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Protein unfolding in the cell

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Protein unfolding is an important step in several cellular processes such as protein degradation by ATP-dependent proteases and protein translocation across some membranes. Recent studies have shown that the mechanisms of protein unfolding *in vivo* differ from those of the spontaneous unfolding *in vitro* measured by solvent denaturation. Proteases and translocases pull at a substrate polypeptide chain and thereby catalyze unraveling by changing the unfolding pathway of that protein. The unfoldases move along the polypeptide chains of their protein substrates. The resistance of a protein to unfolding is then determined by the stability of the region of its structure that is first encountered by the unfoldase. Because unfolding is a necessary step in protein degradation and translocation, the susceptibility of a substrate protein to unfolding contributes to the specificity of these pathways.

Most proteins have well-defined three-dimensional structures that determine their activities in the cell; however, several cellular processes require proteins to unfold from their stable native conformations. Two examples are protein translocation across some membranes and protein degradation by ATP-dependent proteases. Both processes require the polypeptide chains to translocate through narrow channels, and the diameters of these channels are too small to accommodate folded proteins. In the past few years, it has become clear that the translocation and degradation machineries actively induce the denaturation of substrates and this unfolding activity requires energy in the form of ATP or a membrane potential.

In this review, we discuss the unfolding mechanisms used by ATP-dependent proteases and the mitochondrial import machinery. These two well-studied systems form the framework of our current understanding of unfolding in the cell.

Protein degradation by ATP-dependent proteases

ATP-dependent proteases degrade regulatory proteins and thereby control processes such as the cell cycle, gene transcription and signal transduction. In addition, these proteases degrade misfolded or damaged polypeptides and produce many of the antigenic peptides that are displayed at the cell surface for an immune response [1,2]. In eukaryotes, this degradation activity is provided mainly by the proteasome [1]. In prokaryotes and eukaryotic organelles, similar functions are performed by analogs of the proteasome such as the ClpAP, ClpXP, HslUV, Lon and FtsH proteases [2]. The proteasome is highly selective for its substrates, despite the fact that the active sites of proteolysis themselves show very little sequence specificity. Discrimination is achieved by sequestering the proteolytic sites within the structure of these proteases and by tightly controlling access to these sites.

The proteasome is a large multimeric cylindrical complex that consists of two parts: a central core particle that forms the proteolytic chamber, and a regulatory particle that flanks either end of the core (Figure 1) [3,4]. The proteolytic sites in the central core are accessible only through a narrow channel that runs along the long axes of the particle [5]. The width of the degradation channel is 10–15 Å at its narrowest point, which ensures that even small folded proteins cannot enter the catalytic chamber nonspecifically. The regulatory particles bind to the catalytic core at the entrance of the degradation channel and control access to the proteolytic sites [6]. The regulatory particle is composed of ~17 subunits (six of which are ATPases) and is involved in substrate recognition [7] and unfolding [8], gating of the degradation channel [9] and translocation of the unfolded substrate to the proteolytic sites [10]. Prokaryotic ATP-dependent proteases show little, if any, sequence similarity to the eukaryotic proteasome outside the ATP-binding site but their overall structure resembles that of the proteasome (Figure 1).

Substrate proteins are specifically targeted to the proteasome, most commonly via the covalent attachment of several ubiquitin moieties to lysine residues in the substrate [11]. This modification mediates the association of substrates with the protease [12]. Some proteins are targeted for degradation through adaptors that bind both the substrate and the protease simultaneously [13]. A few proteasome substrates are recognized by the protease directly through specific targeting signals that are encoded in the primary sequence of the protein [14]. The latter two recognition mechanisms are similar to the way in which most prokaryotic substrates bind their proteases [2].

Initiation of degradation

Although covalent modification with ubiquitin enables a substrate protein to bind to the proteasome, effective degradation requires the presence of a second signal in the substrate: namely, an unstructured region that acts as the initiation site for degradation [15]. Degradation begins with proteolysis of this initiation site and is followed by sequential hydrolysis of the rest of the protein (Figure 2). The presence of an unstructured region is required for the degradation of tightly folded proteins, and presumably this

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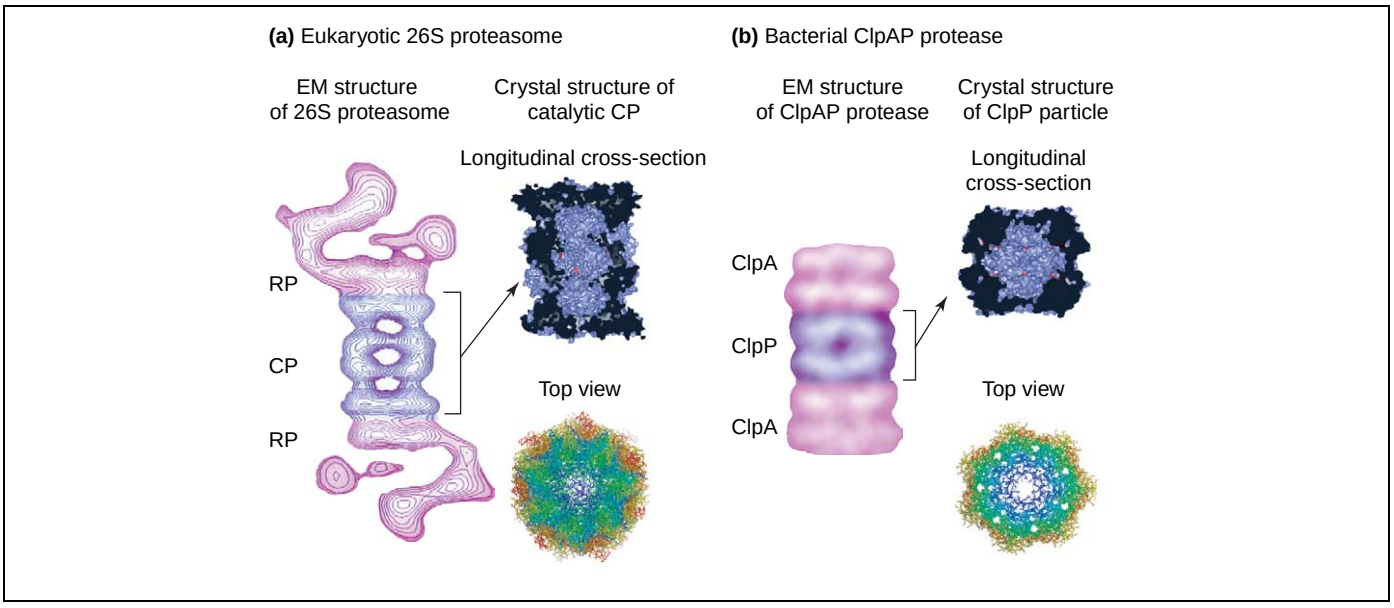

