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Regulation by Proteolysis: Energy-Dependent Proteases and Their Targets

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INTRODUCTION

The ability to adjust the availability and activity of cellular proteins, particularly regulatory proteins and key metabolic enzymes, is essential to allow balanced growth of cells and to enable cells to respond to external stress and developmental signals. Protein activity is regulated by a myriad of well-studied mechanisms, including reversible covalent modification, changes in localization, interactions with other proteins and small molecule effectors, and proteolytic processing. Protein availability is controlled by often complex transcriptional and translational regulation of synthesis. In addition, a less well appreciated but important contributor to

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protein availability is the regulation of the stability of proteins. It has been recognized for some time that intracellular protein degradation plays a role in modulating the levels of metabolic enzymes and in removing damaged and abnormal proteins from cells (reviewed in references 74, 75, and 201). Studies in recent years have revealed the important regulatory roles that a subset of rapidly degraded proteins play in metabolic and developmental circuits in both prokaryotes and eukaryotes.

Although most proteins are relatively stable in vivo (75, 163), a subset of carefully regulated proteins have extremely short half-lives. We have used the term "timing proteins" to refer to proteins that are required for limited periods or under specific metabolic or developmental conditions but are unnecessary or even detrimental at other times or under other conditions (79). Emergency response proteins that carry out necessary functions under conditions of stress and proteins that participate in developmental signaling or initiate regulatory cascades are examples of timing proteins. This definition of timing proteins is meant to imply the role of these extremely short-lived proteins in providing the extra level of control to maximize the performance of a regulatory circuit, the way an actor's timing makes a scene a success. In some but not all cases, timing proteins also serve clocklike "timer" functions. We expect essentially all naturally short-lived proteins with half-lives significantly shorter than the doubling time of the cell to be timing proteins. Abnormal proteins, which are frequently rapidly degraded by the same proteolytic systems that destroy timing proteins, are excluded from the definition because they are generally nonfunctional.

This review describes some of the situations in which timing proteins and their selective degradation have been implicated in regulatory circuits and discusses some of the distinguishing characteristics of the timing proteins and the proteases that degrade them.

We focus here on proteolysis that occurs in the nonlysosomal intracellular compartments, primarily in the cytoplasm. This review will therefore not discuss such important proteases as calpains, lysosomal proteases, and the various extracellular proteases; recent reviews of these subjects are available (19, 20, 37, 119). A more comprehensive treatment of the proteases of *Escherichia coli* can be found in reference 149. Because general protein turnover and many instances of specific protein turnover have been shown to be energy dependent in vivo, we deal primarily with ATP-dependent degradative systems.

Although limited proteolysis or proteolytic processing plays an important role in regulation of enzyme activation and transport, processing is not directly addressed in this review. Proteolytic processing involves cleavage at a limited number of specific sites in the protein to yield an active or mature form of the protein. This limited proteolysis can occur either because the processing proteases are quite specific, cutting at specific well-defined sequences in the target protein, or because potential targets and processing proteases are colocalized for limited times or in limited contexts only, as in the secretory apparatus for protein export (159, 164, 174).

Protein degradation or proteolysis, as opposed to proteolytic processing, involves the cleavage of multiple sites within a target protein and the eventual complete turnover of the protein to amino acids. Degradation is generally complete enough in vivo that intermediates and degradation products are not detected. For biologically active proteins, proteolysis generally results in loss of activity. The most important cleavage affecting the biological activity of the protein may be the initial one, which then may render the protein susceptible to further degradation by other proteases and peptidases. The initiating protease in vivo can be identified as the one that, when mutated, leads to a change in the half-life of the protein, both functionally and chemically.

The next three sections of this introduction outline some of the major issues and possible conclusions currently being considered with respect to degradation of timing proteins. The underlying data supporting these statements are described in greater detail in the body of the review.

Physiological Functions of Protein Degradation

Proteolysis may seem to be a drastic solution to the problem of inactivating a protein, since restoration of the activity requires synthesis of a complete protein. Phosphorylation or other reversible modification, as well as changes in the concentration of noncovalent modulators such as cyclic AMP (cAMP), Ca^{2+}, or others, clearly provides alternative and widely used regulatory mechanisms. Regulatory proteolysis is used in a broad spectrum of biological pathways; we consider below some principles that appear to favor the use of proteolysis as a regulatory mechanism rather than other alternatives.

Obviously, the availability of a short-lived protein present in limiting amounts will be rapidly responsive to changes in its rate of synthesis. The possible advantages to the cell of using an unstable protein, discussed below, all depend on this balance between synthesis and degradation. An implicit assumption is that, to maximize the responsiveness of a system that depends on an unstable protein, the synthesis or activation of that protein will be carefully regulated. In fact, there is a general correlation between the half-life of a protein and the half-life of its mRNA, implying coordinate control over synthesis and removal of proteins in response to physiological signals (94).

(i) If new synthesis of an unstable protein is not continuous but is restricted to specific biological stages, loss of the activity of the unstable protein would provide an effective protein synthesis-dependent checkpoint in the pathway. If all events are proceeding as planned, the signals for new synthesis will be generated and the activity of the timing protein will be restored. The use of unstable proteins in cell cycle control would appear to represent this type of checkpoint control (see below). An unusual but graphic example of a protein synthesis checkpoint is provided by the addiction systems of some plasmids discussed below: when new synthesis of an antidote is blocked by loss of the plasmid DNA template, the result is rapid cell death.

(ii) When new synthesis of the unstable protein is prevented, protein degradation also provides a mechanism for commitment to a metabolic or developmental cycle. For a developmental pathway in which an unstable protein plays a role only during a limited period, there is no particular advantage to a reversible modification, since the protein will not be needed again. Loss of the protein may be important to avoid potentially damaging activity under the new conditions. If the unstable protein is an inhibitor whose synthesis ceases at a given point in the developmental pathway, the cell could be poised with a high level of an inhibited function, ready for a burst of activity when the inhibitor decays. Some proteins made as part of an emergency response can be toxic to cells under normal conditions. The rapid degradation of such proteins ensures that the protein will persist only as

long as the emergency lasts (see the discussion of SulA below).

(iii) Protein degradation is often one level of control exerted on processes that are redundantly regulated. For critically important functions, the cell may use every regulatory trick at its disposal. To put it another way, global regulatory networks must be able to integrate and respond to diverse signals. The regulation of the cell cycle in eukaryotic cells is dependent on not only the rapid and programmed degradation of cyclin but also a complex pattern of reversible protein phosphorylations and dephosphorylations, as well as regulation of synthesis of some of the key components (165). The heat shock sigma factor of *E. coli* is subject to transcriptional, translational, and posttranslational control, both by degradation and by another, not yet defined, mechanism (36, 52, 123, 168, 219, 220, 225).

In addition to biologically active proteins, many "abnormal" proteins are rapidly degraded in cells. Abnormal proteins include incomplete or incorrect proteins resulting from transcription or translation errors, misassembled or misfolded proteins, improperly transported proteins, and heat-denatured proteins. Thus, abnormal proteins can be empirically defined as proteins that do not have a native conformation and thus have altered solvent-exposed surfaces.

The description of proteins as abnormal proteins is a conditional description in many cases, since proteins that are perfectly functional in one context may be viewed by the cell as abnormal in another context. For instance, secreted or membrane proteins in *E. coli* that are designed to survive outside the cytoplasm are frequently inactive and unstable when mutations in export signals cause them to remain inside the cell (59, 160, 185, 222). Similar degradation of misdirected proteins that are unable to leave the endoplasmic reticulum has been found in eukaryotes (130). In addition to misdirected proteins, mutant proteins and proteins that are normally part of multiprotein complexes may be subject to rapid degradation when they are present as separated subunits.

Energy-Dependent Degradation of Unstable Proteins

It has been known for some time that the rate-limiting step in the degradation of proteins in both prokaryotic and eukaryotic cells is energy dependent. Inhibition of ATP production in vivo dramatically inhibits protein degradation; the degradation of abnormal proteins during growth (173) and the degradation of normal proteins during starvation (75, 152) are reduced by about 90% following depletion of ATP. The energy dependence of protein degradation in vivo is also observed for the turnover of specific proteins, such as SulA in *E. coli* (23, 230) and tyrosine aminotransferase (101) and p53 (86) in eukaryotic cells.

E. coli has been the source of two of the best characterized of these energy-dependent proteolytic systems. Lon protease (also called La) (24, 30) and Clp protease (also called Ti) (111, 125, 126) both require ATP for protein degradation in vitro, and both have been shown to contribute to energy-dependent degradation in vivo. In recent years, progress in defining the eukaryotic proteases and the systems that help in identifying their targets has suggested a number of parallels between the organization and possibly the mechanism of action of the prokaryotic and eukaryotic proteases. We will discuss the *E. coli* proteases and compare them with the eukaryotic proteolytic enzymes below.

Why expend energy to degrade proteins? The major energy cost of inactivating proteins via degradation is the ATP consumed in resynthesizing the protein. At least three molecules of ATP are consumed per peptide bond synthesized (250). In comparison, the ATP consumed in energy-dependent proteolysis is relatively modest. For *E. coli* Lon protease, about two ATPs are used per peptide bond cleaved, but only 5% of the available peptide bonds in proteins are cleaved (158). Thus, the cost of energy-dependent proteolysis for at least this energy-dependent protease is probably less than 0.1 ATP per peptide bond, 3% of the total energy cost.

Does the use of energy-dependent proteases provide an additional control on the selectivity of these proteases, or does it provide a mechanism for degrading completely a folded protein with a single site of protease recognition? Consideration of the mechanism of action of these proteases suggests that both may be true. We would like to propose that ATP-dependent proteases carry out what could be referred to as energy-dependent scanning and energy-dependent presentation of substrates, rather than energy-dependent proteolysis as such. Alberts and Miake-Lye (3) have recently described three functions that nucleoside triphosphate-hydrolyzing protein machines might perform: (i) providing a clock or delay mechanism that can increase the time available for improving the accuracy of a process, (ii) acting as a motor to translocate proteins, and (iii) allowing recycling of dead-end protein complexes. The ATP-dependent proteases may be intelligent machines that operate as clocks or switches in the initial capture of substrates and as motors for processive degradation. Energy is expended both to increase the information content of the interactions between the proteases and potential substrates and to do the work required to retain appropriate substrates and complete the degradation of proteins once the process is initiated. In the section on energy-dependent proteases we discuss the characteristics of these proteases that lead to this model.

