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Protein targeting to ATP-dependent proteases

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Abstract

ATP-dependent proteases control diverse cellular processes by degrading specific regulatory proteins. Understanding how these regulatory proteins are targeted to ATP-dependent proteases is of central importance to understanding their biological role as regulators. Recent work has shown that protein substrates are specifically transferred to ATP-dependent proteases through different routes. These routes can function in parallel or independently. In all of these targeting mechanisms it can be useful to separate two steps: substrate binding to the protease and initiation of degradation.

To be active, newly synthesized protein chains must fold into three-dimensional structures, but regulated unfolding is also critically important in some biological processes, such as protein degradation by ATP-dependent proteases and protein translocation across membranes [1]. Unfolding is required during degradation because the proteolytic sites of the ATP-dependent proteases are sequestered deep inside the proteases' structures and accessible only through narrow openings. Similarly, unfolding is required during several translocation processes because the protein import channels in some organelles are not wide enough for native proteins to fit through them. The mechanisms of unfolding in both types of processes are similar to each other but different from that of unfolding induced by heat or chemical denaturants. Here we discuss how the requirement for protein unfolding during degradation affects the way ATP-dependent proteases select their substrates.

ATP-dependent proteases

ATP-dependent proteases degrade short-lived regulatory proteins and thereby control cellular processes such as signal transduction, cell cycle, and gene transcription. The proteases also clear misfolded and aggregated proteins from the cell and produce some of the peptides to be displayed at cell surface as part of adaptive immune response. In eukaryotes, these functions are performed mainly by the proteasome. In prokaryotes and the organelles of eukaryotes, the functions are fulfilled by analogues of the proteasome, such as the ClpAP, ClpXP, HslUV, FtsH, and Lon proteases. Although ATP-dependent proteases show only relatively little sequence identity, they share a common architecture [2].

The ATP-dependent proteases all form large multisubunit particles (Figure 1). In the simplest case, FtsH protease, the particle consists 6 copies of a 71 kDa subunit forming a complex of approximately 425 kDa, and in the most complex case, the proteasome, the particle consists of some 40 different subunits forming a complex of 2 MDa molecular weight [3,4]. The subunits are mostly arranged in six or seven subunit rings that stack on top of each other to

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form cylindrical structures [2]. The proteolytic sites in all of these proteases are buried deep inside the particles and are accessible only through channels that are too narrow to allow folded proteins to pass through them [2,5]. This arrangement prevents the unintentional degradation of proteins. The ATPase subunits sit at the entrance to the proteolytic channels where they gate the channels and select and unfold substrates for degradation [2,5] (Figure 1).

Unfolding presumably occurs at the surface of the protease and the subsequent proteolysis proceeds sequentially along the substrate's polypeptide chain [6] (Figure 2). Unfolding during degradation can be much faster than spontaneous global unfolding, and the susceptibility of a protein to unfolding by the proteases is largely determined by the stability of its local structure first encountered by the protease and not the stability of the overall structure against global unfolding [6]. Proteins are more easily unraveled from surface α -helices and loops than from buried β -strands [6]. In the simplest model, the proteases catalyze unfolding by pulling at the polypeptide chain, perhaps simply as a consequence of the translocation of the polypeptide chain into the degradation channel [1]. Once the protein reaches the proteolytic sites, it is hydrolysed into 3–30 amino acids-long peptides [7,8] (Figure 2).

Besides their role in protein degradation, some ATP-dependent proteases are involved in nonproteolytic functions and most regulatory ATPase complexes show chaperone-like activity. Unfolding of a misfolded protein by ATP-dependent proteases can disrupt inappropriate intermolecular interactions and thus assist proper protein folding if it is uncoupled from degradation. The proteasome also functions as a regulator of a variety of cellular processes including gene transcription, DNA repair, and chromatin remodeling [9]. The chaperone-like activity of the proteasome ATPase ring may also induce conformational changes in the targeted factors involved in such cellular processes.

Substrate targeting to proteases

The proteases' proteolytic sites show little intrinsic sequence preference [10] and instead substrate specificity is conferred by the regulatory complexes selecting the proteins to unfold and translocate to the degradation sites. There are three main pathways by which substrate proteins are targeted to the different proteases (Figure 3).