Figure 1. Structures of the eukaryotic proteasome and its bacterial analog ClpAP. **(a)** Electron microscopy (EM) structure of the 26S proteasome from *Xenopus laevis* and **(b)** ClpAP protease from *Escherichia coli*. The protease subunits comprising the 'core particle' (CP) are shown in blue, the ATPase subunits comprising the 'regulatory particle' (RP) are shown in pink. A side-on cross-section of the crystal structure of the proteasome CP from *Saccharomyces cerevisiae* [Protein Data Bank (PDB) accession code 1RYP; (a)] and of the proteolytic core of ClpAP [PDB 1TYF; (b)] reveals the active site of proteolysis (red dots) in the catalytic chamber and the degradation channel that connects the active site to the exterior of the protease. End-on views of the yeast proteasome CP [top view in (a)] and ClpP [top view in (b)] of *E. Coli* show their sevenfold axis of symmetry. Note that the axial pores of the yeast proteasome [top view in (a)] are in a closed conformation, whereas the ClpP axial pores [top view in (b)] are in an open conformation. Individual subunits of the proteolytic particle are color-coded in shades of the visible light spectrum from the N terminus in blue to the C terminus in red. The EM reconstructions of the proteasome and ClpAP (a,b) are reproduced, with permission, from Ref. [5]. Structures of the cross-sections and top views (a,b) were produced by PyMOL [77].

region functions to engage the unfolding machinery of the proteasome [15]. The initiation site can be either located next to the ubiquitination site or separated from it by a folded domain, but both sites must be close to each other in space.

This mechanism of initiation is similar to the way in which some prokaryotic ATP-dependent proteases engage their substrates through adaptor proteins such as UmuD or SspB [16,17]. For example, the ClpXP protease binds its substrate UmuD′ indirectly by using UmuD as an adaptor and then engages the substrate itself through a defined initiation site [17].

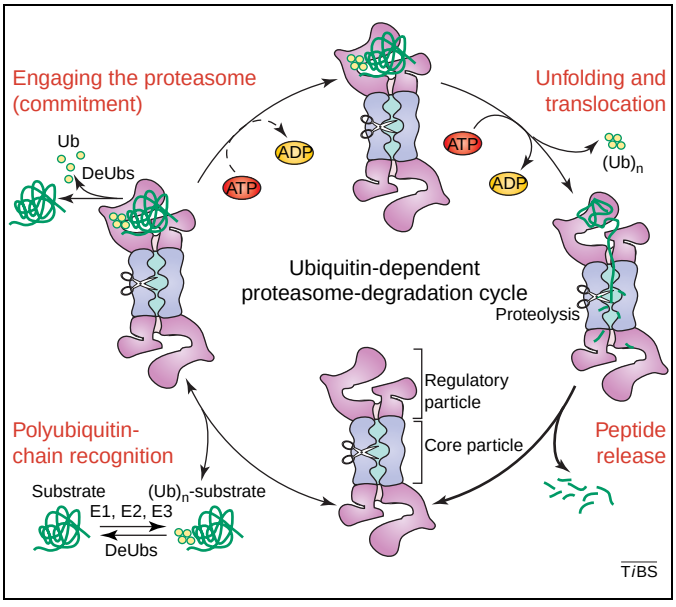

Figure 2. The degradation cycle of the proteasome. Polyubiquitinated (Ub_n) proteins bind to the proteasome through the ubiquitin chain (bottom left). Unfolding and degradation (top right) ensue only after the substrate has engaged the proteasome through an unstructured domain or the degradation initiation site (top left). Once the substrate is engaged, it is degraded sequentially along the polypeptide chain from its degradation initiation site (bottom right). Abbreviation: DeUbs, deubiquitinases.

Unfolding by ATP-dependent proteases

Native proteins must be in an unfolded conformation during degradation, and stabilizing a substrate protein against unfolding can prevent its proteolysis [18,19]. ATP-dependent proteases can accelerate the denaturation of their substrates. It has been shown, for example, that ClpAP can unfold green fluorescent protein at a rate that is six orders of magnitude faster than the rate at which the protein would unfold spontaneously [20]. Subsequent studies have found that the proteasome, ClpXP, Lon and the archebacterial proteasome also actively unfold their substrates [8,19,21–23].

The proteasome catalyzes the unfolding of its substrates by sequentially unraveling them from their degradation initiation sites [19]. In a multidomain protein, the domain that is closest to the degradation initiation site is degraded first, whereas domains located further away are degraded subsequently (Figure 3) [15,19]. Stabilizing a domain close to the initiation site protects downstream domains from degradation but not vice versa [15,19]. Experiments using fluorescence resonance energy transfer have shown that the bacterial protease ClpAP also translocates its substrate to its proteolytic site in a sequential manner [24]. ClpXP and FtsH have also been shown to degrade their substrates sequentially along the polypeptide chain [19,25].