Selecting Targets for Proteolysis: General Considerations

In both prokaryotes and eukaryotes, most cellular protein is stable in vivo, although a significant fraction (5 to 15%) of newly synthesized protein in both eukaryotes and prokaryotes is turned over within a few hours (182, 248). When individual protein half-lives have been measured, the data again suggest that the majority of proteins in growing cells are relatively stable (163). Therefore, the few highly unstable proteins must be distinguished from the majority of stable proteins. An important unanswered question is how the degradative systems recognize proteins that are programmed for rapid degradation and avoid damaging those that are needed and should not be degraded. Do proteases recognize specific sequence motifs, either local or dispersed, in naturally unstable proteins? An added degree of complexity arises because normally stable proteins, when mutated or when made or placed in abnormal environments, become unstable. Such "abnormal proteins" often have primary sequences identical or nearly identical to those in the native form, suggesting that proteolytic susceptibility is dependent on secondary or tertiary properties of the proteins as well.

Genetic evidence suggests that the proteases that degrade these abnormal proteins are identical to those that selectively degrade naturally unstable timing proteins. Therefore, the degradation signals within naturally unstable proteins could be composed of elements found in many stable proteins, where they either are not normally accessible or are altered in some way. This would argue that a recognition

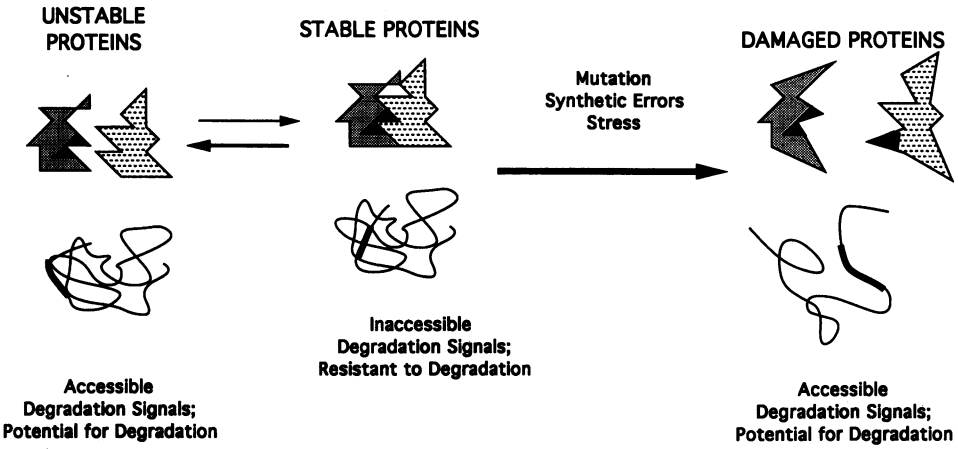

FIG. 1. Substrates for rapid degradation. The heavy black regions indicate potential degradation signals. Such signals may be recognition sites for tagging systems such as ubiquitin conjugation (see text) or protease recognition regions. These motifs may be inaccessible to the protease recognition systems in some multimeric proteins (top figure of Stable Proteins panel) unless the multimers dissociate naturally (top figure of Unstable Proteins panel) or are damaged to interfere with association by mutation, high temperature, or other stress (top figure of Damaged Proteins panel). In most proteins, which are naturally stable, protease recognition motifs probably are inaccessible to the protease recognition system unless the protein is partially unfolded (bottom figure of Damaged Proteins panel); it seems possible that naturally unstable proteins have more extended conformations which allow access of the protease (or other recognition systems) to degradation signals (bottom figure of Unstable Proteins panel).

signal is not sufficient to ensure destruction of a protein. The location of the signal within a potential target protein—accessible to the protease recognition apparatus in a naturally short-lived protein and inaccessible to these degradative components in most undamaged wild-type proteins—will also play an important role in the ultimate fate of the protein (Fig. 1).

Because interactions with other proteins can be extremely important in determining the degradation rate of a protein, analysis of motifs that seem to affect degradation must distinguish between those that serve as direct signals for specific proteolysis and those that work indirectly, for example by improving protein-protein interactions, and thus make degradation signals less accessible. Of course, the protein-protein interaction domains themselves, which are not normally accessible within a properly folded protein or complex, may in fact contain motifs recognized by the protease as degradation signals (Fig. 1).

The information for determining the specificity for degradation can be deciphered by the protease itself, as seems to be the case in prokaryotes, or by a tagging system that identifies proteins for the protease, as the ubiquitin tagging system seems to do in eukaryotic cells. It is not clear that the use of these different levels of recognition necessarily imposes any qualitative differences in the types of signals that are recognized. For instance, amino acids at the N terminus of proteins can serve to target proteins for rapid destruction in both bacteria and eukaryotic cells (the N-end rule; see below and reference 234). While N-end recognition in eukaryotes is mediated through the ubiquitin system, N-end recognition in bacteria, which contain no ubiquitin system, is a function of Clp protease (77, 228, 234).

ENERGY-DEPENDENT PROTEASES

Use of ATP for Protein Degradation

Biochemical and genetic studies of the enzymatic basis for energy-dependent protein degradation have identified three ATP-dependent systems involved in protein degradation. First, all prokaryotic and eukaryotic cells possess proteases that require the binding and hydrolysis of ATP for proteolytic activity. Two such proteases, Lon and Clp, have been identified in *E. coli* (24, 30, 111, 126). ATP-dependent proteases have also been identified and purified from extracts of various eukaryotic cells; these proteases tend to be complex high-molecular-weight enzymes composed of many nonidentical subunits.

Second, eukaryotic, but thus far no prokaryotic, organisms possess an ATP-dependent system for marking proteins for degradation by the covalent attachment of ubiquitin to potential target proteins, which are then degraded by at least one protease that appears to be ATP dependent (reviewed in references 99 and 186). The ubiquitin-conjugating system is highly specific and appears designed to increase fidelity and provide additional flexibility to the degradative system by separating the recognition and degradative processes.

The least well defined ATP-dependent system involved in protein degradation is dependent on the molecular chaperones, which are members of the heat shock protein families. Chaperone proteins are known to promote the folding or unfolding of polypeptides in an ATP-dependent manner (196). In *E. coli*, mutations in genes for the chaperone proteins such as DnaJ and DnaK can completely abolish energy-dependent degradation of abnormal proteins and certain mutant proteins (127, 208, 218), although their direct involvement in protein degradation has not thus far been demonstrated in vitro. It is possible that the presentation of abnormal proteins to the proteases requires their recognition (and unfolding?) by the heat shock chaperone proteins (208). Interpretation of the in vivo role in protein degradation of these chaperone proteins is complicated by their multiple cellular functions, including a role in the induction of the heat shock response itself (36). It has been suggested that the regulatory subunits of the *E. coli* energy-dependent Clp protease may have chaperonelike functions (in presenting protein substrates to the protease or to other cellular proteins), since homologs of the ClpA regulatory subunit include the *E. coli* heat shock protein, ClpB, and an essential

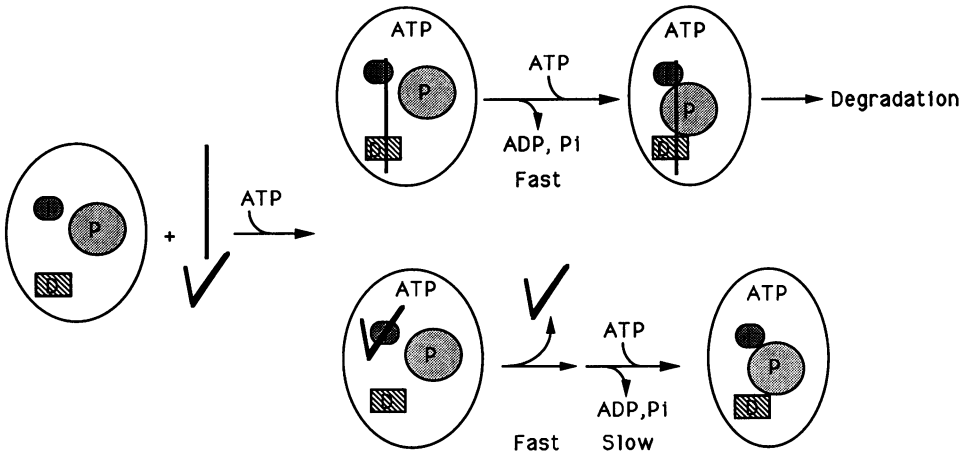

FIG. 2. Initiation of protein degradation by capture of the substrate by an ATP-dependent protease. The proteolytic active site (P) is in the open conformation when ATP is bound to the protease (73, 149, 157). Proteins (vertical bar, good substrate; hinged bar, poor substrate) initially bind to a nonspecific allosteric site, the initiator site (I), and most proteins dissociate again without being captured or cleaved (lower pathway). A few proteins might bind at I in a conformation that allows them to be cleaved to a limited extent (not shown). Proteins that also bind at a second allosteric site, the discriminator site (D), are retained long enough for ATP hydrolysis to promote a change in the conformation of the protease that results in retention of the protein and positioning for efficient cleavage (upper pathway). Substrates binding at D may in fact activate ATP hydrolysis and facilitate their own capture.

heat shock protein from *Saccharomyces cerevisiae* HSP104 (129, 178, 213, 214; see below). In eukaryotic cells, at least one molecular chaperone appears to be involved in targeting proteins to the lysosome for degradation (28, 45). The unanswered questions about these intriguing functions are currently under intense investigation.

The existence of three diverse energy-dependent systems affecting protein turnover in vivo underscores the complex regulatory control maintained over this essential but potentially harmful process in vivo. How these systems interact or act in concert is largely unknown, but some of the molecular details of these processes are beginning to be understood. This review will focus primarily on the first of these systems, the energy-dependent proteases.

ATP-Dependent Proteases: Energy-Dependent Scanning for Proteolysis

ATP hydrolysis is not required to drive peptide bond cleavage to completion. Therefore, the ATP consumed by ATP-dependent proteases must then be used either to overcome some unfavorable step in the overall process of protein turnover or to introduce additional levels of control to the proteolysis. Unfavorable steps in the process might include binding reactions between the substrates or products and the protease, alteration of secondary or tertiary interactions in the substrates, and changes in the conformations of the proteases themselves needed for expression of enzymatic activity and translocation of the protein substrate for multiple-site (processive) cleavage. Goldberg has proposed that ATP hydrolysis provides the energy for processivity (73). As discussed below for specific proteases, particularly Lon, there is considerable evidence that ATP binding and hydrolysis alters the conformation of the proteases and the accessibility of their active sites. Proteolytic activity is restricted to a time window during the ATP binding and hydrolysis steps, since ADP formed by hydrolysis produces an inactive conformation of the protein (76, 146, 157).

