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Protein unfolding — an important process *in vivo*?

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Protein unfolding is an important step in several cellular processes, most interestingly protein degradation by ATP-dependent proteases and protein translocation across some membranes. Unfolding can be catalyzed when the unfoldases change the unfolding pathway of substrate proteins by pulling at their polypeptide chains. The resistance of a protein to unraveling during these processes is not determined by the protein's stability against global unfolding, as measured by temperature or solvent denaturation *in vitro*. Instead, resistance to unfolding is determined by the local structure that the unfoldase encounters first as it follows the substrate's polypeptide chain from the targeting signal. As unfolding is a necessary step in protein degradation and translocation, the susceptibility to unfolding of substrate proteins contributes to the specificity of these important cellular processes.

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Abbreviations

BPTI	bovine pancreatic trypsin inhibitor
DHFR	dihydrofolate reductase
ER	endoplasmic reticulum
WASP	Wiskott–Aldrich syndrome protein

Introduction

Most proteins fold into well-defined three-dimensional structures to be active. However, unfolding plays a critically important role in at least three biological processes: protein translocation across some membranes; protein degradation by ATP-dependent proteases; and the passive elasticity of striated muscle (Figure 1). The mechanisms of unfolding in these three processes resemble each other much more closely than they resemble protein global unfolding induced by temperature or solvents such as urea. In all three cases, it appears that unfolding is induced by the cellular machinery pulling at the polypeptide chain to unravel the native domains. In this review, I will describe the experimental strategies that led to these conclusions. I will also discuss how protein unfolding appears to play an important role in conferring specificity to protein translocation and degradation in the cell.

Protein translocation

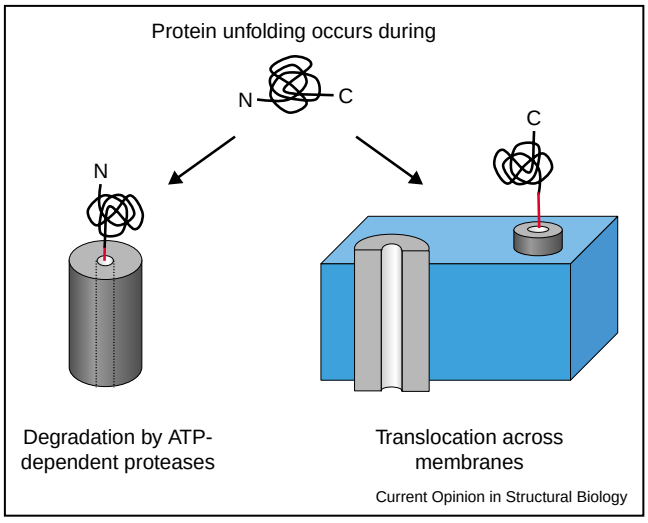
Membranes subdivide the cytoplasm of eukaryotic cells into compartments with well-defined protein compositions. Many of the proteins found in the various compartments are synthesized in the cytosol and then transported to their different destinations. Protein import into mitochondria, chloroplasts, peroxisomes and the endoplasmic reticulum (ER) occurs through membrane channels, which are lined by proteins. For some of these compartments, proteins can only translocate through the import channels in an unfolded conformation, presumably because of the size of the pores in the import channels. I will begin by discussing protein import into mitochondria, specifically protein transport into the mitochondrial matrix, because this system is well characterized and the role of protein unfolding is clear.

Mitochondria

Mitochondria are surrounded by two membranes (Figure 2). Proteins targeted to the innermost compartment, the matrix, are synthesized in the cytosol as precursors with N-terminal targeting sequences. These precursors have to travel to the matrix via two membrane channels. The channel in the outer membrane has a diameter of 20–26 Å and is rigid [1–3]. The channel across the inner membrane is flexible, but its maximum diameter is smaller than that of the outer membrane channel [3–5]. The steric constraints imposed by the import channels require even small proteins to be in an unfolded conformation to pass through the channels. Import of dihydrofolate reductase (DHFR) is blocked when the enzyme is stabilized by binding the high-affinity ligand methotrexate [6] and bovine pancreatic trypsin inhibitor (BPTI) can only be imported into the matrix when its disulfide bridges are reduced [7]. Translocating proteins appear to be in a fully unfolded conformation normally because even a single disulfide bridge introduced into an importing precursor inhibits import to a small but significant extent [4].

The next question is whether proteins fold in the cytosol before translocation. Proteins can be imported into mitochondria post-translationally, both *in vivo* and *in vitro* [8,9], but, traditionally, it has been assumed that precursor proteins remain unstructured until they reach the matrix, probably for three reasons. First, there was no precedent for a cellular unfolding activity and the simplest explanation for import was that precursor proteins never folded. Second, protein import requires chaperones and ATP