A consequence of this sequential unraveling mechanism is that the susceptibility of a protein to unfolding by

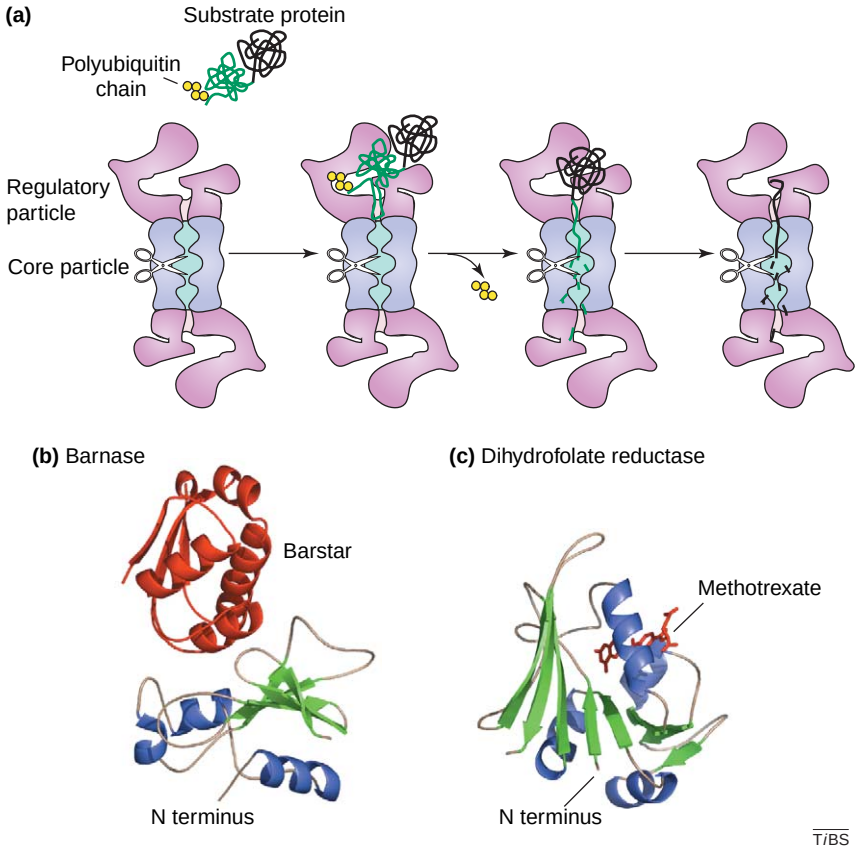

Figure 3. Sequential proteolysis by the proteasome. **(a)** The substrate-linked polyubiquitin chain mediates the interaction between the substrate and the proteasome. Degradation starts once the unstructured degradation initiation site of the substrate is engaged by the proteasome. The polypeptide chain is translocated sequentially into the protease beginning at the initiation site. As a result, the domain close to the initiation site is unfolded and degraded first, and then the downstream domains are degraded. **(b,c)** Two substrate proteins that show that susceptibility to unfolding by the proteasome is determined by the stability of the local structure adjacent to the degradation initiation site. Shown are the structures of barnase (b) and dihydrofolate reductase (c), with their respective tightly binding ligands barstar and methotrexate in red. Barnase is easily unraveled by the proteasome from its N-terminal structure even when it is stabilized against spontaneous unfolding by barstar binding. Methotrexate-stabilized dihydrofolate reductase is not unfolded or degraded from its N-terminal β-strand by the proteasome. Panels (b,c) were produced by PyMOL [77].

the protease is determined by the stability of its local structure adjacent to the degradation initiation site, rather than the stability of its whole structure to global unfolding [15,19,26]. Proteins are readily unfolded when their structure next to the initiation site forms an α-helix or irregular loop on the surface of the protein, even when the proteins are greatly stabilized (Figure 3). Proteins are more difficult to unravel when the degradation initiation site is followed by buried β-strand [19] (Figure 3).

The observation that the local structure of the protein adjacent to the degradation initiation site influences its susceptibility to denaturation by proteases suggests that the protein unravels from its point of attachment to the protease [15]. Unfolding of proteins *in vitro* by chemical denaturants, such as urea, does not follow such a pathway. ATP-dependent proteases therefore seem to catalyze unfolding by changing the unfolding pathway in the same way that mitochondria unravel precursor proteins (see later) and that many enzymes catalyze chemical reactions by changing the reaction mechanism. Equivalent observations have been made for ClpAP, ClpXP and FtsH, suggesting that all ATP-dependent proteases function by a similar mechanism [19,25]. However, the proteases differ in the strengths of their unfolding machine [25,26].

In a plausible mechanism, the ATP-dependent protease traps local unfolding fluctuations next to the initiation site and translocates the trapped part of the polypeptide into the protease particle. Because protein unfolding is a highly cooperative process, once the structure next to the initiation site is unfolded, the rest of the protein unfolds rapidly. During degradation, unfolding occurs together with translocation of the polypeptide chain into the degradation channel, which suggests that the ATP-dependent protease might catalyze unfolding by pulling at the polypeptide chain.

In this model, translocation and unfolding are part of the same overall process. Indeed, susceptibility of proteins to unfolding by proteases correlates more closely with their susceptibility to mechanical unfolding by physical pulling, as measured by atomic force microscopy (AFM) experiments, than with their susceptibility to unfolding by chemical and heat denaturation [19,26]. Despite this similarity, the unfolding produced in AFM experiments and the unfolding mediated by ATP-dependent proteases show clear differences. In AFM experiments, a protein is unfolded by stretching it from both ends of the polypeptide chain, whereas the protease might pull its substrate from one end towards the entrance to the degradation channel.

Assistance of the regulatory particle

Unfolding of native substrates by the proteasome requires hydrolysis of ATP by the regulatory particle [27]. The active sites of ATP hydrolysis are located in the base of the regulatory particle. The base is composed of six subunits, each containing a conserved AAA ATP-binding motif [27]. There are no high-resolution structures of the ATPase subunits in the proteasome, but these data are available for HslU, which is the regulatory subunit of prokaryotic protease HslUV [28].

HslU is a member of AAA+ ATPases and has a characteristic fold of a large α/β amino (N) domain, followed by a small α-helical carboxyl (C) domain [28]. In the hexamer, the nucleotide-binding pocket is located at the subunit–subunit interface and residues in adjacent monomers interact with the nucleotide [28]. ATP binding, ATP hydrolysis, and ADP and inorganic phosphate release induce conformational changes in the subunits, which are presumably translated into concerted movements in the ATPase hexamer and the associated protease [29]. These conformational changes might generate the mechanical force that pulls the bound polypeptide towards the translocation pore. In this way, the regulatory subunit would also assist translocation of the polypeptide chain into the degradation chamber of the associated protease [30].