The function of ATP in proteolysis can be considered separately for the formation of the initial substrate-protease complex (capture) and for the subsequent multiple-site cleavage reactions (processive degradation). Figure 2 illustrates how these steps might proceed with an ATP-dependent protease or protease complex. The role of ATP in the capture step might be analogous to kinetic proofreading (107), with the added feature that "good" substrates activate the ATPase activity of the protease and thus accelerate the transition to the activated complex (upper pathway, Fig. 2). Poorly binding proteins (nonsubstrates) would dissociate before ATP hydrolysis occurs (lower pathway, Fig. 2). It is easiest to envision this mechanism providing added discrimination if the good substrates must interact with the protease at a second allosteric discriminator site (indicated as a striped box in Fig. 2) following the initial weak interaction. The initial site of interaction on the protease could be a low-affinity, nondiscriminating allosteric site (shaded in Fig. 2), which may or may not be a part of an extended active site (large circle in Fig. 2). Substrates that interact at both sites are retained long enough for ATP hydrolysis to shift the complex to the activated state. Evidence for allosteric sites for protein binding on both Lon and Clp proteases will be discussed below. This use of ATP hydrolysis to provide an opportunity to assess the interaction of substrates with a discriminator site is akin to the ATP-driven "clock" described by Alberts and Miake-Lye (3).

The functions of ATP during the processive steps of degradation should encompass a second role in retention or alteration of the protein substrate (suggested by the change in relative positions of the two allosteric sites in Fig. 3) as well as the transition of the complex to the activated state as for initiation (movement of the active site in Fig. 3). Changes in the interactions between proteins and the proteases in response to ATP binding and hydrolysis could result in alteration of the structures of the substrates as in the folding-unfolding reactions carried out by molecular chaperones, making more sites on the protein available for binding or cleavage (indicated by an increase in discernible features in the previously uniform protein in Fig. 3). ATP hydrolysis could also enable the protease to function as a motor in translocation of the bound substrates on the protease, allow-

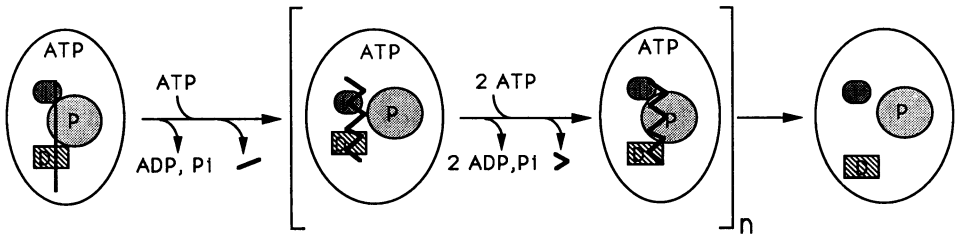

FIG. 3. ATP-dependent processive cleavage of proteins. The initial cleavage of proteins is accompanied by, although it may not require (50), ATP hydrolysis. Successive degradation steps occur without release of the bound protein and with the hydrolysis of two or more ATPs per peptide bond cleaved (146, 158). Processive cleavage requires changes in the dynamic interactions between the protein substrate and the protease, resulting in conformational changes in and translocation of the protein within or between active sites. For some proteins, these changes are facilitated by, but do not absolutely require (147), ATP hydrolysis. Pi, inorganic phosphate.

ing the proteins to be retained and cleaved multiple times before release of the products. Both Lon and Clp proteases from *E. coli*, in fact, appear to degrade proteins in such a processive manner.

We suggest calling the use of ATP to screen potential substrates and provide processivity for appropriate substrates "energy-dependent scanning" (Fig. 2). This proposed use of ATP hydrolysis to amplify the information content of interactions between proteases and their targets is analogous in many ways to the use of ATP hydrolysis in a variety of other systems for similar energy-dependent scanning of protein, DNA, or other macromolecular targets. Table 1 lists some of the energy-dependent surveillance functions that are required in *E. coli* cells and the enzymes that carry out these functions. Because these proteins can bind to both appropriate and inappropriate targets, the ability to reverse the binding interactions via ATP-induced conformational transformations is essential for both selecting substrates (scanning) and facilitating subsequent factor-mediated changes in the substrate. The concept of scanning is most easily envisioned with proteins such as UvrA, whose target is damaged DNA and which binds to undamaged DNA and moves along the DNA until the higher-affinity damaged sites are encountered (226). Proteins such as ClpA would scan by random collisions with proteins until higher-affinity substrates (presumably with two or more potential interaction sites, as in Fig. 2) are met.

Properties of ATP-Dependent Proteases

Three ATP-dependent proteases have now been identified and characterized in some detail: the Lon (La) and Clp (Ti) proteases from *E. coli* and the 26S protease from reticulocytes. Comparisons of the structural and enzymological properties of these proteases reveal considerable similarities and allow several generalizations to be made about ATP-dependent proteases. (i) ATP-dependent proteases are high-molecular-weight multimeric enzymes containing multiple active sites per protomer. (ii) The active sites of the energy-dependent proteases do not resemble the classical serine protease sites. Each of the energy-dependent proteases thus far analyzed seems to have a unique sequence around the essential active-site residues. (iii) Proteolytic active sites reside in distinct subunits (or in distinct domains in the case of Lon) separate from the ATPase subunit (or domain). We will define the ATPase region as the A-domain or A protein. (iv) ATP is an allosteric effector of the enzyme, promoting a conformational change in the A protein (A-domain), which consequently interacts with the proteolytic component (P-domain) to alter the conformation at the proteolytic active site. (v) The proteolytic component can degrade small peptides in the absence of nucleotide and, for multicomponent proteases, in the absence of the A protein. (vi) Binding of ATP (or a nonhydrolyzable analog) is sufficient to promote increased peptide hydrolysis, increased access to the proteolytic active site for larger polypeptides, and processive proteolysis of some protein substrates. (vii) The requirement for ATP hydrolysis is dependent on the substrate protein and appears to be most stringent for high-molecular-weight and more tightly binding proteins. Thus, ATP hydrolysis is required to alter the interaction between the protease and its substrates and not to catalyze peptide bond cleavage.

TABLE 1. Scanning functions in *E. coli*

Chaperone/ scanning protein	Target	Role	Reference(s)
DnaK	Misfolded proteins	Promotion of refolding of proteins	36
	Lambda replication complex	Restructuring of initiation complex	254
	Phage P1 replication complex	Monomerization and alteration of RepA	243
GroEL	Secreted proteins	Presentation to secretory apparatus	17, 18
	Lambda head and tail proteins	Assembly of heads and tails	63
SecB	Secreted and membrane proteins	Retardation of folding to promote translocation	59, 242
Trigger factor	Secreted proteins	Retardation of folding	90, 136
	Unknown protein	Assembly of septation apparatus	90
RecA	Single-stranded DNA	Recombination	35
ClpA	Unfolded proteins	Direction of degradation by ClpP	111, 112, 125, 126, 244
ClpB	Unfolded proteins	Recovery from heat-induced damage	178, 213
UvrA	Damaged DNA	Direction of repair enzymes UvrBC to site of damage in DNA	226

In the next section, specific details about the different energy-dependent proteolytic systems are described.

Lon protease of *E. coli*. The first ATP-dependent protease to be identified and purified was the *E. coli* Lon protease (24, 30), which may turn out to be the simplest model of an ATP-dependent protease. The *lon* gene product is a protein of 783 amino acids (subunit M_r, 87,000) (29), which is probably a tetramer in its native site (M_r, 450,000) (240).

Lon (called La by Goldberg and his coworkers) appears to be a serine protease (239, 240), although, as with many of the ATP-dependent proteases, the serine reacts very slowly with diisopropylfluorophosphate (DFP) compared with the reaction in classical serine proteases. Serine 679 of Lon is in a highly conserved region in Lon homologs recently identified in *Bacillus brevis* (114) and *Myxococcus xanthus* (68). A mutation in Ser-679 of *E. coli* Lon protease abolishes proteolytic activity, suggesting that this serine may be the active-site residue (249).

The amino acid sequence of *E. coli* Lon reveals a single ATP-binding consensus sequence (29), which is also highly conserved in the other bacterial Lon proteases (68, 114). Lon has an intrinsic ATPase activity, which is activated in the presence of protein substrates (239).

Lon is an endoprotease, cleaving protein substrates at multiple sites to yield peptide products 5 to 20 amino acids long but few, if any, free amino acids (147, 158). The rate of peptide bond cleavage by Lon is different for different substrates (76), but under optimal conditions the turnover number is rather low, about 6 min^{-1}, compared with that in classical serine proteases (>600 min^{-1}). Interestingly, the λ N protein, which is a physiological substrate for Lon, is degraded in vitro much faster (~60 min^{-1}) than are other proteins (147), suggesting that there may be characteristics of "true" substrates not present in the currently used model substrates such as casein.

Lon can degrade proteins only in the presence of ATP or an analog of ATP (76, 147, 241). For many protein substrates, maximum stimulation of degradation by Lon requires continuous hydrolysis of ATP, but cleavage of smaller proteins, such as oxidized insulin B chain, and fluorogenic peptides proceeds quite readily in the presence of nonhydrolyzable analogs (147, 241). In fact, the λ N protein (M_r, 12,000) was degraded in the presence of adenylyl-5′-imidodiphosphate (AMPPNP) at 25 to 50% the rate seen with ATP (147), and proteins, such as casein and globin, are degraded by Lon in the presence of AMPPNP or even PP_i at 5 to 10% of the maximum rate seen with ATP (76). The need for ATP hydrolysis tends to be greater for high-molecular-weight proteins (158) and for proteins with higher apparent affinity for Lon. N protein, which has poor affinity for Lon ($K_m \approx$ 200 μM) and polypeptides such as insulin B chain and glutaryl-Ala-Ala-Phe-methoxynaphthylamine, which also have high K_ms, are all cleaved readily in the presence of AMPPNP. Since the proteolytic activity of Lon does not require ATP hydrolysis and since short peptides can be cleaved even in the absence of ATP, ATP cannot be involved in the catalytic mechanism of peptide bond cleavage at the active site (147, 240, 241).

Lon has a basal ATPase activity in the absence of protein substrates, and protein degradation stimulates the rate of ATP hydrolysis. When both ATP and protein are saturating, there is an increase of 2.0 to 2.5 ATP hydrolyzed per peptide bond cleaved, compared with the rate without substrate present (158). This ratio is similar for proteins with different rates of degradation and was also found for insulin B chain, although maximal rates of degradation of this polypeptide can be achieved with nonhydrolyzable analogs of ATP. Half-maximal stimulation of ATPase activity occurs with lower concentrations of proteins (about 25%) than those required for half-maximal proteolysis (241). Thus, the acceleration of ATP hydrolysis is not linked to peptide bond cleavage per se but to some other step in the catalytic cycle.