Figure 1

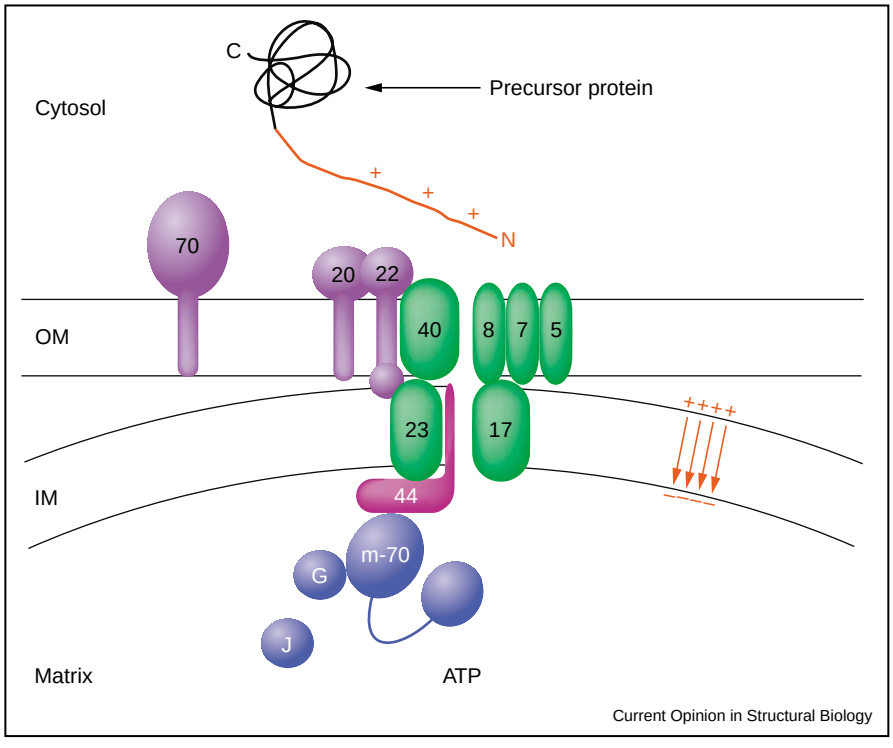


Protein unfolding occurs during translocation across some membranes and during degradation by ATP-dependent proteases (the targeting signal is shown in red).

outside mitochondria, which were assumed to keep proteins in unfolded conformations [10,11]. Third, evidence from work on one of the bacterial protein export systems showed that the bacterial export sequence destabilizes proteins before translocation [12]. However, over the past five years, a consensus has slowly developed that mitochondria can actively unfold proteins, although differences on the proposed mechanism of unfolding remain [13]. Therefore, it is necessary to revisit the question whether proteins fold before translocation.

The folding state of precursors before import has been analyzed directly *in vivo* and in both cases the proteins were found to be in the native conformation [14,15]. In the first study, DHFR targeted to mitochondria was expressed in yeast cells. Import of the DHFR precursor was blocked when a substrate analog was introduced into the cytosol [14]. In the second study, *in vivo* import of the heme-binding domain of cytochrome b_2 was blocked when the unfolding activity in the mitochondrial inner membrane was disengaged [15]. It appears likely that many other precursor proteins will fold before import. Generally speaking, protein folding in the eukaryotic

Figure 2



The mitochondrial protein import machinery. Proteins in the outer/inner membrane are called Tom/Tim, followed by the number indicated in the figure. The number reflects their approximate molecular weight. During import, precursor proteins first interact with the Tom20 and Tom22 receptors through their targeting sequence. The Tom70 receptor binds precursors associated with cytosolic chaperones. Targeting sequences insert into the Tom40 channel and pass through the Tom23 complex into the matrix. Import into the matrix always requires an electrical potential across the inner membrane and the ATP-dependent action of mtHsp70. mtHsp70 is found bound to the import machinery through Tim44 and free in the matrix. Precursors begin to interact with mtHsp70 while they are still associated with the import channels. G, J: mGrpE, Mdj1 — two co-chaperones of Hsp70; IM/OM: inner/outer membrane; m-70: mtHsp70.

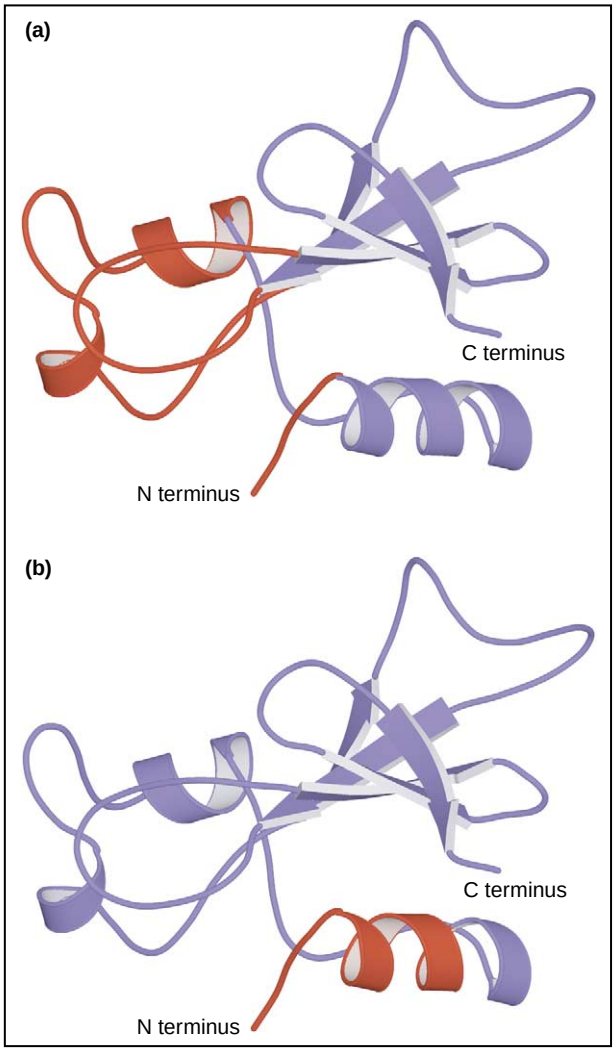
cytosol appears to occur soon after translation. In eukaryotic cells, N-terminal domains of nascent proteins fold before synthesis of the C-terminal domains is complete [16,17]. Other than the presence of the targeting sequence, there is no obvious difference between a cytosolic protein and a mitochondrial precursor protein. Indeed, there are examples of cytosolic and mitochondrial proteins with identical amino acid sequences outside the targeting sequence. In the three proteins analyzed, the mitochondrial targeting sequences do not affect the structure or folding of their passenger proteins [18–20]. Thus, it appears likely that many precursor proteins will fold soon after translation.