In eukaryotes, most substrate proteins are targeted to the proteasome by the covalent attachment of many copies of the small protein ubiquitin. Ubiquitination is carried out by a cascade of three enzymes, E1, E2, and E3, which act sequentially to attach the ubiquitin moieties to the acceptor protein. Typically, the C-terminus of ubiquitin forms an isopeptide bond with the ϵ -amino group of lysine residues in the substrate protein but in some rare cases ubiquitin may also be conjugated through the substrate's N-terminus or a cysteine side chain [11–13]. Yeast encodes a single E1, a few dozen E2s, and hundreds of E3 enzymes. The enzymes pass the ubiquitin from the E1 to one of the E2s and on to the substrates, which are recognized by an E3 enzyme. Once the first ubiquitin is attached to substrate, the E3 can continue to function and attach more and more ubiquitins to lysines in the first ubiquitin. However, in some cases, further extension of the polyubiquitin chain is mediated by an additional conjugating factor (E4), which binds to preformed ubiquitin chain and catalyze multiubiquitin chain assembly in conjunction with E1, E2, and E3 [14]. The minimal proteasome targeting signal or degron consists of four ubiquitin moieties linked to each other by isopeptide bonds between carboxy termini and Lys48 [15]. This polyubiquitin degron is recognized by the 19S regulatory particle of the proteasome through two surfaces formed by the ATPase subunits Rpn10 and Rpt5 [16, 17].

Once attached, a polyubiquitin chain keeps being modified and can grow and shrink [18]. The length of the polyubiquitin chains affects degradation [19]. For example, the E3 anaphase-promoting complex (APC) coordinates the order of substrate degradation during the cell cycle

and the timing by which substrates are degraded depends on the processivity of their ubiquitination by APC [19]. Substrates that acquire long ubiquitination chains quickly are degraded earlier than substrates that are ubiquitinated slowly [19].

During degradation, the polyubiquitin chain must be removed from the substrate because the proteasome cannot translocate more than two or three polypeptide chains through the degradation channel at the same time. Cells contain a large number deubiquitinating enzymes (DUBs) [20] and at least two of them, Rpn11 and Ubp6, are located in the 19S regulatory particle and as such components of the proteasome [3,4,21–23]. Rpn11 removes entire ubiquitin chains from the substrate by cleaving the isopeptide bond between the substrate and the first ubiquitin to recycle ubiquitin and to allow substrate's degradation [22,23]. Ubp6 trims the chain from the free end and may serve as a timer [24]: when the ubiquitinated substrate binds to the proteasome, the proteasome will try to engage its substrate while Ubp6 shortens the ubiquitin chains from their distal end. If the ubiquitin chain has been removed before the proteasome has begun to degrade the protein, it escapes until it is ubiquitinated again and rebinds the proteasome.

The length of the ubiquitin chain appears to be regulated further and it was found recently that the E3 ligase Hul5 associates with the DUB Ubp6 on the 19S regulatory particle [4,25]. The ubiquitin ligase activity of Hul5 promotes degradation by extending the number of ubiquitin moieties in the tag on substrates whereas the deubiquitinating activity of Ubp6 antagonizes degradation by trimming ubiquitin from the tag [25]. In other word, Hul5 activity adds back to the Ubp6 timer and thereby increases the chance of the degradation before it drop off the proteasome. The balance between these two opposing activities may fine tune the proteasome's substrate specificity and thus regulate degradation [26].

The way the ubiquitin moieties are attached to each other also matters. Some polyubiquitin chains are linked through the Lys11 and Lys63 residues of ubiquitin but the extent to which they are involved in proteasome degradation is unclear [27]. Chains linked through Lys63 can serve as a nonproteolytic signaling, such as endocytosis, DNA repair, and protein sorting and trafficking. However, at least *in vitro* Lys63 linked ubiquitin chains also target proteins to the proteasome. Ubiquitin modifications linked through Lys6, 27, 29, 33 are rare [28]. In addition, many proteins are modified by single ubiquitin molecules and these modifications are involved in a wide range of processes unrelated to proteasomal degradation, such as endocytosis, virus budding, and chromatin structure [29].