Energy requirements

ATP hydrolysis powers the unfolding of protein substrates and their translocation from the substrate-binding site to the proteolytic chamber. Analysis of ATP hydrolysis by the bacterial protease ClpXP has shown that the protease can hydrolyze ATP at different rates depending on its interaction with the substrate [26]. The protease hydrolyzes ATP at a high rate when it is fully engaged in the degradation of an unfolded substrate, and the polypeptide is translocated rapidly.

During the degradation of folded substrates, ATP is burnt at a slower rate. The protease undergoes several cycles of hydrolysis at the slow rate until the substrate unravels. In this manner, the protease seems to unfold its substrates by repeatedly tugging at the polypeptide chain [26]. The total amount of ATP consumed in the process is considerable. For unfolded substrates approximately one molecule of ATP is hydrolyzed per amino acid, and for folded substrates it can be an order of magnitude more [26].

Specificity of degradation

Most proteins are targeted to the proteasome via their covalent modification with ubiquitin molecules. The requirement for the unfolding of a substrate protein adds several levels of complexity at which degradation can be regulated. Some proteins, such as the cell division activator Cdc34 and the transcription factor Met4, are appropriately ubiquitinated but not degraded by the proteasome [31,32]. Similarly, the adaptor proteins Rad23 and Dsk2 bind both the proteasome and the substrate but avoid degradation themselves [33,34].

A possible explanation for this lack of degradation is that the unstructured potential proteasome initiation sites in these proteins are located too far away from the ubiquitination sites or the proteasome interaction domains to function as effective degradation initiation sites [15]. A second explanation has been proposed for the degradation of the cell division inhibitor Sic1 [35]. This protein can be ubiquitinated at any of its 20 lysine residues that are distributed throughout the protein; however, only ubiquitination near its N terminus leads to rapid degradation of the protein [35]. It is possible that the folded domain of Sic1 is easier to unravel from its N terminus; if so, then only the N-terminal ubiquitination sites would orient the substrate appropriately. A third explanation is that the effectiveness of the degradation initiation site might depend on the amino acid composition of this site.

Although most of the substrates are degraded completely into small peptides by the proteasome, there are a few surprising exceptions to this rule. Some proteins are ubiquitinated and targeted to the proteasome, but are degraded only partially. Partial degradation or processing is used as a mean to regulate the transcription factors NF-κB precursor protein p105, Cubitus interruptus, Spt23 and Mga2 [36–38]. This partial processing is best understood for activation of a subunit of the transcription factor NF-κB. This subunit is synthesized as a larger precursor protein, which is then processed by the proteasome to yield a smaller active fragment [36]. The partial degradation is dependent on the stability of a folded domain in the protein, because destabilizing this domain leads to complete degradation of the full-length protein and prevents accumulation of the active fragment [19].

In addition to the stability of the folded domain, partial degradation of the NF-κB subunit also requires the presence of a second component in the processing signal – a glycine-rich region [39]. The effect of the glycine-rich region on the proteasome is not completely clear. The glycine-rich region is similar to the glycine-alanine repeat sequences found in the Epstein–Barr viral protein EBNA-1. The glycine-alanine repeat has been shown to interrupt proteasomal degradation [40,41]. It is possible that the glycine-rich region in the NF-κB subunit attenuates the unfolding activity of the proteasome by weakening the association between the protease and the substrate.

In summary, protein degradation by ATP-dependent proteases requires unfolding of the substrate protein. Susceptibility to unfolding is determined by the structure and stability of the substrate protein. By making proteolysis dependent on properties of the folded domains in the substrate, the cell has gained additional ways of regulating the specificity of the degradation process.

Protein translocation across membranes

The eukaryotic cytoplasm is divided into several functionally distinct compartments or organelles that contain unique sets of proteins. Most proteins found in these organelles are synthesized in the cytosol and subsequently imported into their appropriate compartments. In some cases, such as protein import into mitochondria, endoplasmic reticulum and perhaps chloroplasts, proteins are transported across the intracellular membranes into these compartments through narrow translocation channels. The size of these channels is such that native proteins do

not to fit through them; therefore, proteins must be in an unfolded conformation during translocation [42]. Nevertheless, some proteins fold before import and these proteins must unravel to pass through the channels. Protein unfolding during translocation is best understood for protein import into mitochondria, which, surprisingly, resembles the unfolding that occurs during protein degradation.

Protein import into the mitochondrial matrix

Most proteins of the mitochondrial matrix are synthesized in the cytosol as precursor proteins containing positively charged N-terminal targeting signals. The mitochondrial import machinery contains translocases or 'TOM proteins' in the outer mitochondrial membrane, translocases or 'TIM proteins' in the inner mitochondrial membrane, and additional components in the matrix (Figure 4) [43]. Complexes of TIM and TOM proteins associate to form a contiguous translocation channel that connects the cytosol to the mitochondrial matrix. The channel in the outer membrane is rigid and has an internal diameter of 22–26 Å [44–46]. The inner membrane channel seems to be flexible, but its maximum diameter is smaller than that of the outer membrane channel [46,47].