If mitochondrial precursors fold in the cytosol, why are chaperones required for the import of some proteins? The involvement of cytosolic chaperones in import has been studied primarily using cell-free assays. Precursors are synthesized by *in vitro* transcription and translation in a reticulocyte lysate, which contains the factors necessary for efficient protein import into mitochondria [11]. When a variety of authentic and engineered precursors were examined, it was found a subset requires extramitochondrial ATP for matrix import [10], presumably for the action of cytosolic chaperones [11]. The precursors that required external ATP were either membrane proteins or subunits of multimeric protein complexes [10]. Thus, cytosolic chaperones probably facilitate import of precursors that are unable to fold in the cytosol and therefore are prone to aggregation. Also, it is well established that mitochondria can import chemically pure folded precursor proteins (e.g. [6,21]). In conclusion, mitochondria are presented with folded precursor proteins and therefore must somehow unfold these proteins during import.

Catalyzing precursor unfolding

Proteins are in an unfolded conformation during translocation, but mitochondria can import folded proteins (e.g. [6,21]). More importantly, mitochondria can import folded proteins many hundred times faster than these proteins unfold spontaneously, which reveals a mitochondrial unfolding activity [22]. Indeed, it was found that mitochondria can catalyze unfolding by changing the unfolding pathway of a model precursor protein, just like enzymes catalyze some chemical reactions by changing the reaction pathway [18]. The model protein consisted of the ribonuclease barnase with a series of targeting sequences attached to its N terminus. The unfolding pathway of barnase during import into yeast mitochondria differs substantially from the pathway of spontaneous global unfolding observed in free solution. During import, the model protein is unraveled from its N terminus. Once the process has been initiated at the N terminus, the rest of the precursor protein denatures rapidly [18]. By contrast, spontaneous unfolding of barnase in free solution begins as a global process, with a large part of the structure opening up during the first steps [23] (Figure 3).

Figure 3



Unfolding pathways of barnase [18]. Sketches of the structure of barnase, color coded according to the order in which structure is lost **(a)** during spontaneous global unfolding *in vitro* and **(b)** during import into mitochondria. The parts of the structure shown in red unfold early, whereas those shown in blue unfold late. Figure reproduced from [18] with permission.

How do mitochondria unravel precursor proteins? The mitochondrial surface itself does not seem to catalyze unfolding [24], although it is able to trap unfolded protein in the denatured state [25]. Instead, precursor proteins that are inserted into the import machinery appear to engage the unfolding machinery when the targeting sequence is long enough to reach the inner mitochondrial membrane [18,22,26,27•]. Two factors can induce unfolding, mtHsp70, a homolog of the chaperone Hsp70 found in the mitochondrial matrix [8,13], and the electrical potential across the inner membrane [27•]. However, many precursors are not able to interact with mtHsp70 before import because their targeting sequences are too

short to reach across the two mitochondrial membranes (Figure 2). The shortest precursor found to be able to interact with mtHsp70 before its mature domain unfolds at the mitochondrial surface has a 52 amino acid targeting sequence [26]. It is difficult to determine the length of the unstructured N-terminal regions of mitochondrial precursor proteins. A review of all the yeast proteins listed as mitochondrial in the yeast proteome database (YPD) shows that the average length of the N-terminal part of precursor proteins preceding the processing site is 31 amino acids (standard deviation 18 amino acids). Therefore, most targeting sequences are probably too short to allow a native precursor to engage mtHsp70.

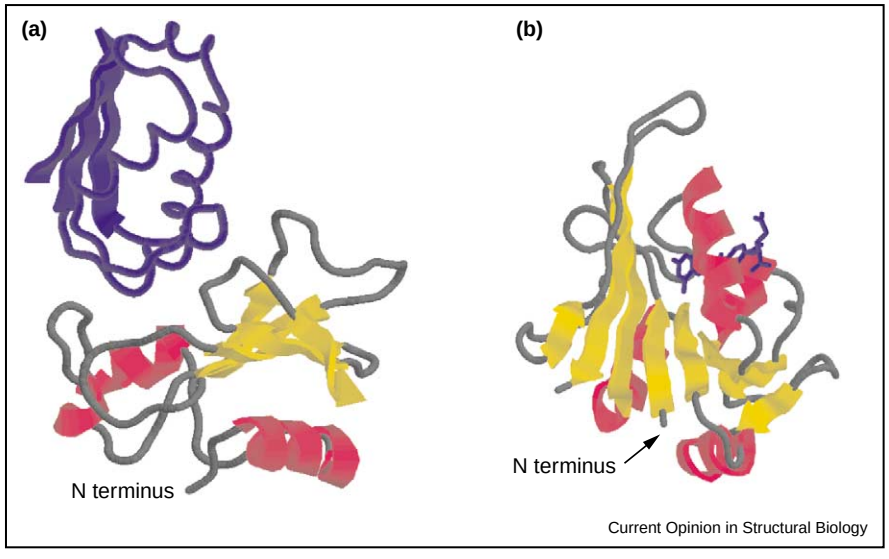
The electrical potential across the inner membrane is able to act on precursors before they reach mtHsp70 because of its physical location (Figure 2). Respiring yeast mitochondria maintain an electrical potential of approximately 150 mV across the inner membrane, which is positive at the outer (intermembrane space) surface and negative at the inner (matrix) surface of the membrane. Because mitochondrial matrix targeting sequences are positively charged, a targeting sequence in the import channel should be pulled towards the matrix. Indeed, experiments demonstrated that passenger proteins are unfolded at the mitochondrial surface when the electrical potential acts directly on positively charged amino acid sidechains of the targeting sequence [27•]. The electrical potential has two functions in addition to protein unfolding. It supports the insertion of the targeting sequences into the import channels [27•,28,29] and it maintains the appropriate dimerization state of a component of the inner membrane import channel [30].