A second step in ubiquitin-dependent proteasome targeting

Ubiquitination by itself does not always lead to rapid degradation [30,31] and effective proteolysis of a folded protein requires the presence of an unstructured region in the substrate [32,33], either at the ends of the polypeptide chain or internally [32,34]. The unstructured regions serves as the degradation initiation site and proteolysis continues from there along the polypeptide chain. Thus, ubiquitin tagging allows the protease to recognize its substrate proteins, and degradation then begins with proteolysis of initiation site [32]. The unstructured region functions to engage the unfolding machinery of the proteasome and is indispensable for the degradation of folded proteins. Thus, protein targeting to the proteasome appears to have two steps: recognition of the ubiquitin modification and initiation at the unstructured region; proteasome degrons have two components: a ubiquitination signal and an initiation site (Figure 2).

This initiation step in protein targeting could play an important role in substrate selection by the proteasome. Even relatively small differences in initiation may affect degradation efficiency if one takes into account the dynamic nature of the ubiquitin modification discussed above. If the ubiquitin modification is disassembled by the proteasome's deubiquitinating

enzymes before the substrate is fully engaged through its initiation site, the substrate will escape degradation until it is ubiquitinated again and the proteasome makes a new attempt at proteolysis.

Some proteins can bind to the proteasome yet escape proteolysis, presumably because the proteasome cannot initiate degradation on the substrate. In some cases the reason may simply be that the substrate lacks a suitable unstructured region. For example, the cyclin dependent kinase cdk2 folds into a compact structure devoid of disordered regions that could serve as initiation sites [35]. However, in other cases, proteins that are targeted to the proteasome contain long unstructured regions yet remain stable [36–39]. The E2 enzyme CDC34 autoubiquitinates on a long C-terminal unstructured region but does not get proteolysed [36, 37]. Similarly, proteasome-targeting adapters such as Rad23 (see below) bind to the proteasome and contain unstructured regions yet remain stable [38,39]. These findings suggest that not all unstructured regions can serve as initiation sites. Some data indicate that an unstructured region has to be of a certain minimal length and be located close to the ubiquitin tag to allow the proteasome to engage its substrate effectively [32] but the relationship between the two components of the degradation signal needs to be analyzed further. Intriguingly, the proteasome may also have preferences for the amino acid sequence of the initiation site and it appears that sequences with a strongly biased amino acid composition do not serve as efficient degradation initiation sites [30,40]. Indeed, the unstructured regions in CDC34 and Rad23 consist of simple or low complexity amino acid sequences [41] and may therefore not function well as initiation site. What could the biochemical basis be for the effect of sequence composition on proteasome binding? Presumably, the proteasome recognizes certain, yet to be defined, sequence motifs in its substrates when it binds to them during initiation and degradation after removal of the ubiquitin modification. These motifs will be less well represented the simpler the amino acid composition of a peptide sequence and thus, regions with simpler amino acid composition may function less well as degradation initiation sites.

The selection of the initiation site will be of particular importance when a proteasome substrate is part of larger complex. The proteasome is able to remodel these complexes by degrading specific subunits without affecting other components [42,43]. For example, several steps in the cell cycle are controlled by the degradation of cyclins while they are bound to cyclin-dependent kinase subunit [44] or the degradation of cdk inhibitor while it is bound to the cyclin cdk complex [36]. The removal of the cyclin stops the kinase from functioning until a new cyclin binds whereas degradation of the inhibitor releases the kinase activity of the cyclin cdk complex. Similarly, the transcription factor NFκB is inhibited when bound to a IκB [45]. During activation, the IκB is degraded by the proteasome without affecting the other subunits of NFκB [46]. The explanation for these observations seemed to be that degradation begins specifically at the ubiquitination site but we now know that this mechanism may not always apply [32,33]. It will be interesting to determine whether the two components of the targeting signal could work together when separated onto two different polypeptides chains in a complex.