Lon binds up to four molecules of ATP per tetramer, with two high-affinity ($K_d \approx 0.1$ μM) and two lower-affinity ($K_d \approx$ 15 μM) sites (156). Binding of ATP (or an analog) to the high-affinity sites is sufficient to activate cleavage of short peptides. On the other hand, ADP binding inhibits peptide cleavage. Thus, ATP (or an analog) promotes an "open" state for one or more active sites and ADP promotes a "closed" state; hydrolysis of ATP during each catalytic cycle results in conversion of active sites from the open (active) conformation to the closed (inactive) conformation. Since nonhydrolyzable analogs cannot promote degradation of many high-molecular-weight proteins, conversion of active sites to the open conformation is not sufficient for degradation and hence the transition to the activated state promoted by ATP hydrolysis (that is, a capture step as described in Fig. 2) appears to be necessary for degradation of these high-molecular-weight proteins. Degradation of α-casein in the presence of the nonhydrolyzable analog AMPPNP leads to greater accumulation of partially degraded polypeptides than is seen with ATP (50), suggesting that ATP hydrolysis also affects the binding or release of proteins either at the active site or at an allosteric site and facilitates processive cleavage of entire substrate molecules (Fig. 3).

Evidence that Lon has an allosteric binding site for proteins comes from two findings: protein substrates, but not peptide substrates, promote the release of tightly bound ADP from Lon (157), and protein substrates activate the peptidase activity of Lon in either the absence or the presence of ATP (241). Even at relatively high concentrations, proteins do not inhibit peptidase activity but in fact stimulate it as much as 10-fold. Thus, protein binding at the allosteric site (discriminator site in Fig. 2) also induces a subunit conformation such that at least one active site for peptide cleavage is open. Since the protein itself is not degraded in the absence of nucleotides, the cleavable regions of the protein must not have access to this open site. In the presence of ATP, when both protein and peptide degradation occur simultaneously, the protein substrate must be cleaved at an active site other than the one at which peptide hydrolysis occurs.

The proposed allosteric site on Lon could be either another (closed) active site within the tetramer or a separate protein-binding site within the same subunit. In either event, the nature of the binding to this allosteric site should be different from the binding of the protein at an open site where the protein can be cleaved. The necessity for proteins to interact at two sites on Lon should increase the specificity of the interaction considerably and provide a mechanism by which Lon can discriminate between potential substrates. Since protein binding at the allosteric site promotes ADP release, ATP binding might in turn disrupt protein binding, in a manner analogous to that of heat shock proteins that mediate protein folding (196). Lon might be able to bind several different conformations of a protein substrate. Release and rebinding of the substrate in response to ATP binding and hydrolysis also would allow the cleaved protein to alter its position on the enzyme, allowing new sites to be positioned for cleavage at the active site.

Generally, large proteins become better substrates when

they are denatured (158, 241), suggesting that proteins must have an extended polypeptide structure or flexibility to interact with the substrate sites on the enzyme. Most proteins are cleaved at multiple sites, yielding small peptides without the appearance of partially degraded intermediates (50, 147). Lon cleaves proteins at peptide bonds between a large number of different pairs of amino acids, indicating that the binding pocket for substrates can accommodate a variety of side chains (147). Lon shows some preference for a hydrophobic amino acid in the P1 position (the position carboxy terminal to the cleavage site) and, in most cases, for a basic residue in one position between P1 and P4 (147). Cleavage of short peptidyl naphthylamides occurs preferentially with hydrophobic amino acids in positions P1 to P3 and an acidic blocking group on the amino terminus (240).

It does not seem likely that these data are indicative of how Lon selects substrates in vivo. Since protein substrates do not inhibit cleavage of peptidyl amide bonds (241), the primary interaction between potential protein substrates and Lon should be at the allosteric site (Fig. 2). So far, the nature of the interactions at that site is not known. It is possible that the allosteric site of Lon binds an extended motif composed of particular types of amino acids rather than a unique sequence motif. It seems possible also that the allosteric site is composed of more than one subsite which must interact simultaneously with the potential substrate or that more than one allosteric site in the Lon multimer must be occupied for a proper degradation complex to form. Thus, a hallmark of Lon substrates might be the existence of several recognition signals disposed to interact at several sites on Lon, or sufficient flexibility to bring such sites into alignment with more than one binding site on Lon. Since Lon-dependent degradation of abnormal proteins in vivo is partially dependent on the heat shock proteins DnaK and DnaJ, molecular chaperones may be capable of presenting specific sequence motifs or proper conformation of polypeptides to Lon (127, 208, 218).

Clp protease of *E. coli*. Clp is a protease composed of two components, ClpA and ClpP, that degrades casein and other proteins only in the presence of ATP (111, 126). Clp is called Ti by Chung, Goldberg, and their coworkers (111). The two components of Clp are functionally distinct proteins and are the products of separate genes. ClpA has a subunit M_r of 83,000 and possesses an intrinsic ATPase activity that is increased in the presence of ClpP and substrates (112, 125). The amino acid sequence of ClpA, derived from the DNA sequence, contains two consensus sequences for ATP-binding sites (81, 83). The two sites are in separate regions of the protein and probably correspond to separate structural or functional domains. ClpP has a subunit M_r of 21,500 and is the proteolytic component of Clp (112, 151, 244). ClpP is inhibited by DFP (111), and Ser-111 has been identified as the site of modification (151). Site-directed mutagenesis of Ser-111 and of His-135 identified these residues as elements of the catalytic triad expected for a classical serine protease (151).

ClpA and ClpP are highly conserved in many if not all organisms. ClpA is a member of a family of proteins found in microorganisms, plants, and animals and includes a recently identified heat shock gene of *S. cerevisiae*, *HSP104*, necessary for thermotolerance (83, 178, 199, 213). The high degree of conservation within the family strongly suggests that the proteins have conserved the function of ATP-dependent proteolysis (83), although this has not yet been demonstrated. The prominent features of the protein sequences are two large domains, each of which is highly conserved within the family and each of which contains an ATP-binding consensus sequence. The two domains show almost no homology with each other, suggesting that each domain has a functionally distinct role in the enzyme. As yet, ClpP sequences have been identified only in *E. coli* and plant chloroplasts, but immunochemical screening indicates that proteins with conserved structures similar to ClpP are present in a number of prokaryotic and eukaryotic organisms (151). Conservation of the ClpP amino acid sequences in homologous plant chloroplast proteins is also quite extensive: the various chloroplast and *E. coli* proteins share 36 to 46% identical and an additional 24 to 34% similar amino acids throughout the entire lengths of the proteins. The active-site serine and histidine residues lie in very well conserved regions, lending support to the idea that the ClpP homologs are also proteolytic enzymes.

ClpA and ClpP are readily separated from each other during purification. ClpP has a native molecular weight of 240,000 (112, 150), and electron micrographs of ClpP (150) reveal that ClpP subunits are arranged in two hexameric rings which are superimposed to form a dodecamer. ClpA is purified as a monomer-dimer mixture, but addition of MgATP or analogs of ATP promotes association of ClpA to a hexamer with M_r 450,000 to 500,000 (148). ClpP does not interact with ClpA in the absence of ATP but binds rapidly and tightly to the ATP-promoted hexamer of ClpA. The M_r of the ClpA-ClpP complex is approximately 750,000 (148, 146), which would correspond to an association of one hexamer of ClpA with one dodecamer of ClpP. Since the dissociation of the ClpA-ClpP complex is slow and the ratio of ATP to ADP in the cell favors association, the complex of ClpA and ClpP should be the predominant form in vivo.

ATP hydrolysis is not required for association between ClpA and ClpP; nonhydrolyzable analogs such as AMPPNP and adenosine-5′-thiotriphosphate (ATPγS) promote self-association of ClpA and formation of the ClpA-ClpP complex (148). Since these analogs do not activate proteolysis, it is clear that ATP has two functions in activating Clp, one requiring binding only and the second requiring hydrolysis. ClpA has a basal ATPase activity that is activated 80 to 100% in the presence of ClpP and appropriate substrates. At saturating concentrations of ATP and protein substrates, a total of 6 to 8 ATP molecules are hydrolyzed per peptide bond cleaved (146). These two roles for ATP are somewhat analogous to the two roles of ATP for Lon protease: one role for ATP is primarily as an allosteric activator, inducing the open conformation of the proteolytic active site by promoting binding of ClpA to ClpP, and the second role is less well understood but should involve conformational changes in the enzyme that facilitate the capture of appropriate substrates and the unfolding or translocation of the protein substrate (Fig. 2 and 3) (73, 149).

ClpP alone can rapidly cleave short (3- to 6-amino-acid) peptides and will also cleave longer unstructured polypeptides, such as oxidized insulin B chain, at about 2% of the rate seen with ClpA and ClpP. Peptide degradation by ClpP alone is not inhibited by casein or other protein substrates, but when ClpA and ATP are added to ClpP, casein can inhibit the peptidase activity of ClpP (146). It thus appears that the active site of ClpP is accessible to peptides but that accessibility to protein substrates requires an ATP-ClpA-promoted conformational change. The specificity of peptide bond cleavage by ClpP is not altered by ClpA (227). ClpA might act directly on ClpP as an allosteric regulator to induce the required opening up of the active site to larger substrates, or ClpA could function as a molecular chaperone in

presenting appropriate conformations of substrate proteins to ClpP. The ATPase activity of ClpA is modulated by numerous proteins and peptides, suggesting that ClpA must interact with these substrates directly, as would be expected if it presents substrates to ClpP (227).

Clp protease degrades proteins into a large number of short acid-soluble peptides. As with Lon, Clp degrades protein substrates to small peptides even in the presence of excess protein substrate, indicating a processive mechanism in which multiple peptide bonds are cleaved in the same substrate without the release of large intermediates. The turnover number for peptide bond cleavage of reductively methylated α-casein is $>20\ min^{-1}$, somewhat higher than for Lon protease (227). The proteolytic activity of Clp against large proteins is completely dependent on ATP; no other ribonucleotide triphosphates or nonhydrolyzable analogs of ATP can activate the protease (126). In vitro, Clp shows a preference for cutting peptide bonds after amino acids with hydrophobic side chains, although other sites are also cleaved. Since Clp protease appears to be able to cleave a wide variety of peptide bonds in proteins, it is likely that, as with Lon protease, the specificity for protein degradation by Clp depends on interactions at an allosteric site as well as at the proteolytic active site.

ClpA is a member of a family of proteins that includes the yeast HSP104 protein, which is required for acute thermotolerance (178, 199). *E. coli* itself has two members of the ClpA family, ClpA and ClpB (83). ClpB is also a heat shock protein, and mutations in ClpB cause thermosensitivity (129, 214). The ATPase activities on ClpA and ClpB, the interactions of both proteins with peptides and proteins (227), and the heat shock regulation of expression of the Clp proteins all suggest that these proteins function as molecular chaperones (213). Parsell et al. (178) have suggested calling this the HSP100 family of proteins.