What is the function of mtHsp70? mtHsp70 interacts directly with translocating precursors [31,32] and is required for the matrix import of all proteins, whether they contain folded domains or not [10,31,33]. In addition, mtHsp70 is directly involved in the unfolding of precursors with very long targeting sequences, as demonstrated by the identification of a specific mutation in mtHsp70 that affects the import only of precursors that contain a folded domain [34,35]. Thus, mtHsp70 unfolds precursors with very long targeting sequences and C-terminal domains in multidomain precursors. Most other matrix-bound precursors are probably unfolded by the electrical potential [27•]. In addition, all precursors may require mtHsp70 to overcome friction between translocating proteins and the import channel [36].

How effective is the unfolding activity?

Barnase precursors are efficiently unfolded by mitochondria, even when greatly stabilized by tightly binding ligands [18]. There appears to be no maximum stability that cannot be overcome by the import machinery. By contrast, import of DHFR precursors can be blocked when the protein is stabilized by ligand binding [6,18,37]. Thus, although mitochondria can catalyze the unfolding of DHFR precursors [22], DHFR can be too stable to be unfolded by mitochondria [18]. This observation is at first surprising because barnase is considerably more stable than DHFR against unfolding *in vitro* and binds its ligand with similar affinity. However, the difference can be rationalized by a comparison of the structures of these proteins (Figure 4). The first 12 amino acids of barnase's globular domain form an α helix at the surface of the structure. The protein collapses when the mito-

Figure 4



Sketches of the structures of **(a)** barnase and **(b)** DHFR, with their tightly binding ligands barstar and methotrexate, respectively, shown in purple.

chondria peel the N-terminal α helix off the surface of the structure by pulling at the targeting sequence [18,27•]. In the case of DHFR, the N-terminal stretch of the polypeptide chain forms an internal β strand in a β sheet that is sandwiched between two layers of α helices (open sheet α/β fold, which is the most common protein fold [38]). Presumably, the buried region cannot be unraveled until the protein unfolds globally to release its N terminus. Authentic mitochondrial proteins with both types of structural features exist. Cytochrome b_2 and citrate synthase resemble barnase in that their compact domains begin with α helices. As predicted, cytochrome b_2 is efficiently unfolded by mitochondria when stabilized by its heme cofactor [39,40]. By contrast, the folds of mtHsp70 and aldehyde dehydrogenase are more similar to DHFR. In conclusion, the susceptibility of proteins to being unfolded by mitochondria depends not only on the proteins' stability against global spontaneous unfolding, but primarily on their local structure adjacent to the targeting signal [18,41•].

Protein sorting

Clearly, the fidelity of protein sorting is critically important for cellular survival. For protein import into the mitochondrial matrix, specificity is, to a large extent, conferred by the N-terminal targeting sequences [42,43]. However, it appears that not all the sorting information is encoded in these N-terminal extensions. When a collection of random peptides was attached to the N terminus of subunit IV of cytochrome oxidase (COX IV), approximately a quarter of these constructs were imported into mitochondria [44]. In addition, there have been some reports that the mature domain affects import efficiency [45,46]. Indeed, when proteins of different stabilities and folds are attached to the same targeting sequence, the import efficiencies of these constructs vary considerably, with precursors that are more difficult to unfold importing less well [18,22,47,48]. Together, these results suggest that the susceptibility of the mature domain of precursor proteins to unfolding contributes to the specificity of protein import into mitochondria. As the length [18,22] and charge [27•] of the targeting sequence determine how well a precursor protein can engage the unfolding machinery, effective sorting to the mitochondrial matrix requires that the targeting sequence and mature domain are properly matched to each other. In other words, sorting information is located in both the targeting sequence and the mature domain.

Protein transport into other compartments

Protein unfolding may also play a role in protein import into chloroplasts and the ER. Chloroplasts are surrounded by two membranes and are divided into two compartments, the stroma and the thylakoids. Protein import into chloroplasts is also thought to occur post-translationally and, following the reasoning above, presumably some precursors will fold before translocation. However, it is less clear whether proteins must always unfold during import. It appears that the small protein BPTI can translocate across the envelope with intact disulfide bridges [49], whereas the somewhat larger DHFR unfolds during translocation [50,51]. Similarly, large enzymes or protein complexes can block translocation across the envelope when they are stabilized against unfolding [52–54]. Translocation of proteins from the stroma into the thylakoids occurs through two different translocation machineries, which seem to impose different steric requirements on their substrates. The ΔpH-dependent pathway tolerates folded proteins [49], whereas the Sec-related pathway is blocked when proteins are prevented from unfolding [55].