Targeting signals in the primary sequence of the substrates

Most proteins are targeted to prokaryotic ATP-dependent proteases by sequence motifs present in their primary structure from the moment that they are synthesized. However, at least one substrate tagging system also exists in prokaryotes in the form of the SsrA RNA quality control system in *E. coli* [47]. SsrA is a small RNA that enters the A-site of ribosomes stalled at the 3' end of damaged mRNA. The ribosome switches template to the SsrA and becomes programmed to add an 11 residue tag to the C-terminus of the nascent polypeptide before encountering a stop codon. The ssrA tag targets the substrate for quick degradation by ATP-dependent proteases [47,48]. Several other consensus motifs have been defined for degradation signals [49,50]. Some of these motifs appear to be specific for particular proteases, others can

target proteins to several different proteases at the same time. For example, the C-terminal ssrA tag is recognized by ClpAP, ClpXP and FtsH proteases [47,50,51] and the signal in UmuD's N-terminus targets the protein to both ClpXP and Lon [52,53].

The consensus motifs in the degradation tags are relatively short (around 10 amino acids long) and they seem to be able to perform both functions of the two components of the proteasome targeting signal: they allow the protease to recognize its substrate and to initiate degradation. However, in some circumstances, initiation and binding sites can be separated. For example, an artificial substrate protein containing an internal ClpAP targeting tag requires an additional sequence tag at its C terminus for efficient degradation by ClpAP [54]. In this case, the internal targeting tag appears to tether the substrate to the protease and the C-terminal sequence serves as the initiation site [54].

The targeting signals are recognized by the proteases through loops in the central channel of the ATPase ring [55–58]. The best characterized of these is a loop containing the sequence GYVG, which is highly conserved in most proteolytic AAA+ ATPases and has been implicated directly in protein unfolding and translocation [55]. Other loops facing the central channel, such as an RKH loop in ClpX and two loops in ClpA D1 domain, also participate in the signal recognition [57,58]. The cooperation between the various loops probably allows ATP-dependent proteases to interact with the broad range of substrates.

In eukaryotes too some proteins are targeted to the proteasome directly by sequence motifs in their primary structure [59,60]. Degradation of thymidylate synthase (TS) and ornithine decarboxylase (ODC) by the proteasome is mediated by specific sequences, at the N terminus for TS and at the C terminus for ODC, and does not depend on ubiquitin [59,60]. These degradation signals also serve as both the protease binding site and the degradation initiation site. Finally, the proteasome can interact with misfolded or natively unfolded proteins lacking any known targeting signals in an ubiquitin-independent manner [61,62]. However, the relevance of this interaction to protein degradation is not clear.

Adapter proteins

The separation between protease binding and degradation initiation is clearest when proteolysis is mediated by adaptor proteins that bind both protease and substrate but escape degradation themselves. In eukaryotes, the DNA repair proteins Rad23 and Dsk2 can target ubiquitinated proteins for degradation [63,64]. They interact with the proteasome through a ubiquitin-like domain (UBL) and with the ubiquitin modification in substrates through two ubiquitin-association domains (UBAs) [37,38,63,65–67]. Thus, UBL-UBA proteins appear to deliver ubiquitinated proteins to the proteasome where they are subsequently degraded. Some E3 ubiquitin ligases have also been implicated in substrate delivery to the proteasome [68]. These E3 ligases interact with 26S proteasome directly or via other adapter proteins and the association could promote substrate degradation either directly by increasing the local concentration of substrate at the proteasome, or indirectly by increasing the length of polyubiquitin chain and thereby enhancing the affinity of the substrate for the proteasome. For some substrates, targeting can be even more complicated and lead through an additional ATPase ring complex called Cdc48 or p97 [69]. Cdc48/p97 can interact with both E3s and DUBs and may unfold proteins prior to proteasome degradation [68]. Some ubiquitinated proteins appear to be recruited to CDC48 by adapter proteins similar to Rad23 and Dsk2 but containing UBX domains instead of the UBL domain [68].

