Regulatory Subunits of Energy-Dependent Proteases

Susan Gottesman,¹ Michael R. Maurizi,¹ and Sue Wickner*
*Laboratory of Molecular Biology
¹Laboratory of Cell Biology
National Cancer Institute
Bethesda, Maryland 20892-4255

Intracellular protein degradation is a tightly controlled and highly regulated process. Within the cytosol, nucleus, and mitochondria of eukaryotic cells and within the cytoplasm of bacterial cells, a major portion of protein degradation is carried out by high molecular weight, multimeric ATP-dependent proteases. These proteases have proteolytic cores able to cleave a broad range of peptide bonds, and yet their activity is directed against only abnormal proteins and a limited number of native proteins with short half-lives. The regulation and specificity of proteolysis by ATP-dependent proteases depends on accessory or regulatory components, which may include as many as six different ATPases within a single complex. What roles these ATPases and other accessory components have in protein degradation and how they do it are exciting questions for which answers are quickly becoming clearer. Information from biochemical and genetic studies, inferences from structural studies, and parallels from structurally or functionally related proteins suggest a number of ways in which accessory factors may be involved in regulating degradation: (1) They may bind specific substrates and thereby target them for degradation by keeping them in the vicinity of the proteolytic components. (2) They may unfold the substrate such that it can pass through the access channels. (3) They may actively aid in translocating the bound protein into the proteolytic cavity. (4) They may allosterically affect the proteolytic active sites and hence the rate or specificity of peptide bond cleavage. In addition to effects on proteolysis, the accessory components may interact with other macromolecules or cellular structures, raising the possibility that they have additional functions not directly dependent on the proteolytic activity of the complex.

Much of our current knowledge about specific steps in energy-dependent degradation and the role of accessory factors comes from studies of the prokaryotic ATP-dependent proteases, ClpAP and ClpXP, which are complexes of separately encoded ATPase and peptidase subunits, and the homomeric Lon and FtsH proteases, in which both functions are encoded within single polypeptide chains (reviewed in Gottesman, 1996; see Figure 1 for model of ATP-dependent degradation pathway). Information from these bacterial systems can help to define the possible pathway of degradation and specific functions of the significantly more complex regulatory components of the eukaryotic 26S protease. As with the Clp proteases, the 26S protease consists of a proteasomal core and accessory ATPases, but both the core (not discussed in detail here; see Larsen and Finley, 1997 [this issue of Cell]) and the regulatory subunits include multiple types of proteins. For one defined regulatory complex of the 26S protease, PA700, six different ATPases as well as a large number of additional, non-ATPase polypeptides have been defined (Hochstrasser, 1996). However, the general organization of the proteolytic cores and the regulatory components appears to be amazingly conserved (see Figure 1 in Larsen and Finley, 1997). We would like to propose that the similarity in structure between the ClpAP or ClpXP proteases and the 26S proteasome, despite their apparent evolutionary unrelatedness, reflects underlying similarities in the biochemical mechanism of protein degradation. The existence of ClpYQ (HsiUV), a hybrid between a protease homologous to the proteasome and a Clp ATPase, reinforces this suggestion.

Substrate Recognition and Binding

Because ATP-dependent proteases must select out abnormal proteins and specific proteins whose activities are regulated by degradation from the bulk of cytoplasmic proteins, there must be some degree of specificity in recognition of targets. For ClpP-dependent proteases, substrate specificity is determined by which ATPase component, ClpA or ClpX, associates with ClpP, confirming that the ATPases not only have catalytic functions but are responsible for substrate selection. What effects does ATP have on that selection? This question is complicated for the Clp ATPases since ATP binding, but not hydrolysis, is necessary for ATPase assembly into multimeric rings and for ClpA/X to interact with ClpP (Figure 1, steps 1 and 2). Studies with RepA, a ClpAP substrate, show that RepA binds to unassembled ClpA and assembled ClpA and ClpAP, indicating that substrate binding is independent of nucleotide binding and ClpP (Pack and Wickner, 1997) (Figure 1, steps 3 and 3a). There may be a proofreading step that follows initial binding of protein substrates that would provide an opportunity for regulating proteolysis by release of some proteins before they are irreversibly committed to degradation. For example, at 0°C and in the presence of a nonhydrolyzable ATP analog, RepA and ClpA form unstable complexes that can be displaced by competing substrates. After incubation at higher temperature, the complexes become stable and RepA acquires resistance to displacement.

We would expect the regulatory subunits of the 26S protease to be responsible for the initial interactions and selection of substrates. A major difference between this system and the Clp proteases discussed above is that selection of substrates in eukaryotic cells is helped by a highly regulated and discriminating ubiquitin-tagging mechanism (Hochstrasser, 1996). Since ubiquitin tagging is almost universally required for recognition by the 26S proteasome, a ubiquitin recognition component is presumably among the regulatory components. At least one ubiquitin binding protein has been shown to associate with the 19S regulatory complex of the 26S protease, although there is evidence that this is either not the sole or the primary ubiquitin receptor (van Nocker...
Figure 1. Steps for Regulation of ATP-Dependent Protein Degradation

A model for the ATP-dependent degradation pathway is shown for a generic Clp-like protease, composed of a Clp ATPase component (shown in blue) and a proteolytic component (shown in green). The substrate is shown in red. The alternate pathway of protein remodeling by the ATPase component is also shown. We would predict similarities in the degradation pathways for both the 26S protease and the homomeric Lon and FtsH families of proteases. See the text for full discussion of these steps.