Most protein translocation into the ER occurs co-translationally and therefore most preproteins do not fold before translocation. However, some translocation into the ER does occur post-translationally, especially in yeast, and it is possible that some of those precursor proteins will fold in the cytosol [56]. The internal diameter of the import channel into the ER has been estimated to be 20–40 Å at its narrowest point [57–59]. Some experimental results suggest that proteins have to be in an unfolded conformation to fit through the translocation channel [60]. Thus, for proteins imported into the ER post-translationally, unfolding at the entrance of the translocation channel may occur.

One striking similarity between the mitochondrial, chloroplast and ER import systems is the presence of homologs of the chaperone Hsp70 at the lumenal side of the translocation channel [61]. Thus, protein unfolding during import into chloroplasts and during post-translational import into the ER may play a similar role as during import into mitochondria.

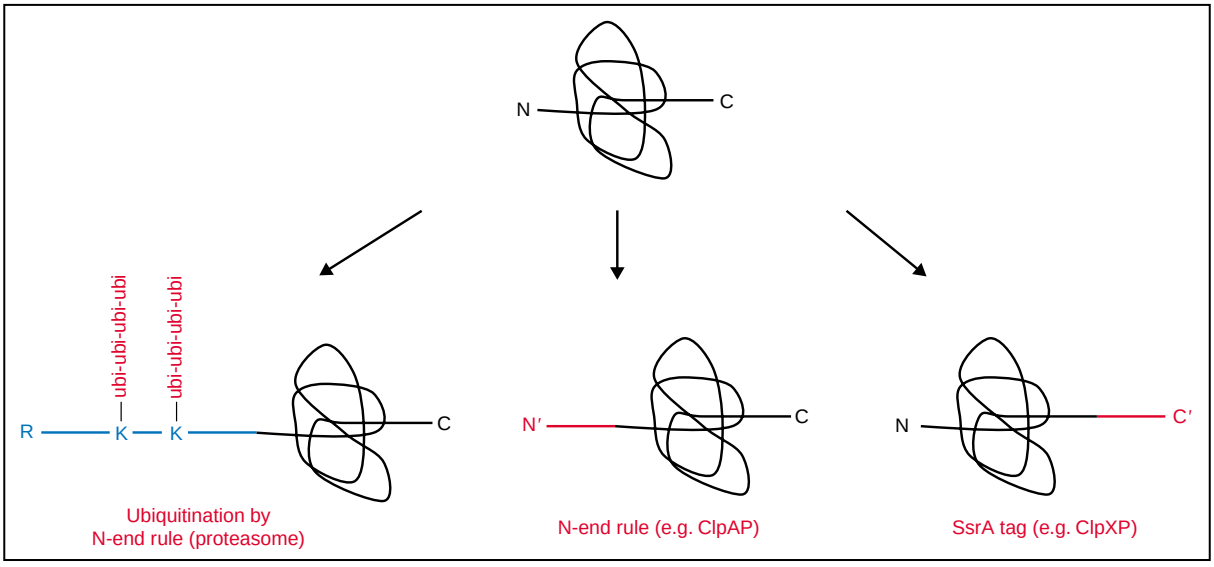
Protein degradation by ATP-dependent proteases

Protein degradation by ATP-dependent proteases at first seems to be entirely unrelated to protein translocation. However, it turns out that the mechanisms of protein unfolding in each process are surprisingly similar.

Protein degradation by ATP-dependent proteases is a critical step in the control of many cellular processes [62]. In eukaryotic cells, the most important of these proteases is the proteasome, which is located in the cytosol and nucleus. The proteasome is responsible for the degradation of short-lived regulatory proteins and abnormal polypeptides, and for the production of peptides for antigen presentation [63]. Degradation by the proteasome generally involves two consecutive steps: targeting the substrate to the proteasome by the attachment of multiple ubiquitin moieties to lysine residues (Figure 5) and degradation of the modified protein by the protease [64].

The substrate specificity of the proteasome is tightly controlled by the sequestration of the proteolytic active

Figure 5



Protein targeting to ATP-dependent proteases. Most substrates are targeted for degradation by the proteasome through the covalent attachment of multiple ubiquitin molecules to lysine residues in the substrate. The first ubiquitin molecule is attached through an isopeptide bond between the C-terminal carboxyl of ubiquitin and the ϵ amino group of the lysine residue. Many substrates of ATP-dependent proteases of prokaryotic origin are targeted for degradation by N- or C-terminal degradation signals. For example, the 11 amino acid SsrA tag is attached to the C terminus of proteins if their translation is prematurely arrested on a damaged mRNA. The modified proteins can be recognized by ClpAP and ClpXP. Another well-characterized targeting mechanism is the bacterial N-end rule pathway. Here, the identity of the first amino acid of a protein in the context of an unstructured N terminus determines the life-time of the protein by targeting it to ClpAP. Different N-terminal residues have different targeting efficiencies.

site deeply within the structure of the protease [63] (Figure 6). The proteasome is a large cylindrical particle formed by a central proteolytic core with regulatory caps at either end. The active sites of ATP hydrolysis are located in the caps. The proteolytic sites are in the central core and are accessible only through a narrow channel that runs along the long axis of the particle. In the isolated yeast core particle, the entrance to the degradation channel is blocked by the N termini of the α subunits at the small ends of the cylindrical particle. The channel opens when the regulatory caps bind to the core [65•]. At its narrowest point, the opened degradation channel is approximately 13 Å wide [65•,66•]. This constriction is too small for folded proteins to fit through it and substrate proteins must unfold to gain access to the proteolytic sites [67]. Indeed, stabilizing a substrate protein against unfolding can protect it from degradation [41•,68].