The major difference between ClpA and ClpB is the presence in the latter of a 120-amino-acid spacer region between the ATP-binding domains. The central spacer is highly conserved in some members of the family but is not present in all members, and thus it may serve to further divide the family into ClpA-like and ClpB-like subfamilies (83, 213). It is possible that these subfamilies have evolved different catalytic functions which are reflected in the central regions or in the less-well-conserved amino- and carboxy-terminal portions of the proteins. It seems more likely that the Clp family members are all subunits of proteases and that the differences reflect the specificities of the ATPase subunits for their corresponding proteases or for the types of protein substrates with which they interact. If this is true, ClpB may be more specific for directing proteolysis at the types of abnormal proteins that arise under heat shock conditions or ClpB may have specific regulatory targets under conditions of stress.

The function of the central domain is a particularly intriguing question. One possibility is that the central domain contains a proteolytic activity of the ClpB subfamily members, although the sequence of this region is not homologous to that of any known proteases. Another possibility is that the central region is a binding domain, involved in interaction with specific targets or perhaps in localization of the ClpB proteins within the cell. Comparison of the functions of ClpA and ClpB should provide valuable insights into the specificity of action and function of intracellular proteases.

Eukaryotic energy-dependent proteolysis. While a variety of ATP-dependent protease activities in eukaryotic cells have been described, the only system which thus far has been implicated in the degradation of specific unstable timing proteins is that described below: the tagging of unstable proteins by ATP-dependent ubiquitin conjugation followed by degradation by an ATP-dependent protease complex, most probably the 26S protease.

(i) Ubiquitin system for protein tagging. The ubiquitin system is a highly complex enzymatic system that covalently modifies selected proteins by attachment of the 8-kDa protein, ubiquitin, by way of an isopeptide linkage between the carboxy-terminal glycine of ubiquitin and the ε-amino group of an internal lysine of the target protein. The ubiquitin system has been reviewed recently (60, 99) and will be described very briefly here. Ubiquitin and the enzymatic components for ubiquitination of proteins have been observed only in eukaryotic cells, and this system is not believed to exist in bacterial cells. Ubiquitination is involved in a number of important functions, such as cell cycle control and DNA repair; at least one function of ubiquitin is essential for cell survival, since mutations which block ubiquitin conjugation are lethal in *S. cerevisiae* and mice (61, 153). Considerable data have now been amassed indicating that ubiquitination of some proteins allows them to be efficiently recognized and degraded by intracellular proteases and that, indeed, it may be a prerequisite for degradation of many proteins within eukaryotic cells. Targeting of certain proteins for degradation requires attachment of not just a single ubiquitin molecule to the protein but multiple addition of ubiquitin molecules linked via the carboxy terminus of each molecule to lysine 48 of the preceding one, attached at a single site on the substrate protein (25). The presence of a polyubiquitin chain appears to be both necessary and sufficient to target these proteins for degradation (85). The function of single ubiquitin additions to proteins is not so clearly understood, although conjugation of methylated ubiquitin (which blocks polyubiquitination) to several different sites on some proteins can also lead to their rapid degradation (91). It is not known whether the essential function of ubiquitin is related to targeting proteins for degradation, but only a fraction of the pool of ubiquitin conjugates in cells are targeted for degradation, and there is evidence for involvement of ubiquitinated proteins in diverse cellular functions (92; reviewed in reference 60).

Ubiquitination of proteins requires three different classes of proteins, E1, E2, and, in some cases, E3 (60, 99). Ubiquitin is conjugated via a thiol-ester linkage to E1 in an ATP-dependent reaction and is then transferred to one of several possible E2s, a diverse class of ubiquitin carrier proteins, to which it is attached via another thiol ester linkage. Depending on the type of E2 and on some factors that are not yet understood, ubiquitin is transferred from the E2 either directly to the target protein or to the target protein complexed with one of another class of binding proteins called E3s or ubiquitin protein ligases. E1 has been shown to be essential in both yeast and mammalian cells (61, 153). Temperature-conditional mutations in E1 cause a severe defect in ubiquitin conjugating activity in vivo and in vitro, and the mutant cells are defective in protein degradation under nonpermissive conditions (33, 61). It is not known whether the lethality is related to the defect in protein degradation or to the loss of one of the other possible functions for ubiquitinated proteins (33, 61, 153). The functions of the E2 proteins are diverse and redundant. Thus, mutations in either *UBC4* or *UBC5* in *S. cerevisiae* which code for 16-kDa E2 proteins, have little effect on protein degradation, but the double mutant has reduced turnover of proteins and reduced amounts of ubiquitinated proteins

(205). Yeast *UBC2* (*RAD6*) mutants are highly pleiotropic, with defects in DNA repair, sporulation, and the degradation of specific N-end rule substrates (see below for a description of the N-end rule) (116, 221). Mutations in the yeast gene for E3α (*UBR1*) are defective in the degradation of these N-end rule substrates also, but they do not display the sporulation defect or other phenotypes of *RAD6* mutants (11). Thus, RAD6 presumably carries out both E3α-dependent and E3α-independent ubiquitination. Another yeast gene, *UBC3* or *CDC34*, codes for an E2 that will monoubiquitinate histones (71) and also polyubiquitinate proteins in an E3α-dependent manner. *UBC3* (*CDC34*) is essential for chromosome replication, but E3α is not (11, 71).

The E3 proteins are the least well understood components of the ubiquitin system. For one, E3 proteins have at least one function that is independent of ubiquitin, that is, the ability to bind certain proteins. E3α and E3β have binding sites for proteins with different amino-terminal amino acids (34, 77, 99, 187). Ubiquitination of the E3-bound proteins will occur in the presence of some but not all E2 proteins, if a lysine is available at an appropriate position in the protein (8). Several proteins, including cyclins and N^{α}-acetylated proteins, are ubiquitinated in vitro and degraded in ubiquitin-dependent reactions, although these proteins do not necessarily have amino termini that can be recognized by E3α or E3β (69, 78, 105). Whether there exist other E3 proteins which recognize features of proteins other than their amino termini and present them to E2-ubiquitin for conjugation or whether there are specific E2 proteins that can interact directly with these N-end-independent substrates remains to be demonstrated.

In general, polyubiquitinated proteins, but not monoubiquitinated proteins, are degraded in an ATP-dependent reaction, probably by the 26S protease (see below). Presumably, some component of the 26S protease has a binding site for polyubiquitin, which allows the protein to be positioned for degradation. Alternatively, the attached ubiquitin may simply keep the protein in a protease-sensitive state. Ubiquitin itself is not degraded but is removed from the protein or the peptide products by one of a number of isopeptidases and recycled. The binding site for polyubiquitin on the 26S protease has not been defined, and the mechanism by which the protein gets into the protease active site and is degraded is not understood.

(ii) 26S protease. Numerous researchers have detected and partially characterized ATP-dependent proteolytic activities in extracts of eukaryotic cells. Efforts to identify the degradative system for ubiquitinated proteins led to the discovery that degradation of ubiquitin conjugates is dependent on at least one of these ATP-dependent proteases, called the 26S protease (99). The 26S protease, in one form or another, has also been referred to as UCDEN (237), the 1,500-kDa proteolytic complex (48, 62), megapain (109), and simply the ubiquitin conjugate-degrading protease (73). The identities of all the components of the 26S protease are still uncertain and controversial, but what is clear is that the protease is a complex of complexes, consisting of at least three multisubunit components. The 26S protease was originally identified as an ATP-dependent activity that could degrade ubiquitinated proteins (108, 109, 237). The protease is composed of a number of proteins ranging in size on sodium dodecyl sulfate-gels from 35 to 110 kDa, and, in addition, it contains an array of subunits that were similar in size and distribution to those previously identified in a 20S complex called the proteasome, the 20S protease, or the multicatalytic protease complex. The presence of proteasomes in similarly large ATP-dependent proteases was suggested by several groups, who showed that antibodies directed against proteasome subunits could inhibit proteolytic activity against ubiquitin conjugates in their preparations (42, 145). Electron micrographs of purified 26S protease reveals dumbbell-shaped particles that have a central barrel of four layers of subunits, resembling proteasomes, flanked at the top and bottom by rectangular structures (113). We summarize the properties of the proteasome later in this review.

Other researchers have obtained high-molecular-weight, ATP-dependent, ubiquitin-degrading proteases by reconstitution from separated protein fractions. Eytan et al. (55) and later Driscoll and Goldberg (48) reported that a protease similar to the 26S protease could be reconstituted from reticulocyte proteasomes (in a fraction called CF3) and two proteins called CF1 (600 kDa) and CF2 (250 kDa). Assembly of the protease from the different components requires ATP, and it was proposed that the 26S protease had not been isolated intact because the cells used had been depleted of ATP prior to extraction. The presence of the proteasome subunits in these assembled proteases was assayed by enzymatic activity, and the stoichiometric ratios of proteasomes to the regulatory factors were not reported. In fact, only a small fraction of the proteasomes were recovered in the high-molecular-weight complex. Seelig et al. (203) suggested that the proteasomes are not found in the same fractions with the ATP-dependent protease activity of similar complexes reconstituted from extracts of either reticulocytes or liver, but this result appears to be contradicted by the findings of Orino et al. (176), who used silver staining to detect both proteasome subunits and the 35- to 110-kDa proteins of the 26S protease in similar assembled complexes. Further experiments are required to explain the differences in these results.

Strong support for the role of proteasomes in the 26S ubiquitin conjugate-degrading protease has come from mutational studies in *Saccharomyces cerevisiae*. *S. cerevisiae* mutants defective in the proteasome subunit PRE1 are sensitive to canavanine, display slower turnover of abnormal proteins, and accumulate ubiquitin conjugates in vivo (96). These PRE1 mutants were shown to be defective in degrading certain β-galactosidase fusion proteins that are known to be ubiquitinated in vivo (189, 206). Since ubiquitinated proteins are degraded by the 26S protease in vitro, these results strongly implicate the proteasome in 26S protease function.

More recently, the 250-kDa component of the reconstituted 26S protease, CF2, has been shown to interact with the proteasome and inhibit peptidase activity (46). CF2 resembles to some extent the 200-kDa proteasome inhibitor reported by Li et al. (137) but appears to be different from an inhibitor recently reported by Chu-Ping et al. (31). CF2 may bind ATP, since it is stabilized at 42°C by addition of ATP or AMPPNP. Addition of CF1 and ATP overcomes the inhibitory action of CF2 and potentiates an ATP-dependent proteolytic activity in the complex (46). The ATP-dependent assembly of the ubiquitin conjugate-degrading protease is analogous to the ATP-dependent assembly of Clp from ClpA and ClpP (Fig. 4). One difference is that ATP hydrolysis is required for assembly of the 26S protease whereas the assembly of Clp occurs in the presence of noncleavable ATP analogs. Possibly formation of the 26S protease requires an energy-dependent folding step, whereas ATP acts as an allosteric effector in promoting ClpA and ClpP association.