Prokaryotes also use adapter proteins for their ATP-dependent proteases [70,71]. SspB is one such adapter and it promotes degradation of several substrates, including that of ssrA-tagged proteins by ClpXP [72,73]. SspB interacts with residues in the ssrA tag as well as with ClpX, thereby increasing the effective local concentration of the substrate at the protease and

facilitating its degradation [74,75]. Some bacterial adapter proteins alter substrate preferences. For example, the ClpS adapter protein specifically inhibits the degradation of ssrA-tagged substrates by ClpAP but stimulates ClpAP to degrade aggregated proteins and possibly N-end rule substrates [76–78]. The use of adaptors allows for an additional level of regulation of degradation. The alternative σ factor σ^S controls the expression of many stress response genes in *E. coli* and during exponential growth in the absence of stress its concentration is kept low by ClpXP. However, σ^S is not recognized by ClpXP and its degradation requires the adaptor protein RssB [79]. The antiadaptor IraP controls σ^S concentration by binding directly to RssB [80].

Additional layers of substrate targeting

The various routes to degradation described above overlap (Figure 3). For example, degradation of several proteasome substrates including p21/Cip1, c-Jun, c-Fos, p53, and RPN4, are mediated by both ubiquitin-dependent and ubiquitin-independent routes [81–83]. Although these proteins are usually ubiquitinated, they are degraded even when their ubiquitination is inhibited. The ubiquitin-dependent pathways themselves also show overlap. In the yeast, polyubiquitinated proteins are recognized by the proteasome subunit Rpn10 directly and by adapter proteins, such as Rad23 and Dsk2 [37,68,84,85]. Cells lacking one of these polyubiquitin receptors are viable, but double or triple deletions of these receptors have synthetic defect in the recognition and degradation of ubiquitinated substrates. This observation indicates that Rpn10, Rad23 and Dsk2 may represent distinct receptor pathways that link ubiquitinated substrates to the proteasome. Other ubiquitin receptor factors, such as an intrinsic Rpt5 subunit and Cdc48 adapter complex, may also participate in the transfer pathways [68].

Cooperative targeting with adapter proteins is also observed in bacterial proteases. Bacterial targeting signals, such as ssrA motif, are often recognized by the adapter protein. Thus, bacterial proteases can recognize their substrates either directly or via adapter proteins [78,86,87]. These multiple pathways work in parallel with and independently from one another and converge at the initiation step. The pathways are modulated depending on cellular condition and may help the cell fine tune the levels of individual proteins.

Conclusions

In summary, protein substrates are specifically targeted to the ATP-dependent proteases through many different routes. The pathway taken by any substrate may change in response to the cellular environment. For the proteasome, targeting appears to have two components: substrate binding, which for most substrates is mediated by the ubiquitin modification, and initiation of degradation at a separate site. In prokaryotic proteases, the distinction between binding and initiation sites is less clear cut but can be demonstrated in a few cases. In both proteasome degradation and degradation mediated by prokaryotic proteases, the binding step can be mediated by adaptor proteins. We propose that for folded proteins the availability of initiation sites contributes to substrate selection. Thus, studying the way in which degradation is initiated may provide useful insights into the specificity of degradation.

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HslUV protease

Figure 1.

Structures of the bacterial ATP-dependent protease HslUV (PDB 1G3I). The protease subunits HslV are shown in yellow, the ATPase subunits HslU are shown in blue. A side-on cross section 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 view shows the sixfold axis of symmetry. Structures were produced by PyMOL.

Figure 2. Pathways regulating the transfer of substrate to proteases in eukaryotes (a) and bacteria (b). Subunits of 26S proteasome bind to the polyubiquitin chain of modified substrate (2) or to exposed polypeptide sequence of the substrate (3). Alternatively, adaptor proteins that bind the polyubiquitin chain and the proteasome simultaneously can mediate targeting (1). Bacterial proteases recognize substrate via exposed sequence tags (3) or via adaptor proteins (4).

Figure 3.

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) occur only after the substrate has engaged the proteasome through an unstructured region (red strings) (top left). Once the substrate is engaged, it is degraded sequentially along the polypeptide chain from its unstructured initiation site (bottom right).