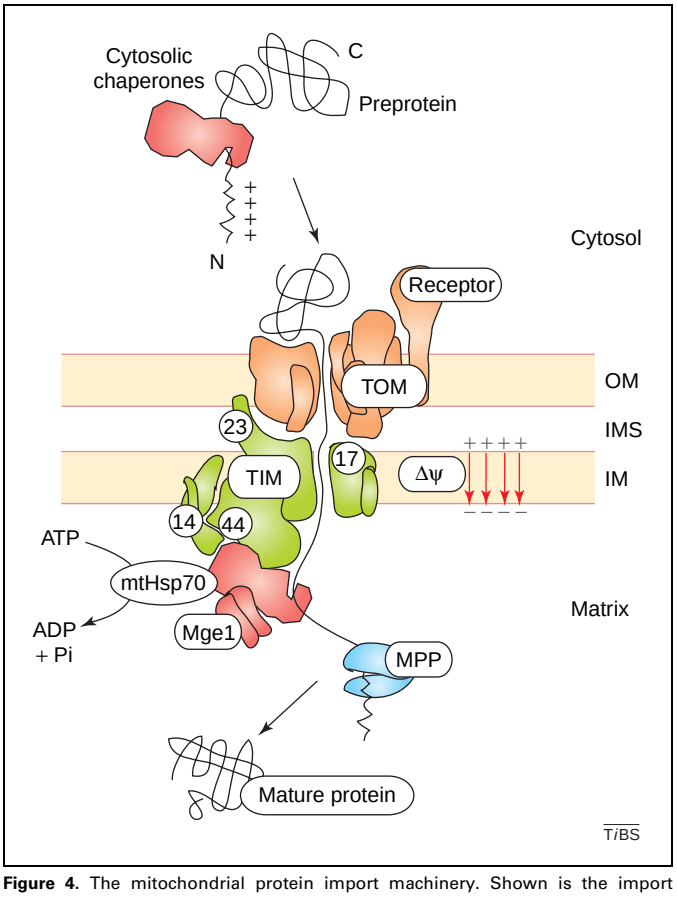

Figure 4. The mitochondrial protein import machinery. Shown is the import pathway of a matrix protein. The preprotein is synthesized in the cytosol with an N-terminal positively charged presequence. Cytosolic chaperones can bind to the preprotein. The translocase of the outer mitochondrial membrane (TOM) contains receptors that recognize the presequence and a general import pore that mediates translocation across the outer membrane (OM). The translocase of the inner membrane (TIM) includes an import channel formed by Tim23 and Tim17 and the peripheral subunit Tim44. The membrane potential ($\Delta\psi$) across the inner membrane (IM) drives translocation of the presequence. Matrix Hsp70 (mtHsp70) binds the preprotein in transit and, together with Tim14, the co-chaperone Mge1 and possibly Tim44, forms an ATP-dependent import motor. The presequence is cleaved off by the mitochondrial processing peptidase (MPP). Abbreviation: IMS, intermembrane space. Reproduced, with permission, from Ref. [67] (www.nature.com).

There has been some debate concerning whether proteins fold before their translocation into mitochondria. Much evidence suggests that they do. Eukaryotic proteins are generally found to fold cotranslationally [48]. In addition, *in vivo* studies have shown that two specific precursor proteins are present in a folded conformation before translocation [49,50]. Furthermore, *in vitro* experiments on isolated mitochondria have shown that these organelles can unfold and import native precursor proteins [42,51,52]. However, cytosolic chaperones and ATP located outside the mitochondria are also required for protein import [53,54]. The chaperones might function to retain proteins that cannot fold in the cytosol, such as mitochondrial membrane proteins and subunits of larger macromolecular complexes, in a soluble state.

Mitochondria catalyze precursor unfolding

Mitochondria can induce precursor proteins to unfold at a rate that is many hundred times faster than the rate at which these proteins would unfold spontaneously in free solution [55]. Unfolding during import has been studied in detail using a model precursor protein comprising the ribonuclease barnase as the mature domain and an authentic N-terminal mitochondrial targeting signal. Site-directed mutagenesis experiments on barnase have elucidated the unfolding pathway both in free solution and during import. The two unfolding pathways differ from each other substantially [56,57]. Spontaneous denaturation of barnase in free solution begins with the concerted unfolding of a large portion of the structure [56] (Figure 5). During mitochondrial import, by contrast, only the N-terminal part of the structure adjacent to the targeting signal is unfolded at first (Figure 5). This unraveling is followed by the cooperative collapse of the rest of the protein [57]. Mitochondria therefore unfold substrates by unraveling them from the targeting signal and changing the unfolding pathway.

It seems that mitochondria can unfold some structures more easily than others. For example, they can efficiently unravel barnase even when the protein is greatly stabilized against spontaneous unfolding [57]. By contrast, mitochondrial unfolding of dihydrofolate reductase, which is less stable than barnase, is completely blocked when this protein is stabilized against spontaneous unfolding by ligand binding [42,57]. It seems that the susceptibility of a protein to unfolding during import is determined primarily by the stability of the local structure adjacent to the presequence and not by the stability of a precursor protein to global unfolding [57]. Thus, the same rules govern both protein unfolding during degradation and protein unfolding during mitochondrial import.

Partners in unfolding

The mechanism by which mitochondria induce the unfolding of precursors is better understood than the mechanism by which ATP-dependent proteases unravel their substrates. During import, most mitochondrial

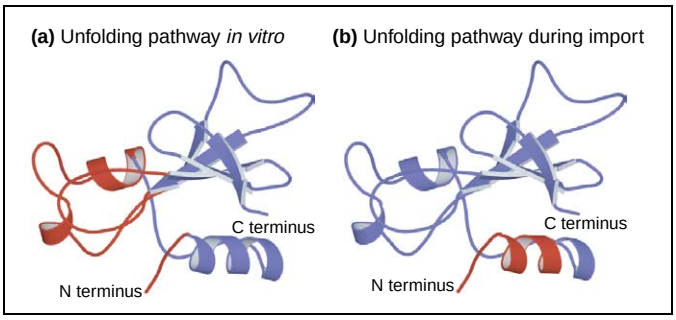

Figure 5. Unfolding pathways of barnase. Shown is the structure of barnase, color-coded according to the order in which structure is lost during spontaneous global unfolding *in vitro* (left) and during import into mitochondria (right). The parts shown in red unfold early, whereas those shown in blue unfold late. Reproduced, with permission, from Ref. [57] (www.nature.com).

proteins are unfolded by the electrical potential across the mitochondrial inner membrane acting directly on positive charges in the targeting sequences [58,59]. The orientation of electrical potential is maintained such that it is positive towards the outside of the membrane and negative towards the mitochondrial matrix. A positively charged targeting signal in this field will therefore experience a force directed towards the matrix. This pulling force will be transmitted through the polypeptide chain to the folded domain at the entrance to the import channel where it unravels. Unfolding and import are inhibited by reducing the electrical potential or by removing the positive charges on the presequence in the membrane [59]. In addition to its role in protein unfolding, the electrical potential is important for insertion of the presequence into the import channel and for dimerization of a protein component of the import channel [58,60].