The substrate specificity of the 26S protease is still an important unresolved issue. For some proteins, such as

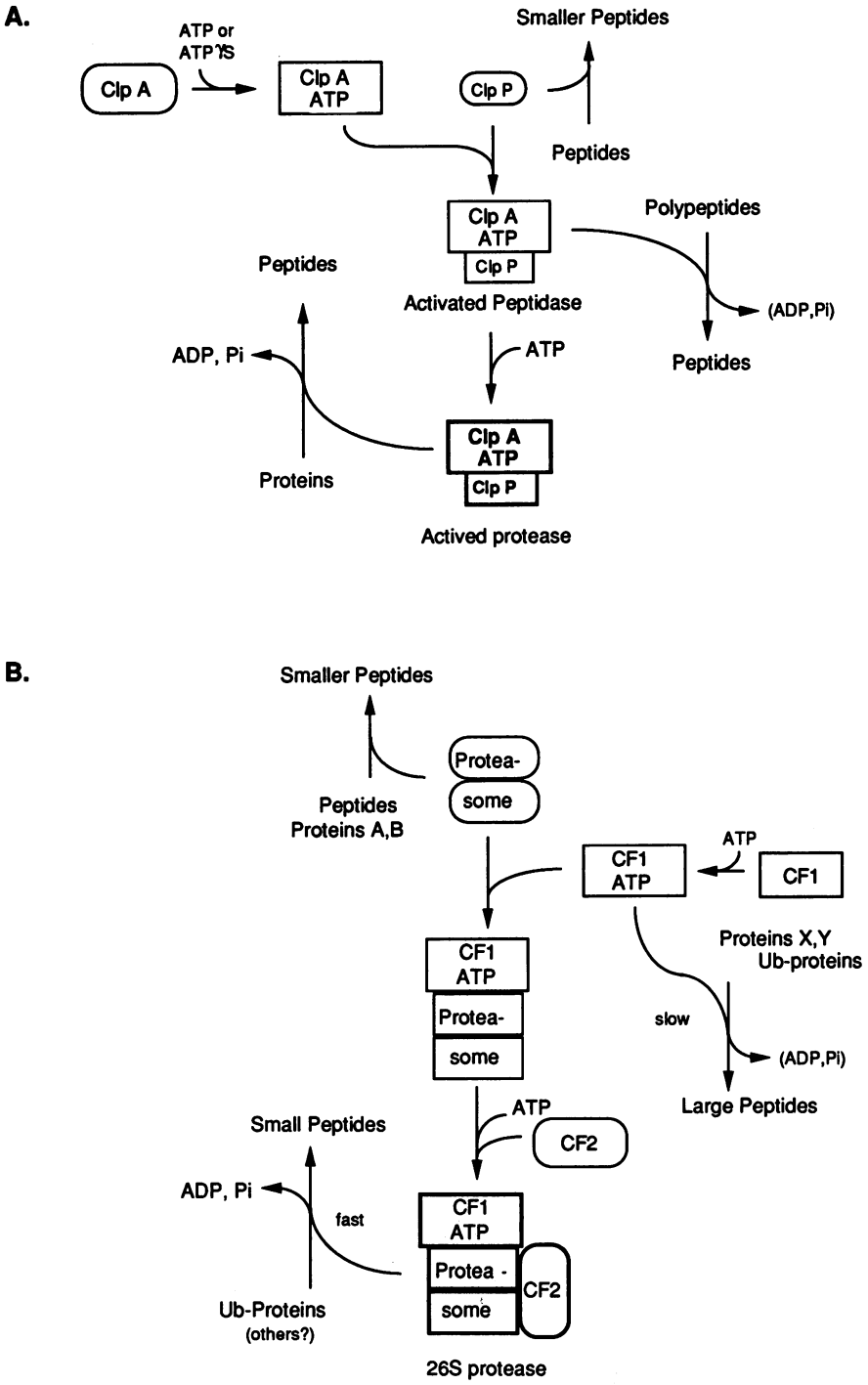

FIG. 4. Comparison between molecular interactions and proteolytic activities of Clp protease and eukaryotic ATP-dependent proteases. ATP-dependent proteases are complex arrays of proteins with distinct enzymatic activities. The proteolytic components are multimers of one or more different proteases and display limited activity with small peptides and possibly a few proteins. Increased proteolytic activity depends on ATP-dependent interactions with one or more multimeric proteins (A proteins). The A proteins themselves may have protein-folding functions and possibly limited proteolytic activity. Protein degradation by the complex is ATP dependent and, depending on the associated factors, may have specificity for selected proteins. (A) *E. coli* Clp protease (112, 125, 126, 148, 149, 244); (B) eukaryotic 26S protease and its components (see text and reviews in references 73, 99, 177, and 191). Pi, inorganic phosphate.

[^{125}I]lysozyme, ubiquitin conjugation appears necessary for degradation by the 26S protease. Likewise, antibodies against the ubiquitin-conjugating enzyme, E1, inhibited degradation of proteins by the 26S protease in BHK cell extracts (42, 154). However, the 26S protease has also been reported to degrade nonubiquitinated proteins, such as RNase and α-casein, and also certain small peptides, all in ATP-dependent reactions (47, 108). Although it is possible that these

latter activities are artifacts of in vitro conditions, it may be that the 26S protease specifically degrades ubiquitin conjugates but also can degrade certain unstructured proteins and polypeptides.

(iii) Proteasomes. The proteasome, also referred to as the multicatalytic proteinase complex, is a high-molecular-weight protease ($M_r \approx 700,000$) found in most eukaryotic cells and in archaebacteria. It is the major neutral intracellular protease found in eukaryotic cells and appears to be located in the cytoplasm and in the nucleus. The reader is referred to several recent reviews for more details of the physical and biochemical properties of proteasomes (99, 177, 191, 224).

Interaction of the proteasome with specific regulatory proteins stimulates an ATP-dependent proteolytic activity against ubiquitinated proteins (see above). The relative abundance of proteasomes (0.5 to 0.1% of cell protein), compared with the regulatory factors making up the 26S protease, suggests that proteasomes, at least, may have other targets in addition to ubiquitin conjugates. In vivo, the proteasome could express ATP-independent proteolytic activity or might possibly interact with other regulatory factors to catalyze ATP-dependent proteolysis of different classes of proteins.

In vitro, the proteasome is capable of a number of proteolytic activities. Purified proteasomes have peptidase activity against three distinct classes of fluorogenic peptides, cleaving on the carboxyl side of residues whose side chains are hydrophobic (chymotrypsinlike), basic (trypsinlike), and acidic (peptidylglutamyl-peptide hydrolyzing). Various studies have indicated that cleavage of different classes of peptides is dependent on different active sites within the proteasome (177, 191). Proteasomes have proteolytic activity against a number of denatured or oxidized proteins (155, 177), but this activity is usually latent in the purified proteasome and must be unmasked by various structural perturbations (e.g., heating or detergent treatment). The latent proteolytic activity and the peptidylglutamyl-peptide-hydrolyzing activities may be carried out by the same subunit.

Proteasomes are composed of 24 to 28 subunits arranged in a cylindrical particle that appears in electron micrographs to be composed of four superimposed rings of subunits, each ring containing either six or seven subunits (6, 57, 253). Eukaryotic proteasomes are composed of a heterogeneous mixture of 12 to 16 types of subunits, differing in size (21 to 35 kDa), isoelectric point (3.5 to 7.5), and elution position from C18 reverse-phase columns. At least seven different human and six different yeast genes for proteasome subunits have been identified to date, and it is conceivable that all 12 to 16 subunits of proteasomes are unique gene products (223, 224, 251). It is not known whether all proteasome particles from a particular tissue or cell type are composed of identical arrays of the different subunits. Recent data do indicate that proteasomes show tissue-specific variations in their subunit composition and in expression of various activities during development (2, 124, 131).

Archaebacteria have a proteasome similar to the cylindrical structure found in eukaryotic cells but made up of only two different types of subunits. The inner rings of the proteasome from *Thermoplasma acidophilum* is composed of β-subunits, and the outer, flanking rings are composed of α-subunits (88, 95). *E. coli* ClpP, which has 12 identical subunits, would appear to resemble the inner core (β-subunit) of the *Thermoplasma* proteasome. It is interesting that the β-subunit of the *Thermoplasma* proteasome is closer in size to ClpP and that it alone has the necessary residues to form a serine protease active site. There is, however, no significant amino acid sequence similarity between ClpP and the β-subunit (233). The amino acid sequences derived from the DNA sequences of the cloned proteasome genes indicate that eukaryotic proteasome subunits constitute an evolutionarily related family of proteins. Although there is considerable divergence in sequences, Zwickl et al. (252) have proposed that all proteasome subunits may be grouped into two subgroups related to either the α- or the β-subunit of the *Thermoplasma* proteasome. These sequence similarities could reflect similar structural or functional roles for the subunits in each subgroup, but there is no evidence for this as yet.

The sequence analyses of proteasomes have not been of much help in identifying functional residues in these proteins, because most proteasome sequences do not contain consensus sequences for any protease families. The one instance in which putative active-site consensus sequences have been identified is with the human *RING10* gene (70), but even here the results are somewhat confusing. Although the sequences of *RING10* and the supposed proteolytic β-subunit of the *Thermoplasma* proteasome align quite well, with 27% identical and 48% similar plus identical amino acids, none of the putative active-site residues from *RING10* are conserved in the *Thermoplasma* β-subunit (70, 252). Apparently, the *Thermoplasma*, as well as most of the eukaryotic, proteasomes represent unique families of proteases for which the consensus is yet to be defined. Also, since only three or four activities have been identified, but there are 12 to 16 eukaryotic proteasome subunits, it is possible that the genes for the proteolytic subunits have not been cloned or sequenced.

Why are there so many proteasome subunits? The existence of relatively few proteolytic activities and the absence of recognizable protease active-site sequences from many of the identified proteasome subunits suggest that, through evolution, many proteasome subunits may have lost critical active-site residues but now have other functions as structural components only, as binding proteins, or possibly as regulatory subunits. In this regard, it is worth noting two other examples of proteolytic subunits undergoing evolutionary changes to become regulatory subunits. The angiotensin-converting enzyme, for example, is composed of a catalytic subunit and a regulatory subunit, the latter having arisen by duplication and mutation of the catalytic subunit (212). The bacterial toxin streptokinase has extensive homology to the trypsin protease family but lacks the critical histidine residue. Rather than cleaving plasminogen directly, streptokinase interacts strongly with plasminogen and activates its autocatalytic conversion to plasmin (170).