**1. Regulatory ATPase assembly (ClpA, ClpX, HslU), requires ATP binding but not hydrolysis**
**2. Protease assembly (ClpP, HslV), requires ATP binding**
**3. Substrate capture (RepA, MuA-DNA, λ O), by binding to assembled protease**
**4. Substrate unfolding by the ATPase and translocation (through ATPase into protease cavity)**
**5. Peptide bond cleavage and release of degradation products**

et al., 1997). Initial interaction with substrate may not be energy-dependent, although as with the prokaryotic Clp ATPases, assembly of the eukaryotic protease requires nucleotide binding. Is it possible that ubiquitin is only one element in substrate recognition by the 19S regulatory complex? Ubiquitin conjugation may help maintain a protein in an unfolded state or promote unfolding (Hochstrasser, 1996); it is possible that exposed motifs in ubiquitinated proteins may act synergistically with the ubiquitin signal to provide additional specificity and stability to the substrate complex with the 19S regulator. Such a bipartite recognition system also would provide an explanation for why some substrates can be directly recognized by the 26S proteasome. Ubiquitin is not required for degradation of at least one protein (ornithine decarboxylase), and it will be interesting to see if additional examples emerge as more substrates for this degradative system are identified.

The coiled-coil domains at the amino termini of the regulatory ATPases have been implicated in protein-protein interactions among the ATPases and proposed as substrate binding domains as well (Richmond et al., 1997). Binding of substrates by the ATPases would be quite parallel to the observed ability of ClpA and ClpX to bind specific substrates and would suggest that, as substrate specificity changes for the Clp proteases with a change in the ATPase subunit from ClpA to ClpX, the 26S protease may use the variety of ATPases at least in part to provide a variety of substrate binding sites. Many of the ATPases of the 26S protease have been independently identified as proteins able to bind specific transcription factors (see Confalonieri and Duguet, 1995 for recent review). It remains to be clarified whether this binding represents a true independent function of these ATPases outside the 26S protease complex or simply reflects the ability of the ATPases to engage in protein-protein interactions.

**Substrate Unfolding for Degradation: Intrinsic Molecular Chaperone Activity of ATPase Components**

Once bound to an ATPase component, not all substrates are destined to be degraded. Instead, recent results have shown that several ATPase components and ATPase domains possess molecular chaperone activities capable of remodeling or reactivating proteins in the absence of proteolytic components. For example, in reactions requiring ATP hydrolysis, ClpA activates the latent DNA binding activity of RepA by converting RepA dimers to monomers and prevents heat inactivation of firefly luciferase; ClpX disassembles MuA-DNA complexes and prevents aggregation of λ O protein; and the yeast Hsp104 Clp homolog (although not a protease component) is involved in resolubilization of heat-induced protein aggregates and reactivation of heat-inactivated luciferase and mRNA splicing activity (Figure 1, steps 3a and 3c; see Schirmer et al., 1996 for recent review). The question of whether or not the ATPase components can act independently of protease components in vivo remains unanswered. Chaperone-like activities have also been attributed to the FtsH family of...
proteases, and Lon can substitute for some of these in S. cerevisiae (Arlt et al., 1996; Rep et al., 1996); the ATPase modules of these proteases are of the same AAA family as the ATPases of the 26S protease.

It is likely that substrate unfolding or remodeling is an intermediate step in energy-dependent degradation, to allow the substrate entry into the proteolytic cavity. In some cases, the chaperone activities of Clp proteins may represent uncoupled unfolding reactions since there are several examples where the substrate specificity of the chaperone is the same as that of the protease; ClpA but not ClpX acts on RepA, and ClpAP but not ClpXP degrades RepA. Similarly, ClpX acts on MuA, and ClpXP degrades MuA. Further evidence that substrate unfolding is an intermediate step in energy-dependent degradation was obtained from studies with Lon protease showing that the requirement for ATP hydrolysis for degradation is determined by the presence of a helical substrate in a cavity, CcdA. At temperatures that disrupt the secondary structure of CcdA or with a truncated form of the protein that lacks stable secondary helical structure, degradation occurs without ATP hydrolysis (van Melderen et al., 1996). Together, these observations suggest that a major function of ATP hydrolysis by regulatory subunits is to unfold the substrate for presentation to the proteolytic component.

The observations that the ATPase components of proteases can function independently as chaperones suggest the interesting possibility that the ATPases may act along with classical molecular chaperones in kinetic partitioning of nonnative proteins between pathways leading to reactivation, degradation, or aggregation. In this way, the relative affinity of a misfolded protein for proteases, chaperones, and other cellular components would determine the fate of the protein.