Protein import into the mitochondrial matrix also requires the function of the mitochondrial Hsp70 homolog mtHsp70, which binds both to the exit of the import channel in the matrix and to the translocating precursor proteins [61–63] (Figure 4). The co-chaperones TIM14 in the inner membrane and Mge1 in the matrix accelerate the ATPase activity of mtHsp70 and regulate its ability to bind and to release the polypeptide chain [43,64]. mtHsp70 can trap an incoming polypeptide chain in the matrix and prevent its retrograde movement. The precursor protein can be thus unfolded by harvesting the spontaneous unfolding fluctuations in the protein. In addition, the ATPase cycle induces conformational changes in mtHsp70 that could be used to generate a power stroke to pull the bound precursor polypeptide chain physically into the matrix [65], thereby denaturing folded precursors as they are forced through the entrance of the translocation channel (reviewed in Refs [66–68]).

Precursor proteins can be unfolded by mtHsp70 if they have a targeting sequence that is long enough (at least 60 amino acids) to reach across both mitochondrial membranes to connect mtHsp70 to the folded protein at the surface of the mitochondria. However, the position of targeting sequence cleavage sites in most authentic precursor proteins indicates that most precursors might be too short to span the double membrane and to engage mtHsp70 before unfolding. Therefore, it seems that the unfolding of most precursors must be catalyzed primarily by the electrical potential. Nevertheless, mtHsp70 is required for the import of both folded and unfolded proteins because it assists the translocation of precursor proteins through the import channel into the matrix and might be required to unfold C-terminal structures.

In summary, the import of precursor proteins begins when the membrane potential unfolds their mature domains by pulling at positive charges in the presequence. Once the polypeptide reaches the inside of the matrix, mtHsp70 takes over, translocating the remainder of the polypeptide chain into the matrix.

Protein sorting

Sorting proteins into their correct subcellular compartments is crucially important for the survival of a cell. The specificity of protein import into the mitochondrial matrix is largely conferred by N- or C-terminal targeting sequences [69,70]. It seems, however, that not all sorting information is encoded in these signals. For example, when random peptides were attached to the N terminus of cytochrome oxidase subunit IV, roughly a quarter of the resulting fusion proteins were imported into mitochondria [71]. Stably folded proteins are imported only inefficiently when an authentic targeting signal is attached to their N termini [72–74]. Their import can be improved by attaching targeting signals that engage the unfolding machinery more effectively, such as targeting signals that are more positively charged or longer [55,59]. Thus, effective import into mitochondria requires a correct combination of the targeting signal and the folded domain in the precursor protein, indicating that sorting information is located both in the targeting signal and in the mature domain.

This bipartite nature of the sorting signal might explain the molecular mechanism of a subset of cases of the inherited disease primary hyperoxaluria type 1. In this disease, the enzyme alanine glyoxylate aminotransferase (AGT) is mistargeted from peroxisomes to mitochondria. The mistargeting is caused by two mutations in the protein. The first mutation is located near the N terminus of the protein and seems to activate a mitochondrial targeting signal in the protein [75]. By itself, however, this mutation does not cause AGT import into mitochondria [76]. Efficient import requires the presence of a second mutation located away from the N-terminal mitochondrial signal. The function of the second mutation might be to destabilize AGT by dissociating the homomeric AGT dimer [76]. The two mutations therefore enhance mitochondrial import by affecting both components of the two-part sorting signal: namely, the targeting signal and the mature domain.

Concluding remarks

Protein unfolding is an essential step in several processes in the cell, such as protein translocation across membranes and protein degradation by ATP-dependent proteases. The mechanisms of unfolding during translocation and degradation resemble each other but differ from that of spontaneous global unfolding. Unfolding occurs concurrently with translocation of the polypeptide chain, and it seems that unraveling is induced by pulling at the

polypeptide chain. During unfolding in the cell, the susceptibility of a protein to unraveling does not depend on its stability to global unfolding but instead depends on the stability of the local structure that is adjacent to the targeting signal. The susceptibility of a protein to unfolding contributes to the specificity of both the translocation and degradation pathways. In this manner, the cell has achieved additional ways in which to regulate these important processes. Future work will determine how widespread these regulatory mechanisms are in the cell.

