff et al. 1994) and the assembly of inner membrane complexes (Tzagoloff et al. 1994; Paul and Tzagoloff 1995). As argued above, these phenotypes may simply be explained by the accumulation of polypeptides that are det-

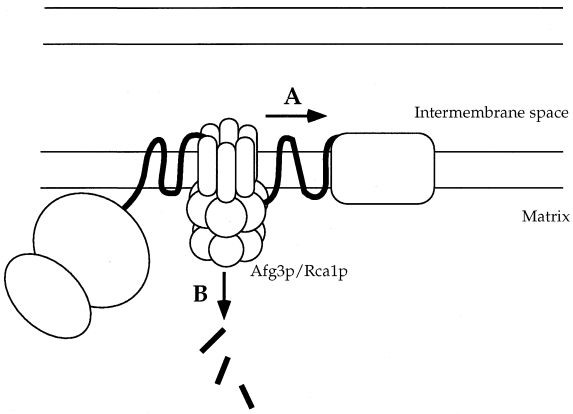

Fig. 2 Quality control of mitochondrial translation products. A complex of Afg3p and Rca1p may function both as a protease and a chaperone (see text). Newly synthesized mitochondrial translation products are either assembled into a complex of the inner membrane (*A*) or broken down (*B*). It is not known if these processes occur co-translationally, as shown in the picture, or only post-translationally, or both. If assembly occurs co-translationally, the Afg3p/Rca1p complex may assist in the attainment of the correct membrane topology by multiple membrane-spanning proteins, as has been suggested for FtsH, the *E. coli* homologue of Afg3p and Rca1p (see text). Degradation of complete mitochondrial translation products in an Afg3p-dependent manner indicates that proteolysis can occur post-translationally (Guélin et al. 1996). Thus, Afg3p/Rca1p may also have a role in the removal of denatured or otherwise damaged proteins from the membrane

rimental to the integrity of the inner membrane. However, inactivation of the proteolytic activity of Afg3p by a point mutation in the protease active site does not affect respiratory growth (Guélin et al. 1996). Complex assembly apparently proceeds normally in this mutant, although degradation of mitochondrial translation products was reduced to a similar extent as in an *AFG3*-deletion mutant. These observations raise the intriguing possibility that at least some ATP-dependent proteases are dual-function proteins, with both chaperone and degradation activities. In one model (Fig. 2), the ATPase domains of Afg3p and Rca1p interact with substrate (membrane proteins) either for degradation by the C-terminal protease domain, or for folding/assembly/integration purposes. Through such a dual function, these ATP-dependent proteases could be part of a quality control system for mitochondrial translation products (Grivell 1995; Nakai et al. 1995).

A similar model may hold for the *E. coli* homologue FtsH, although the question is still unresolved whether this protein is directly involved in the maturation of membrane proteins. Mutations in FtsH affect the membrane translocation of secretory proteins and the topology of membrane proteins in *E. coli* (Tomoyasu et al. 1993b; Akiyama et al. 1994a, b) and cause a different pattern of membrane-associated proteins in *L. lactis* (Nilsson et al. 1994). One possibility is that all these phenotypes result from the stabilization of various host proteins, like the heat-shock transcription factor σ^{32} (Herman et al. 1995). However, some observations are still difficult to explain by such a model. For example, although FtsH is involved in the degradation of unassembled SecY, and the accumulation of SecY inhibits protein translocation (Kihara et al. 1995), the negative effects of *ftsH*$^-$ mutations on the translocation and stop-transfer of trans-membrane segments of the SecY-PhoA fusion proteins are not caused by SecY or SecY-PhoA accumulation (Kihara et al. 1995). Kihara et al. proposed that FtsH is involved in both the degradation of unassembled SecY and its assembly/integration into the membrane. One explanation offered for the effect of FtsH on stop-transfer is that FtsH is necessary for opening of the Sec-channel to allow lateral diffusion of trans-membrane segments (Akiyama et al. 1994a, b). Alternatively, FtsH may bind to the PhoA moiety of the SecY-PhoA fusion protein, a function that could be shared with HtpG, a Hsp90-type chaperone (Shirai et al. 1996).

Yme1p

A combination of chaperone and protease function has also been proposed for Yme1p/Osd1p (Nakai et al. 1995). Mutation of *YME1* yields cells that are respiratory deficient at high temperature (Thorsness et al. 1993) and have reduced activity levels of respiratory chain complexes (Nakai et al. 1995). Moreover, such mutants show additional defects such as intolerance of deletions in mitochondrial DNA (mtDNA) (leading to the absence of mitochondrial translation products), leakiness of mitochondria resulting in mtDNA escaping to the nucleus, and cold-sensitive growth on rich glucose medium. All these phenotypes are thought to result from a morphologically altered inner membrane (Thorsness et al. 1993). Whether this can be fully attributed to the accumulation of membrane proteins that are degraded by Yme1p or indeed involves another function of Yme1p, is still a matter of speculation (Nakai et al. 1995; Weber et al. 1996).