Regulating Accessibility to Proteolytic Sites

In addition to being involved in unfolding substrates for presentation, the ATPase components very likely have a role in regulating protease accessibility by mediating conformational changes that facilitate entry of the substrate into the proteolytic components of the protease. Recent structural studies of the proteolytic components of the 20S proteasome, ClpP, and HsIV (ClpQ) have demonstrated that the proteolytic active sites are located in the interior of a cavity generated by the ring-like structures of the assembled subunits (Bochtler et al., 1997; Lowe et al., 1995; Wang et al., 1997 [this issue of Cell]; see Larsen and Finley, 1997). Proteins and polypeptides of more than 10-15 amino acids are unlikely to be able to enter the cavity through the narrow axial channels without the help of the regulatory components. Electron micrographic images show that the regulatory ATPases bind to the face of the proteolytic rings (Peters et al., 1993; Kessel et al., 1995). Thus, the ATPase component is in an ideal position to regulate the entry of substrates, perhaps by imposing conformational changes in the proteolytic component (Figure 1, step 4). If these conformational changes are coupled to the binding of the substrate to the ATPase component, then appropriate substrates, but not inappropriate ones, will have access to the protease and ultimately be degraded (Figure 1, step 5).

Functional Organization of the Regulatory Subunits

Comparisons between the structures of the Clp ATPases and studies on the effects of mutations in ClpA ATPase sites point to more than one possible mode of achieving control by the ATPases. ClpX has only a single ATPase domain that possesses both chaperone activity and the ability to specifically activate ClpP-dependent degradation, while ClpA has two ATPase domains. Mutations in the ATP binding site consensus of the carboxy-terminal domain of ClpA have drastic effects on ATPase activity and protein degradation without compromising the ability to degrade shorter polypeptides that do not require unfolding or possibly active translocation. Thus, the carboxy-terminal domain of ClpA may be functionally equivalent to ClpX. Mutations in the amino-terminal ATPase site cause only slight defects in catalytic activities, except for some mutants that show inefficient self-association to form the hexameric ring (Singh and Maurizi, 1994). However, the amino-terminal domain may further modulate the selection or activity of ClpA with certain substrates. This additional level of control may be paralleled in the 26S proteasome by some of the accessory factors, which could have a role in substrate selection. Some of the ATPase subunits of the 26S proteasome interact with one another; however, it is not yet known whether they form a ring-like structure, as seen with Clp ATPases, or even if all the ATPases associate in a single subdomain of the 19S regulatory subunit (Richmond et al., 1997). By analogy with the Clp proteases, one would predict that at least some of the ATPases are positioned to interact directly with the 20S proteasome and have analogous functions of unfolding and translocating protein substrates into the proteolytic core.

While very little direct information has yet been published on the specific roles of the 26S ATPases, other members of the family are associated with assembly of membrane protein complexes and membrane fusion (see Confalonieri and Duguet, 1995 for recent review of this family of proteins). NSF, one of the first of the AAA family of proteins to be identified, has two ATP binding domains; mutations in the first abolish activity and can act as dominant-negative mutants for Golgi transport while mutants in the second site reduce activity but do not abolish it; this second domain is required for formation of a cylindrical oligomer (Hanson et al., 1997). Association of NSF with SNAP5 and SNAREs occurs only when ATP is not hydrolyzed; it disassembles when ATP is hydrolyzed. This assembly/disassembly is presumably catalyzed by the ATPase of NSF and is reminiscent of the ATP hydrolysis-dependent activation of RepA by ClpP.

Allosteric Effects on Proteolytic Active Sites

In the prokaryotic proteases, the regulatory subunits modify the behavior of the protease active sites, apparently making them more accessible to and promoting more rapid cleavage of certain oligopeptides while decreasing the degradation rate of other peptide substrates (see Gottesman, 1996). The different proteolytically active subunits of the proteasome, on the other hand, have intrinsic differences in substrate specificity. Peptidase activity is stimulated and specificity may be further modulated by an energy-independent regulatory
factor, PA28, found in cells where proteolysis for antigen production is important (Groettrup et al., 1996). These data indicate that interactions between the regulators and the proteolytic components can lead to allosteric effects on the proteolytic active sites and may be a means of regulating the peptide bond cleavage specificity of the enzyme.

Summary
The regulatory components of the energy-dependent proteases provide controlled access to the proteolytic components, which innately possess broad specificity of peptide bond cleavage. The existence of multiple regulatory complexes capable of interacting with the same proteolytic component (e.g., ClpAP and ClpXP) provides a means of further regulation by increasing the range of substrate specificity of the protease without losing selectivity. Such a combinatorial approach to assembling different types of regulatory complexes may be used to direct degradative activity toward specific proteins or classes of proteins in different cell types or in response to regulatory signals. Once a substrate is recognized, ATP-dependent unfolding and translocation moves it into the proteolytic cavity. Interactions with additional factors can further modify both substrate selection and the specificity of peptide bond cleavage to control not only the proteins targeted but also the peptide output from proteolysis. By controlling these instruments of protein destruction, the cell has added enormously to its ability to regulate the levels and activities of important regulatory proteins.

Selected Reading