Mutational studies with yeast proteasome genes in fact suggest structural or regulatory roles for some proteasome subunits. Heinemeyer et al. (96) reported that mutations in the "chymotrypsin-like" activity of proteasomes fell into two complementation groups, *PRE1* and *PRE2*. If the active site resides in just one of the two proteins, mutations in the other subunit must either disrupt the structure of a neighbor of the proteolytic subunit or affect a regulatory subunit for the protease. Emori et al. (51) reported mutations in a subunit labeled Y13, which did not lower any of the proteolytic activities of the yeast proteasome but eliminated the latency of the casein-degrading activity. Possible regulation of proteolytic activity of proteasomes is suggested by several observations. Proteasomes were reported to be directly activated by ATP and other nucleoside triphosphates. Activity against small peptides was activated by nonhydrolyz-

able analogs of ATP, but activity against proteins required ATP hydrolysis (47). Since none of the core proteasome subunit sequences have ATP-binding consensus sequences, it seems likely that these effects were mediated through an auxiliary protein factor. Proteasomes were also reported to copurify with a protein kinase that phosphorylated 27- and 28-kDa subunits of the proteasome; however, no effects on the enzymatic activity of the proteasomes as a result of these modification were reported (180).

Other ATP-dependent protein degradation. ATP is used as an allosteric effector for RecA, which can promote an unusual self-cleavage reaction in several repressor proteins found in *E. coli*. This topic has been reviewed recently (80, 235) and will be described very briefly. The λ repressor (192), LexA (139), and UmuD (171) are stable in vivo but are inactivated following DNA damage by specific cleavage at an Ala-Gly bond in each protein. In a series of reports, Little and colleagues (138) have documented that degradation of LexA and other "substrates" for RecA occurs via a self-cleaving reaction that is promoted by binding between the protein and activated RecA in the presence of ATP. Since nonhydrolyzable analogs of ATP can be used, ATP must be required only to promote the proper conformation of RecA that can activate the latent proteolytic activity of the repressors. Although self-cleavage is probably rare, the RecA system illustrates several of the basic biochemical features of ATP-dependent proteolysis: ATP-dependent activation of the protease, an induced alteration of protein conformation, and allosteric activation of proteolytic activity.

Eukaryotic ATP-dependent proteases that are clearly distinct from both proteasomes and the 26S protease have been isolated from various sources such as the adrenal cortex (236) and liver (44) mitochondria, murine erythroleukemia cells (238), and chicken (56) and rabbit (53) skeletal muscle. Confusion arises because some of these proteins are similar in size ($M_r \approx$ 500,000 to 700,000) and some have not been purified sufficiently that their subunit compositions and sizes are known. The mitochondrial protease is a hexamer of 110-kDa subunits and resembles *E. coli* Lon in the activation of its protease activity by ATP and the activation of its peptidase activity by polyphosphates (44, 236). The chicken and rabbit skeletal muscle proteases, also called multipain, can degrade both ubiquitinated and nonubiquitinated proteins (53, 54, 56) in ATP-dependent reactions. The rabbit muscle protease resembles CF1, which was not previously reported to have proteolytic activity (53, 62), and can associate with proteasomes to form a proteolytic complex that resembles the 26S protease (53, 54). Whether this complex is identical to the 26S protease isolated from reticulocytes or an entirely different ATP-dependent protease is not completely clear. Finally, Driscoll and Goldberg (47) reported that activity of freshly prepared skeletal muscle or liver proteasomes could be activated by ATP in the absence of CF1 and CF2. Both protease and peptidase activities were affected. Goldberg has suggested that activation factors for ATP-dependent proteolysis by the proteasome may be tissue specific (72). Further characterization of these proteases should clarify some of these issues.

SPECIFIC TARGETS FOR PROTEOLYSIS IN PROKARYOTES AND EUKARYOTES

General Strategies

The number and nature of highly unstable proteins eluded analysis for many years. The very small amounts of these proteins and their accumulation only under particular physiological conditions made direct biochemical detection by general screening procedures difficult. In most cases, protein degradation may not play a significant role in regulating protein levels; in the absence of continued synthesis the intracellular concentration of even a stable protein can decrease relatively rapidly on cell growth. If protein turnover is to be an important contributing mechanism in regulating the availability of a protein, the half-life of the protein must be considerably shorter than the doubling time of the cell. Today we know that many of the highly unstable timing proteins are degraded more than 20 times faster than the doubling time of the cells.

Because instability of a timing protein is a major determinant of expression of its biological activity, interference with its degradation, for example by mutational removal of a protease, is likely to produce a recognizable phenotype for the cell. In prokaryotic cells, for which the analysis of mutations has been relatively straightforward, many of the best-understood examples of regulatory proteolysis have been elucidated by genetic analysis of suppressors of mutations in the protease itself. The targets of the ATP-dependent Lon protease (discussed below) were initially identified during studies of second-site revertants of *lon* mutants. Specific changes in some biological activity resulting from inhibition of new protein synthesis is another observation that may implicate a regulatory proteolytic mechanism. In *E. coli*, for instance, new protein synthesis is necessary for continued replication of bacteriophage lambda. The instability of the λ N and O proteins undoubtedly accounts in part for this requirement (82). Loss of biological activity in eukaryotic cells after brief treatment with inhibitors of protein synthesis has been used to provide evidence for unstable proteins (38).

Although restoration of biological activity is dependent on new protein synthesis following proteolysis, new synthesis would be required after irreversible inactivation by any mechanism. For example, the DNA repair methyltransferase Ada protein of *E. coli* carries out a suicide demethylation reaction so that a single protein molecule is used to repair a single DNA lesion (see reference 198 for recent review of this subject). Since irreversibly inactivated proteins are likely to be degraded in cells, proteases probably play a role, if only an auxillary one, in all irreversible losses of protein activities.

Below we discuss a number of instances in which the rapid degradation of a timing protein is an important aspect of the regulation of its biological activity. The discussion does not include all examples of rapidly degraded proteins but is focused on systems about which the greatest amount of information is available and on systems that best demonstrate the range of biological functions regulated in this manner.

Targets of Lon ATP-Dependent Protease

Mutations in the *E. coli lon* gene, which codes for the ATP-dependent Lon (La) protease, produce two easily detectable phenotypes. Consequently, genetic selection and screening procedures have led rather directly to the identification of the targets for Lon protease and the regulatory circuits and metabolic pathways in which these timing proteins participate. Lon-dependent regulation has been reviewed elsewhere and will be discussed only briefly here to allow comparisons with other systems regulated by proteolysis. In fact, *E. coli* Lon substrates provide a reasonably

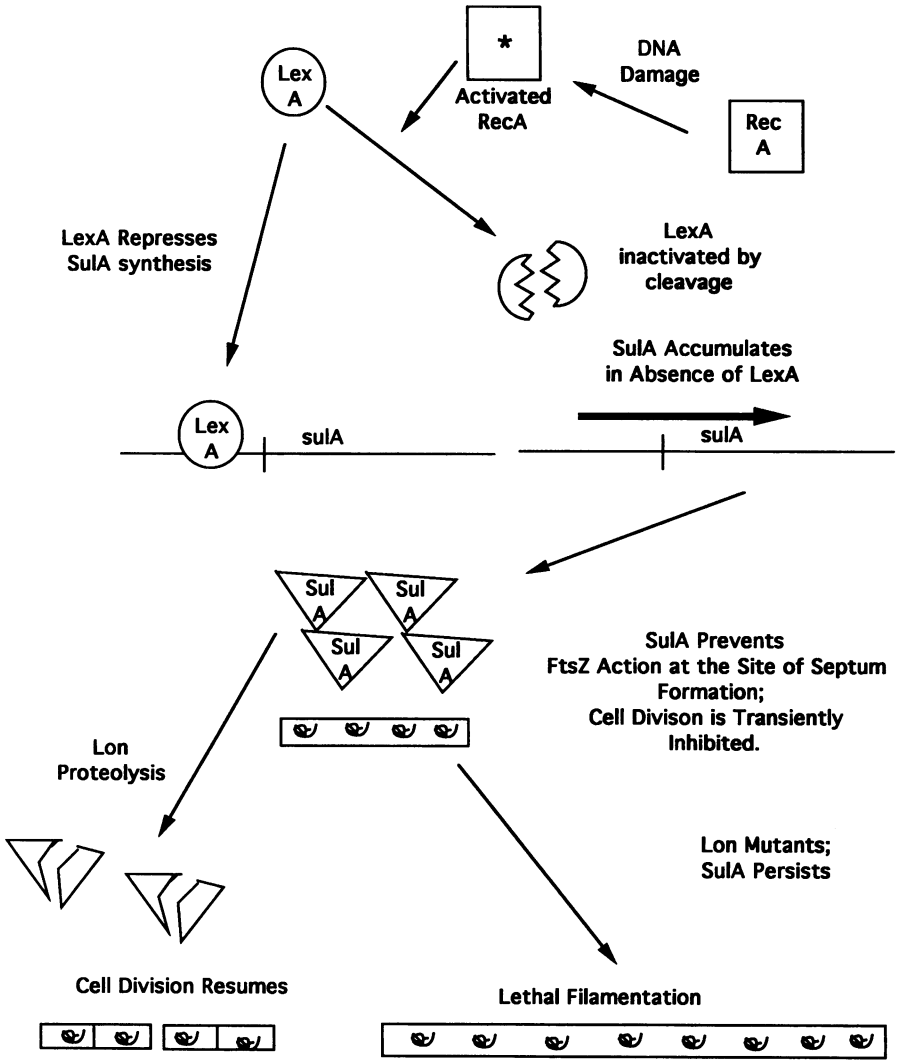

FIG. 5. Role of the unstable division inhibitor, SulA. SulA synthesis is regulated by the LexA repressor, which is itself destroyed by proteolysis after *E. coli* cells are treated with DNA-damaging agents such as UV or mitomycin C. SulA is highly unstable in *lon*$^+$ cells; the half-life of the protein is slightly more than 1 min, so that SulA does not persist long after DNA damage is repaired and new SulA synthesis stops. The SulA half-life in *lon* mutants (right bottom of figure) is significantly longer (more than 20 min), so that SulA inhibition of cell division persists beyond a point at which it becomes irreversible (162).

representative example of the types of proteins targeted for rapid degradation and the functional role of these proteins in cellular regulatory networks of both prokaryotic and eukaryotic cells.

SulA: limiting time of action. One of the phenotypes of *lon* mutants is sensitivity to DNA-damaging agents such as UV light or methyl methanesulfonate. DNA damage is known to induce synthesis of a set of genes regulated by the LexA repressor, as part of the repair process known as the SOS response (see reference 235 for a recent review of this subject). One of the induced genes, SulA (or SfiA) is a reversible inhibitor of cell division; cells that overproduce SulA fail to put septa in elongating cells (110, 202). After several generations of growth, cells producing SulA form long, unseptated filaments; if unreversed, this leads to cell death. Because SulA is extremely unstable, with a half-life of about 1 min in *lon*$^+$ cells, the protein accumulates to inhibitory levels only when synthesis is increased significantly by full induction of the SOS response (162). As DNA damage is repaired in the cell (a function of other SOS-induced genes), repression of the SOS genes is restored. Once SulA synthesis slows, levels of SulA would be expected to fall rapidly, given its very short half-life. In fact, wild-type cells form filaments transiently after UV treatment, but after a few hours, the filaments resolve, septa form in the elongated cells, and normal cell division resumes. In *lon* mutant cells, however, the half-life of SulA is considerably longer (more than 20 min) and the SulA that accumulates during SOS induction persists for much longer times (162). As a result, *lon* mutant cells induced for the SOS response can repair their DNA normally, but they fail to recover from the normal transient filamentation and instead form long, nonviable filaments (Fig. 5). Therefore, SulA is an excellent example of an emergency response timing protein, one whose activity is required for a transient period but is inconsistent with normal cell growth and whose instability is

necessary to allow the cells to recover from the action of the protein.