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References

1 Pickart, C.M. and Cohen, R.E. (2004) Proteasomes and their kin: proteases in the machine age. *Nat. Rev. Mol. Cell Biol.* 5, 177–187
2 Gottesman, S. (2003) Proteolysis in bacterial regulatory circuits. *Annu. Rev. Cell Dev. Biol.* 19, 565–587
3 Groll, M. *et al*. (1997) Structure of 20S proteasome from yeast at 2.4 Å resolution. *Nature* 386, 463–471
4 Baumeister, W. *et al*. (1998) The proteasome: paradigm of a self-compartmentalizing protease. *Cell* 92, 367–380
5 Larsen, C.N. and Finley, D. (1997) Protein translocation channels in the proteasome and other proteases. *Cell* 91, 431–434
6 Horwich, A.L. *et al*. (1999) Chaperone rings in protein folding and degradation. *Proc. Natl. Acad. Sci. U. S. A.* 96, 11033–11040
7 Verma, R. *et al*. (2004) Multiubiquitin chain receptors define a layer of substrate selectivity in the ubiquitin–proteasome system. *Cell* 118, 99–110
8 Braun, B.C. *et al*. (1999) The base of the proteasome regulatory particle exhibits chaperone-like activity. *Nat. Cell Biol.* 1, 221–226
9 Kohler, A. *et al*. (2001) The axial channel of the proteasome core particle is gated by the Rpt2 ATPase and controls both substrate entry and product release. *Mol. Cell* 7, 1143–1152
10 Benaroudj, N. *et al*. (2003) ATP hydrolysis by the proteasome regulatory complex PAN serves multiple functions in protein degradation. *Mol. Cell* 11, 69–78
11 Pickart, C.M. (2001) Mechanisms underlying ubiquitination. *Annu. Rev. Biochem.* 70, 503–533
12 Thrower, J.S. *et al*. (2000) Recognition of the polyubiquitin proteolytic signal. *EMBO J.* 19, 94–102
13 Hartmann-Petersen, R. *et al*. (2003) Transferring substrates to the 26S proteasome. *Trends Biochem. Sci.* 28, 26–31
14 Orlowski, M. and Wilk, S. (2003) Ubiquitin-independent proteolytic functions of the proteasome. *Arch. Biochem. Biophys.* 415, 1–5
15 Prakash, S. *et al*. (2004) An unstructured initiation site is required for efficient proteasome-mediated degradation. *Nat. Struct. Mol. Biol.* 11, 830–237
16 Levchenko, I. *et al*. (2000) A specificity-enhancing factor for the ClpXP degradation machine. *Science* 289, 2354–2356
17 Neher, S.B. *et al*. (2003) Distinct peptide signals in the UmuD and UmuD′ subunits of UmuD/D′ mediate tethering and substrate processing by the ClpXP protease. *Proc. Natl. Acad. Sci. U. S. A.* 100, 13219–13224
18 Johnston, J.A. *et al*. (1995) Methotrexate inhibits proteolysis of dihydrofolate reductase by the N-end rule pathway. *J. Biol. Chem.* 270, 8172–8178
19 Lee, C. *et al*. (2001) ATP-dependent proteases degrade their substrates by processively unraveling them from the degradation signal. *Mol. Cell* 7, 627–637
20 Weber-Ban, E.U. *et al*. (1999) Global unfolding of a substrate protein by the Hsp100 chaperone ClpA. *Nature* 401, 90–93
21 Kim, Y. *et al*. (2000) Dynamics of substrate denaturation and translocation by the ClpXP degradation machine. *Mol. Cell* 5, 639–648
22 Van Melderen, L. *et al*. (1996) ATP-dependent degradation of CcdA by Lon protease. Effects of secondary structure and heterologous subunit interactions. *J. Biol. Chem.* 271, 27730–27738
23 Navon, A. and Goldberg, A.L. (2001) Proteins are unfolded on the surface of the ATPase ring before transport into the proteasome. *Mol. Cell* 8, 1339–1349
24 Reid, B.G. *et al*. (2001) ClpA mediates directional translocation of the substrate proteins into the ClpP protease. *Proc. Natl. Acad. Sci. U. S. A.* 98, 3768–3772
25 Herman, C. *et al*. (2003) Lack of a robust unfoldase activity confers a unique level of substrate specificity to the universal AAA protease FtsH. *Mol. Cell* 11, 659–669
26 Kenniston, J.A. *et al*. (2003) Linkage between ATP consumption and mechanical unfolding during the protein processing reactions of an AAA^+ degradation machine. *Cell* 114, 511–520
27 Glickman, M.H. *et al*. (1998) A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3. *Cell* 94, 615–623
28 Sousa, M.C. *et al*. (2000) Crystal and solution structures of an HslUV protease–chaperone complex. *Cell* 103, 633–643
29 Wang, J. *et al*. (2001) Nucleotide-dependent conformational changes in a protease-associated ATPase HslU. *Structure* 9, 1107–1116
30 Sauer, R.T. *et al*. Sculpting the proteome with AAA+ proteases and desassembly machines. *Cell* (in press)
31 Verma, R. *et al*. (2002) Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome. *Science* 298, 611–615
32 Flick, K. *et al*. (2004) Proteolysis-independent regulation of the transcription factor Met4 by a single Lys48-linked ubiquitin chain. *Nat. Cell Biol.* 6, 634–641
33 Rao, H. and Sastry, A. (2002) Recognition of specific ubiquitin conjugates is important for the proteolytic functions of the ubiquitin-associated domain proteins Dsk2 and Rad23. *J. Biol. Chem.* 277, 11691–11695
34 Kleijnen, M.F. *et al*. (2003) The ubiquitin-associated domain of hPLIC-2 interacts with the proteasome. *Mol. Biol. Cell* 14, 3868–3875
35 Petroski, M.D. and Deshaies, R.J. (2003) Context of multiubiquitin chain attachment influences the rate of Sic1 degradation. *Mol. Cell* 11, 1435–1444
36 Palombella, V.J. *et al*. (1994) The ubiquitin-proteasome pathway is required for processing the NF-κB1 precursor protein and the activation of NF-κB. *Cell* 78, 773–785
37 Jiang, J. and Struhl, G. (1998) Regulation of the Hedgehog and Wingless signalling pathways by the F- box/WD40-repeat protein Slimb. *Nature* 391, 493–496
38 Hoppe, T. *et al*. (2000) Activation of a membrane-bound transcription factor by regulated ubiquitin/proteasome-dependent processing. *Cell* 102, 577–586
39 Lin, L. and Ghosh, S. (1996) A glycine-rich region in NF-κB p105 functions as a processing signal for the generation of the p50 subunit. *Mol. Cell Biol.* 16, 2248–2254
40 Levitskaya, J. *et al*. (1997) Inhibition of ubiquitin/proteasome-dependent protein degradation by the Gly-Ala repeat domain of the Epstein–Barr virus nuclear antigen 1. *Proc. Natl. Acad. Sci. U. S. A.* 94, 12616–12621
41 Zhang, M. and Coffino, P. (2004) Repeat sequence of Epstein–Barr virus-encoded nuclear antigen 1 protein interrupts proteasome substrate processing. *J. Biol. Chem.* 279, 8635–8641
42 Eilers, M. and Schatz, G. (1986) Binding of a specific ligand inhibits import of a purified precursor protein into mitochondria. *Nature* 322, 228–232
43 Rehling, P. *et al*. (2004) Mitochondrial import and the twin-pore translocase. *Nat. Rev. Mol. Cell Biol.* 5, 519–530
44 Kunkele, K.P. *et al*. (1998) The preprotein translocation channel of the outer membrane of mitochondria. *Cell* 93, 1009–1019
45 Hill, K. *et al*. (1998) Tom40 forms the hydrophilic channel of the mitochondrial import pore for preproteins. *Nature* 395, 516–521
46 Schwartz, M.P. and Matouschek, A. (1999) The dimensions of the protein import channels in the outer and inner mitochondrial membranes. *Proc. Natl. Acad. Sci. U. S. A.* 96, 13086–13090
47 Truscott, K.N. *et al*. (2001) A presequence- and voltage-sensitive channel of the mitochondrial preprotein translocase formed by Tim23. *Nat. Struct. Biol.* 8, 1074–1082