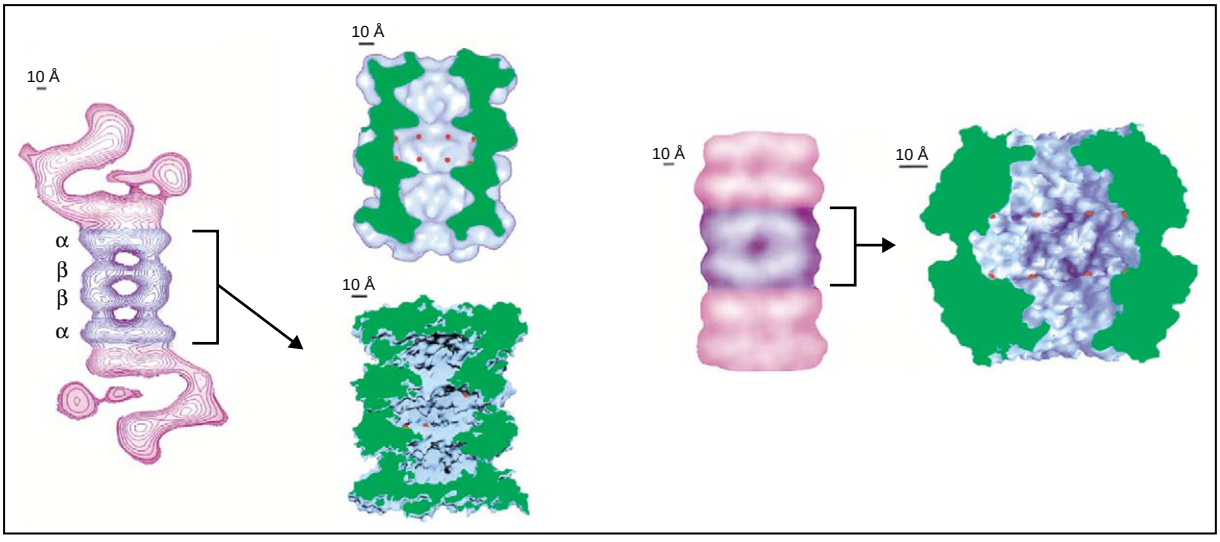
Other ATP-dependent proteases, such as the Lon, ClpXP, ClpAP and HslUV proteases, fulfill functions in organelles and prokaryotes that are similar to those of the proteasome (Figure 6). Although these proteases show no strong sequence homology to the proteasome, their overall architectures resemble each other [69,70]. These proteases also form large cylindrical particles that sequester their active sites of proteolysis in a central cavity. Access to the cavity is controlled by ATPase subunits at the entrance to a narrow degradation channel. Substrate proteins are often targeted to the protease by N- or C-terminal sequences, similar in principle to the targeting sequences found in the translocation systems discussed above (Figure 5). Again, the dimensions of the degradation channel require substrate proteins to be in an unfolded conformation to be degraded [69].

Mechanism of unfolding

As one of the functions of ATP-dependent proteases is the destruction of active regulatory proteins, many of the protease substrates are folded before degradation. An unfolding activity was first demonstrated for ClpAP [71] and it is now clear that the proteasome, ClpXP and an archaebacterial proteasome homolog are also able to unfold proteins [41•,72,73•–77•]. Studies with model proteins revealed that these proteases induce unfolding by unraveling their substrates from their targeting signals, just as mitochondria unfold importing precursor proteins [41•].

The mechanism of protein unfolding by the proteasome was analyzed in experiments that are similar to those described above for mitochondrial import [41•]. Barnase and DHFR were targeted to the proteasome and the Clp proteases, and efficiently degraded *in vitro*. When DHFR is stabilized by ligands, its degradation is blocked; however, stabilizing barnase does not affect its degradation. Following the same reasoning as above for mitochondrial

Figure 6

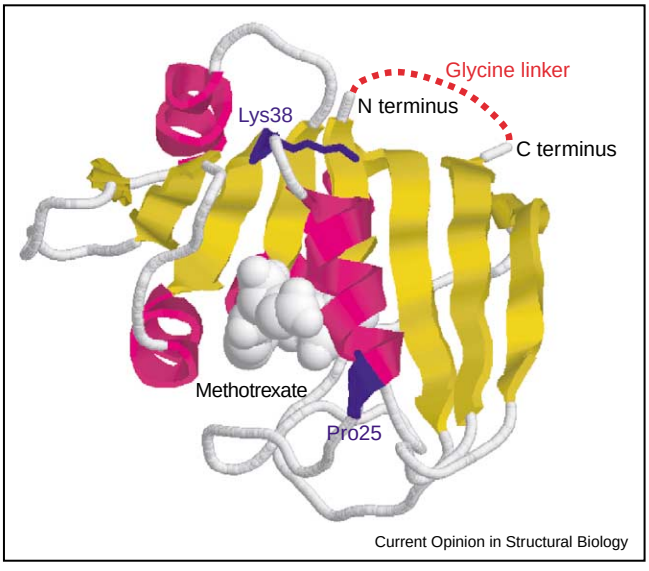


Structures of the proteasome and ClpAP [70]. From left to right: structure of the eukaryotic proteasome holoenzyme from *Xenopus laevis* [107] as determined by electron microscopy, the core particle is shown in blue, the ATPase caps are in pink; medial cut-aways of the proteasome core particles from *T. acidophilum* (top) [108,109] and yeast (bottom) [110] as determined by crystallography, active sites of proteolysis are indicated by red dots, the slice surface is shown in green; structure of the ClpAP holoenzyme as determined by electron microscopy [111]; structure of the ClpP particle, the proteolytic core of ClpAP, as determined by crystallography [112]. Figure reproduced from [70] with permission.