A very interesting but puzzling finding is that an altered form of Ynt1p (Yta2p), a probable regulatory subunit of the 26*s* protease and like Yme1p a member of the AAA family (Confalonieri and Duguet 1995), can suppress all *yme1*-associated phenotypes (Campbell et al. 1994). As it seems unlikely that the 26*s* proteasome, a 1500–2000-kDa protein complex (Fischer et al. 1994), can pass the mitochondrial outer membrane, the altered form of Ynt1p probably acts independently from the proteasome. At least some other AAA-type subunits of the 26*s* proteasome can be part of different subcomplexes and can bind certain proteins as free subunits both in vivo and in vitro (Demartino et al. 1996; Rubin et al. 1996; vom Baur et al. 1996). Significantly, Ynt1p does not have a C-terminal protease domain like Yme1p, suggesting that the Ynt1 mutant protein replaces a non-protease function of Yme1p. However, it can be argued that Ynt1p binds Yme1p substrates and subsequently presents them to the proteasome, or another protease, for degradation.

A chaperone-activity of mitochondrial ATP-dependent proteases is perhaps not so surprising in view of the close functional link between ATP-dependent proteases and chaperones (Squires and Squires 1992; Craig et al. 1994; Horwich 1995). Two *E. coli* proteins, ClpA and ClpX, that

can combine with ClpP subunits to form an ATP-dependent protease (Gottesman and Maurizi 1992; Gottesman et al. 1993), can function independently as chaperones (Wickner et al. 1994; Levchenko et al. 1995; Wawrzynow et al. 1995). Yeast mitochondrial Hsp78p, another member of the Clp-family, can partly substitute for mt-Hsp70 (Schmitt et al. 1995) and/or stabilize mutant forms of the latter (Moczko et al. 1995). Comparison with Clp proteins is significant for two reasons: (1) there is significant homology between members of the Clp-family and the conserved domain of the AAA-family, to which the FtsH subfamily belongs (Dubiel et al. 1992; Gottesman et al. 1993), and (2) there are functional similarities between the *E. coli* Clp system and the eukaryotic 26*s* proteasome, where members of the AAA-family may function like ClpA and ClpX proteases, namely: selection of substrate and presentation to a protease 'core' for degradation (Rechsteiner et al. 1993; Kessel et al. 1995) and/or unfolding of the substrate to allow entry into the interior of the protease (through a 'reverse chaperone' activity) (Peters 1994; Goldberg 1995; Wenzel and Baumeister 1995). A chaperone-like function for two other mitochondrial members of the AAA-family has also been proposed: Bcs1p, involved in the biogenesis of complex III (Nobrega et al. 1992) and Msp1p, an outer-membrane protein whose over-production results in the mislocalization of an outer-membrane reporter protein to the inner membrane (Nakai et al. 1993). Finally, chaperone activity has been proposed for NSF, a AAA-protein involved in vesicle fusion (Morgan and Burgoyne 1995).

In spite of a potential chaperone activity of ATP-dependent proteases, conventional chaperones like Hsp70 or DnaK appear to be necessary for these proteases to degrade at least some of their substrates (Wagner et al. 1994 and references therein). These chaperones may retain the accessibility of denatured proteins by preventing their aggregation. Alternatively, prior binding of a chaperone to a substrate protein may be necessary for recognition by the protease, thus providing an additional layer of control on proteolysis. In yeast mitochondria, proteolysis by Pim1p requires prior binding of the substrate to chaperones Hsp70 and Mdjp (Wagner et al. 1994). Accordingly, degradation of the only mitochondrially encoded non-membrane protein, the small ribosomal subunit protein Var1p, depends on functional Hsp70. This is not true, however, for the membrane protein Atp6p (Herrmann et al. 1994), which is a target of Afg3p (Guélin et al. 1996). In the latter case, either another chaperone is involved or the protease is by itself capable of recognizing its substrate.

The quality control model of mitochondrial translation products (Fig. 2) raises several questions that demand imaginative experimental approaches. One of the main questions is by what mechanism the 'choice' between assembly and degradation is made. Another is what the proposed chaperone function of the FtsH-subfamily proteins is in molecular terms. It must be noted in this respect that while 'assembly' or 'folding' are commonly used when referring to the proposed (second) function of these proteins, ClpA and ClpX promote disaggregation or monomerization (Wickner et al. 1994; Levchenko et al. 1995; Wawrzynow et al. 1995).

Concluding remarks

Our knowledge concerning the turnover of mitochondrial proteins has benefited immensely from the power of the molecular genetics of *S. cerevisiae*. Indeed, the isolation of genes for proteases and the manipulation of individual components is essential to unravel the complicated, entangled processes of assembly, turnover, enzymic activity and membrane physiology. We now know that mitochondria have inherited their ATP-dependent proteases from their endosymbiotic predecessors, as is the case for many other basic components of gene expression. Especially exciting is the recent characterization of a family of ATP-dependent proteases that specifically act on membrane constituents, and the possibility that these are also involved in the building of membrane complexes. Of equal interest is the characterization of the major ATP-dependent protease of the mitochondrial matrix.

The mechanisms of quality control of the inner membrane and its constituents remain to a large extent to be uncovered. One level which is still difficult to access is the higher-order organization of the inner membrane. Are there superstructures of different complexes? Are there sub-regions of the membrane specialized in assembly and/or turnover? With the different factors identified, multiple approaches can be used to resolve these questions.

Looking forward, we can anticipate the gradual uncovering of the division of substrates among the different proteases, and the identification of new proteases and of complexes with protease and/or chaperone activities. With the current acceleration of yeast research due to the recent release of the sequence of the complete yeast genome, new strides forward are expected in the near future. Our understanding of the underlying causes of clinically important phenomena, like the apparent high turnover rates of mitochondrial protein in tumor cells (Luciaková and Kuzela 1992) and the intra-mitochondrial degradation failure of subunit *c* of F_1F_0-ATPase in Batten disease (Ezaki et al. 1995), should increase concomitantly.

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