48 Netzer, W. and Hartl, F. (1997) Recombination of protein domains facilitated by co-translational folding in eukaryotes. *Nature* 388, 343–349
49 Bomer, U. *et al*. (1997) The sorting route of cytochrome b_2 branches from the general mitochondrial import pathway at the preprotein translocase of the inner membrane. *J. Biol. Chem.* 272, 30439–30446
50 Wienhues, U. *et al*. (1991) Protein folding causes an arrest of preprotein translocation into mitochondria *in vivo*. *J. Cell Biol.* 115, 1601–1609
51 Vestweber, D. and Schatz, G. (1988) A chimeric mitochondrial precursor protein with internal disulfide bridges blocks import of authentic precursors into mitochondria and allows quantitation of import sites. *J. Cell Biol.* 107, 2037–2043
52 Schwartz, M.P. *et al*. (1999) The structure of precursor proteins during import into mitochondria. *J. Biol. Chem.* 274, 12759–12764
53 Mihara, K. and Omura, T. (1996) Cytoplasmic chaperones in precursor targeting to mitochondria: the role of MSF and hsp70. *Trends Cell Biol.* 6, 104–108
54 Wachter, C. *et al*. (1994) Protein import into mitochondria: the requirement for external ATP is precursor-specific whereas intramitochondrial ATP is universally needed for translocation into the matrix. *Mol. Biol. Cell* 5, 465–474
55 Matouschek, A. *et al*. (1997) Active unfolding of precursor proteins during mitochondrial protein import. *EMBO J.* 16, 6727–6736
56 Matouschek, A. *et al*. (1989) Mapping the transition state and pathway of protein folding by protein engineering. *Nature* 340, 122–126
57 Huang, S. *et al*. (1999) Mitochondria unfold precursor proteins by unraveling them from their N-termini. *Nat. Struct. Biol.* 6, 1132–1138
58 Martin, J. *et al*. (1991) Role of an energized inner membrane in mitochondrial protein import. $\Delta\Psi$ drives the movement of presequences. *J. Biol. Chem.* 266, 18051–18057
59 Huang, S. *et al*. (2002) Protein unfolding by the mitochondrial membrane potential. *Nat. Struct. Biol.* 9, 301–307
60 Bauer, M.F. *et al*. (1996) Role of Tim23 as voltage sensor and presequence receptor in protein import into mitochondria. *Cell* 87, 33–41
61 Kang, P-L. *et al*. (1990) Requirement for hsp70 in the mitochondrial matrix for translocation and folding of precursor proteins. *Nature* 348, 137–143
62 Schneider, H-C. *et al*. (1994) Mitochondrial Hsp70/MIM44 complex facilitates protein import. *Nature* 371, 768–774
63 Rudiger, S. *et al*. (1997) Interaction of Hsp70 chaperones with substrates. *Nat. Struct. Biol.* 4, 342–349
64 Mayer, M.P. (2004) Timing the catch. *Nat. Struct. Mol. Biol.* 11, 6–8
65 Voisine, C. *et al*. (1999) The protein import motor of mitochondria: unfolding and trapping of preproteins are distinct and separable functions of matrix Hsp70. *Cell* 97, 565–574
66 Neupert, W. (1997) Protein import into mitochondria. *Annu. Rev. Biochem.* 66, 863–917
67 Matouschek, A. *et al*. (2000) Protein unfolding by mitochondria. The Hsp70 import motor. *EMBO Rep.* 1, 404–410
68 Pfanner, N. and Geissler, A. (2001) Versatility of the mitochondrial protein import machinery. *Nat. Rev. Mol. Cell Biol.* 2, 339–349
69 Horwich, A.L. *et al*. (1985) A leader peptide is sufficient to direct mitochondrial import of a chimeric protein. *EMBO J.* 4, 1129–1135
70 Hurt, E.C. *et al*. (1984) The amino-terminal region of an imported mitochondrial precursor polypeptide can direct cytoplasmic dihydrofolate reductase into the mitochondrial matrix. *EMBO J.* 3, 3149–3156
71 Lemire, B.D. *et al*. (1989) The mitochondrial targeting function of randomly generated peptide sequences correlates with predicted helical amphiphilicity. *J. Biol. Chem.* 264, 20206–20215
72 Van Steeg, H. *et al*. (1986) Targeting efficiency of a mitochondrial pre-sequence is dependent on the passenger protein. *EMBO J.* 5, 3643–3650
73 Verner, K. and Lemire, B.D. (1989) Tight folding of a passenger protein can interfere with the targeting function of a mitochondrial presequence. *EMBO J.* 8, 1491–1495
74 Vestweber, D. and Schatz, G. (1988) Point mutations destabilizing a precursor protein enhance its post-translational import into mitochondria. *EMBO J.* 7, 1147–1151
75 Purdue, P.E. *et al*. (1991) Mistargeting of peroxisomal L-alanine:glyoxylate aminotransferase to mitochondria in primary hyperoxaluria patients depends upon activation of a cryptic mitochondrial targeting sequence by a point mutation. *Proc. Natl. Acad. Sci. U. S. A.* 88, 10900–10904
76 Leiper, J.M. *et al*. (1996) Inhibition of alanine:glyoxylate aminotransferase 1 dimerization is a prerequisite for its peroxisome-to-mitochondrion mistargeting in primary hyperoxaluria type 1. *J. Cell Biol.* 135, 939–951
77 DeLano, W.L. (2002) *The PyMOL Molecular Graphics System.* DeLano Scientific (http://www.pymol.org)