import, it seems likely that susceptibility to unfolding by the proteases is also determined by the local substrate structure adjacent to the targeting signal. This conclusion was tested by monitoring the degradation of circular permutants of DHFR. In these permutants, the N and C termini of DHFR are connected by a short glycine linker and new termini are introduced at various positions throughout the structure (Figure 7). The resulting proteins have almost identical structures and differ primarily in the points of attachment of the degradation signals [41•]. However, these circular permutants showed different susceptibilities to unfolding and degradation by the ATP-dependent proteases. Proteins were more easily unfolded when the degradation signal leads into a stretch of the polypeptide chain that forms an α helix or irregular loop at the surface of the folded domain. Substrates are more difficult to unfold and degrade when the degradation signal leads into a β strand. Thus, the local structure next to the degradation signal strongly influences the ability of a protein to be unfolded and degraded by the proteases. This finding suggests that the proteases unravel their substrates from the targeting signal. Indeed, in substrate proteins with more than one folded domain, the proteases first unfolded and degraded the domain at the N terminus adjacent to the targeting signal and then the next domain in the protein. Because the spontaneous global unfolding of barnase follows a different pathway, the proteases can catalyze unfolding by changing the unfolding pathway of their substrates, just as was the case for mitochondrial protein import [41•].

The sequential degradation of substrates by ClpAP was also demonstrated by fluorescence resonance energy transfer experiments [78•]. In these experiments, an energy donor was attached to the protease subunits of

Figure 7



Circular permutants of DHFR. In the permutants, the N and C termini of DHFR are connected by a short glycine linker, and new termini are created at Pro25 or Lys38. The figure also shows methotrexate bound to the active site of DHFR.

ClpAP and an energy acceptor was attached either to the N terminus of the substrate or next to the degradation signal at the C terminus of the substrate. In kinetic degradation experiments, the probe near the degradation signal interacted with the protease probe before the N-terminal probe, indicating that the polypeptide chain is threaded into the protease from the degradation signal [78•]. In addition, biochemical and electron microscopy experiments demonstrated the sequential interaction of substrate proteins first with the ATPase subunits and then with the protease subunits of ClpAP and ClpXP [73•–76•].

One observation has been interpreted to contradict the mechanism described above. The archaebacterial proteasome unfolds proteins even when translocation of the polypeptide chain into the degradation channel is blocked. This finding may indicate that translocation and unfolding are not necessarily coupled [77•]. However, alternative interpretations of these results are also possible.

Specificity of degradation

The mechanism of unfolding proteins by unraveling them from their degradation signals has several consequences. First, it allows the proteases to specifically degrade single subunits of a larger complex without affecting the other components. For example, the proteasome degrades the cell-cycle inhibitor Sic1 while it is associated with the yeast S-phase cyclin–cyclin-dependent kinase (CDK) complex to release active CDK [79•].

Second, the observation that proteins are difficult to unfold from β structures is interesting in the context of diseases that are characterized by the accumulation of large intracellular protein aggregates, such as Parkinson's and Huntington's diseases [80,81]. The protein aggregates are found associated with ubiquitin and components of the proteasome, suggesting that the cell tries to degrade the aggregates, but is unable to do so. The aggregates associated with amyloid diseases are characterized by the accumulation of long fibers with extensive β-sheet character, even if the native structure of the constituent proteins is largely α helical [82–85]. It has been proposed that at least the aggregates found in Huntington's disease are also formed by β structures [86].

Third, differences in the susceptibility to unfolding of domains within a substrate influence the end product of the degradation reaction. The ATP-dependent proteases analyzed degrade their substrate sequentially from the degradation signal and therefore a resistant domain can protect the distal parts of a larger protein from proteolysis [41•]. These two features of degradation provide a mechanism for the processing of proteins by partial degradation. Some experimental evidence suggests that this mechanism explains the activation of the p50 subunit of the transcription factor NFκB [41•], which plays a central role in the regulation of immune and inflammatory responses in mammals [87]. It remains to be seen whether the mechanism is applied more generally in the cell. For example, the transcription factors Cubitus interruptus in *Drosophila* [88], and Spt23 and Mga2 in yeast [89] are activated by processing. This processing could be due to partial degradation by the proteasome using the mechanism described for NFκB [41•,90•].

Unfolding in muscle

Protein unfolding during translocation and degradation are similar because, in both systems, unfolding occurs together with translocation of the polypeptide chain. This mechanism suggests a picture of domains being unraveled by the unfolding machinery pulling at the polypeptide chain. This simple pulling mechanism of unfolding occurs in domains of the muscle protein titin (connectin).

The functional unit of muscle is an elongated structure called the sarcomere. Actin filaments are attached to both ends of the sarcomere and, during contraction, the ends are pulled toward each other by a bundle of myosin fibers in the center of the sarcomere. The sarcomere ends are held together by titin molecules [91,92]. Titins are exceptionally large proteins and single titin molecules span half a sarcomere. One titin polypeptide chain has a relative molecular mass in the range of three to four million Daltons and is more than one micrometre long. The molecule consists mainly of around 300 compact domains with immunoglobulin or fibronectin folds, and only 10% or less consists of unique sequences. Among the unique sequences is the PEVK region, which can be around 1000 amino acids long and consists mainly of proline, glutamic acid, valine and lysine residues. The PEVK region does not fold into a unique globular structure. Together, these features give titin an elongated, string-like shape.

The response of sarcomeres to tension is determined by the properties of the titin molecules. The mechanical properties of single titin molecules have been investigated extensively by atomic force microscopy and optical trapping experiments [93–96]. In these experiments, one end of the titin molecule is attached to a surface and a pulling force is applied to the other end. The experimental set-up allows the recording of the force on the titin molecule as a function of its extension. There are three factors contributing to the elasticity of titin *in vitro*. At the lowest extension forces, the disordered conformation of the string-like molecule resists straightening through entropic effects. If the straightened molecule is then extended further, the PVEK region deforms, which again resists extension through effects largely entropic in nature. As the pulling forces increase, single immunoglobulin and fibronectin domains begin to unfold. This unfolding is reversible and the domains refold if the molecule is relaxed. There is some debate as to which of the three mechanisms makes the most important

contribution to muscle tension *in vivo* (e.g. [97,98]). However, overall, the evidence suggests that unfolding of single immunoglobulin and fibronectin domains in titin contributes to the elasticity of striated muscle *in vivo* [92]. Protein domains vary in their resistance to unfolding by pulling [99–101] and it is possible that the stiffness of various muscle fibers is adjusted through the domain composition of the constituent titin isoforms. As we have come to expect from the observations on protein unfolding during import and degradation, the resistance of a protein against unfolding by pulling is not determined by the protein's thermodynamic stability [99,100].

The molecular elasticity of other proteins with structural roles, such as the extracellular matrix protein tenascin [102] and the intracellular scaffold protein spectrin [99], has also been determined *in vitro*, but it is not clear whether unfolding plays a physiological role in these cases.

Protein unfolding *in vitro* and *in vivo*

Mitochondria and ATP-dependent proteases appear to unfold proteins by unraveling them from their targeting signals. In both processes, the resistance of substrate proteins to unraveling is not primarily determined by the thermodynamic or kinetic stability of the substrate against global unfolding, as measured *in vitro* by heat or solvent denaturation. How do the unfoldases induce denaturation? Unfolding occurs together with the threading of the substrate through a channel. In one simple mechanism, the unfoldases would trap local unfolding fluctuations in the part of the substrate structure that follows the targeting signal, effectively pulling at the polypeptide chain. Pulling by the unfoldase may also contribute to denaturation by lowering the energy barrier of the local unfolding transitions. Sequestering several amino acids of a compact domain will collapse its structure because protein folding is highly cooperative [103]. In this model, structures that are difficult to unfold are those that lack the appropriate local unfolding transitions, so that the proteases are unable to change their unfolding pathways [41•].

This mechanism would predict that the resistance of proteins to unfolding during import and degradation would correlate with their physical stability against pulling in atomic force microscopy (AFM) experiments. There has been no systematic study of the physical properties of the substrates used in the biological experiments and direct comparisons between the two kinds of experiments will be complicated because pulling rates differ by orders of magnitude. However, the existing results are consistent with the mechanism proposed here; the forces required to unfold barnase [100] and the α-helical protein spectrin [99] are lower than the forces required to unfold the β-barrel immunoglobulin domains of titin [96]. Unsurprisingly, even titin domains can be unfolded by mitochondria [104].

Other unfolding transitions in the cell

In all the examples of protein unfolding in the cell discussed so far, denaturation appears to be induced by pulling. There are of course processes in the cell in which unfolding occurs by different mechanisms. Two examples follow.

Amyloid diseases, such as Alzheimer's disease, involve the accumulation of large fibrous aggregates of specific proteins [80,81]. The aggregates can form from folded globular proteins and it is thought that the critical step in fiber formation in these cases is the initial spontaneous unfolding of the native structure [105].

The activity of some proteins is modulated by conformational changes and some of these changes can be so considerable that they effectively involve the unfolding of whole domains. For example, WASP (Wiskott–Aldrich syndrome protein) family proteins regulate actin cytoskeleton formation by providing a nucleation platform for actin fibers. In the inactive form, WASP proteins form an autoinhibitory structure in which the actin interaction domain is occluded by a G-protein-binding domain from the same polypeptide chain. Release of the actin interaction domain occurs when the G protein Cdc42 binds to the WASP protein and causes dramatic structural rearrangements [106•].

Conclusions

Protein unfolding is a critical step in several processes in the cell. Here, I discussed three examples, translocation, degradation and extension, in which the mechanisms of unfolding are very similar to each other, but differ from spontaneous global unfolding in the cell. Unfolding can be catalyzed and the collapse of the folded structure occurs together with the translocation of the polypeptide chain. The proteins unravel when the unfolding machinery pulls at the polypeptide chain. As a consequence of this mechanism, the susceptibility of a protein to unfolding depends on its structure, as well as its stability. By making protein degradation and import dependent on properties of the substrate itself, as well as its targeting signal, the cell has gained additional ways of controlling the specificity of these processes.

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