Genetic and biochemical studies indicate that the target of SulA action is FtsZ, an essential cell division protein. During cell division, FtsZ accumulates in annular rings at the point of septation and is thought to be necessary for proper septum formation (15). Specific mutations in *ftsZ* or overexpression of wild-type FtsZ protein renders cell division resistant to SulA action (122, 142, 143). In addition, the in vivo stability of SulA is increased in the presence of excess FtsZ (13, 121). These results suggest that SulA interacts directly with FtsZ and inhibits septation by blocking binding of FtsZ to the septation apparatus or otherwise interfering with the activity of FtsZ at the point of septation. The normal rapid reversibility of filamentation suggests that SulA does not irreversibly modify or inactivate FtsZ (144).

Reversal of FtsZ inhibition after SulA synthesis stops presumably depends on the degradation of both free SulA and SulA complexed with FtsZ. SulA from a SulA-FtsZ complex will be degraded rapidly if the complex is in rapid equilibrium or more slowly if SulA remains in the complex (somewhat less accessible to Lon). If the SulA remains in the complex until it is degraded, the timing of the resolution of filaments might be controlled separately from the timing of control over the synthesis of SulA and other SOS functions.

Comparison of SulA as a transient inhibitor of cell division with two other recently identified cell division inhibitors, MinC and MinD, which are encoded by the minicell *minB* locus of *E. coli*, and *dicB*, which is encoded by part of a cryptic lambdoid prophage in *E. coli*, provides some perspective on the choice between unstable and stable regulators. The *minB* locus, which encodes three proteins, is responsible for targeting septation in growing cells to a new central septum rather than to previously used polar septum sites (39; reviewed in reference 195). In the absence of the Min functions, septa form at the poles as well as in the center of the cell, giving rise to DNA-less cells (minicells). The *min* genes work in a peculiar fashion: *minC* and *minD* encode an inhibitor of septation, which may, as with SulA, have FtsZ as its target, and MinE, the third protein encoded by the locus, blocks the inhibition specifically at the central septum site, leaving septation at the polar sites inhibited (14). DicB seems to act as an analog of MinD, to activate MinC for septation inhibition; however, the inhibition of septation by DicB/MinC is irreversible at all septum sites (MinE cannot act), so that cells form filaments and die (40). As with SulA, DicB is not normally expressed by growing cells (12). There is no indication that the MinC-based septation inhibition is reversible, as SulA inhibition is, although mutations in *ftsZ* which lead to resistance to SulA action are also resistant to MinC action (14). An absence of obvious homology between SulA and MinC confuses the situation further, but the similarities in action suggest a number of conclusions: (i) FtsZ is an effective target for blocking septation without interfering with other cellular functions; and (ii) such blockage can be transient, as part of a reversible emergency response, or more permanent, as part of the normal septum placement mechanisms.

The determining factor in the transience or permanence of the septation inhibition is the half-life of the inhibitory protein. MinC, a stable inhibitor, is required continuously to avoid improper septation, whereas SulA, an unstable inhibitor, is required only transiently. Proper septation during normal cell division is accomplished by site-specific blocking of MinC action by MinE. It is not yet clear whether MinE blocks MinC and MinD inhibition at the central septum site by preventing initial access of MinC to its site of action or by reversing the inhibitory action of MinC at that site. Since MinE action is site specific, which is not the case for SulA action, prior interference by MinE binding at the central septum might be the most direct way to provide location specificity.

TABLE 2. Effect of protein-protein interactions on degradation of lon substrates

Substrate	Half-life (min)		
	lon$^+$ cells	*lon* cells	*lon*$^+$ cells with plasmid[a]
SulA[b]	3	30	10–14
RcsA[c]	3	30	10

[a] Turnover of the protein in cells containing a plasmid overproducing the indicated functional protein was measured. For SulA, the functional protein was FtsZ; for RcsA, the functional protein was RcsB.
[b] Data from references 23 and 121.
[c] Data from reference 217.

Might the cell use a transient inhibitor such as SulA to time cell division? SulA itself is not essential for *E. coli* growth and cell division and probably functions only to allow an SOS-induced pause in septation. A similar protein acting as a timer for septation during the normal cell cycle would have to have its synthesis regulated in concert with a cell cycle event (possibly coupled to replication of the timing gene) and/or its degradation regulated in a cell-cycle-specific manner. If synthesis is regulated, we would imagine that the putative unstable timer is made in sufficiently small quantities at specific times to act for only a short period, before its concentration is depleted by degradation. No evidence for such an unstable coupling factor in *E. coli* has been found; however, relatively little is known about the molecular basis for integrating different parts of the *E. coli* cell division cycle. In contrast, the cyclin regulators of the eukaryotic cell cycle show both regulated synthesis and degradation, and they clearly act as important cell cycle timers (see below).

RcsA: unstable regulatory protein. The other obvious phenotype of *lon* mutant cells is overproduction of capsular polysaccharide (see reference 216 for a recent review). The capsule, which is composed of colanic acid, is made at only low levels in wild-type *E. coli*, but its synthesis increases significantly in *lon* mutants, covering the colonies with a viscous, mucoid slime, particularly at low temperatures and on minimal media. The increase in capsule synthesis in *lon* mutants is mediated by accumulation of RcsA, a positive regulator of transcription of the *cps* genes necessary for capsule synthesis. RcsA is essentially undetectable in *lon*$^+$ cells, in which it has a very short half-life, but accumulates to detectable levels in *lon* mutants, in which its half-life is significantly longer (217, 229). Increasing the rate of synthesis of RcsA also results in increased capsule production, further suggesting that capsule synthesis depends on the level of RcsA and that Lon acts solely by limiting the accumulation of RcsA (210).

The ability of RcsA to activate transcription of *cps* genes depends on a second positive regulator, RcsB, a cytoplasmic protein essential for capsule expression. RcsB activity in turn is regulated by a membrane sensor protein, RcsC, which presumably responds to some environmental signal, as yet unknown, to modulate capsule production. RcsA and RcsB probably form a complex to activate *cps* expression. Interaction between RcsA and RcsB is suggested by the ability of overexpressed RcsB to partially protect RcsA from

degradation in vivo, similar to the partial protection of SulA by FtsZ (Table 2) (217). Also, mutations in *rcsA* (RcsA*) which lead to increased expression of the *cps* genes are best explained by a stronger interaction of RcsA* with RcsB, because both increased transcriptional activation and increased stability of RcsA* are dependent on the presence of excess RcsB in the cell.

Changes in capsule synthesis can be mediated through RcsA or RcsB. Although activation of RcsB through the sensor RcsC is probably analogous to other sensor-effector mechanisms (reviewed in references 4 and 215), increasing the activity of RcsA depends on altering the availability of the protein. Changes in the rate of synthesis would affect the concentration of RcsA. Because RcsA is turned over so rapidly, a transient increase in RcsA synthesis will lead only to a transient activation of *cps* synthesis. Signals that modulate RcsA synthesis have not yet been identified, although preliminary evidence suggests the existence of a regulatory cascade for controlling RcsA synthesis (210). Also, RcsC-mediated changes in RcsB might affect its interaction with RcsA, altering RcsA stability and thereby modulating the environmental signal. RcsC-dependent phosphorylation of RcsB, predicted from the homologies between RcsB and RcsC and other sensor-effector pairs, is also likely to be a transient signal. Therefore, the capsule regulators appear to be poised to respond rapidly but transiently to an increased requirement for capsule synthesis.

RcsA is a reasonable model for the unstable regulatory proteins we have called timing proteins; other examples are discussed below. Its interaction with its target, as well as its synthesis, is regulated. Mutations which increase interaction between RcsA and its target result in increased activity and increased stability of RcsA, but only in the presence of the target. Small changes in the interaction of RcsA and RcsB or in RcsA amounts or stability can apparently have large effects on capsule synthesis.

Tn*903* transposase: unstable protein with limited site of action. The transposase encoded by the Tn*903* transposon, in common with similar proteins encoded by other transposons, preferentially acts in *cis*, on the ends of the transposon which encodes it (43). At least some part of the inability to act in *trans*, on transposon ends located elsewhere on the chromosome or on plasmids, is due to the instability of the transposase itself. Diffusion through the cytoplasm to other sites exposes the protein to rapid degradation, presumably limiting its concentration at these other sites. Mutations in *lon* and fusions of the transposase to β-galactosidase both stabilize the protein and increase its ability to act in *trans* (43). The *cis* action of transposase presumably ensures preferential activity at nearby sites of action within the transposon, rather than at other copies of the insertion sequence or transposons elsewhere in the chromosome. Therefore, this short-lived protein is limited in space as well as time.

One can easily imagine similar mechanisms for limiting the range of action of other proteins that are required to act at sites near the genes encoding the proteins. Echols et al. (49), in discussing the instability and apparent preference for *cis* action of a number of lambda proteins, have suggested that part of the characteristic of metabolic instability and *cis* action of these proteins is due to their ability to bind to nonspecific sites on the DNA. Given that we now know that proteins such as transposase are actually degraded, it seems possible that these two characteristics combine to contribute to *cis* action. If, for instance, transposase is degraded more readily when bound to incorrect sites than when bound to its correct sites, the time available for degradation before such proteins reach *trans* sites will include the time spent on the DNA at incorrect sites. If the available space for diffusion is large enough, one might expect a gradient of decreasing protein concentration away from the site of synthesis of such an unstable protein; in this case, we may not need to invoke other mechanisms for slowing the travel of such proteins. Such gradients have in fact been observed for the unstable eukaryotic developmental regulators, such as Ftz, discussed below.

Additional roles for Lon. The *lon* targets described here undoubtedly do not represent the full range of its natural substrates. Winkler has recently described an effect of *lon* mutations in *E. coli* on the growth of cells containing insertions which are polar on a gene of unknown function, named *dpj*, which is best explained by assuming that a Lon target protein can substitute for the Dpj product (133). Gill et al. have identified a homolog of *E. coli* Lon with a role in the regulation of sporulation and fruiting-body formation in the gram-negative bacterium *M. xanthus* (68). The *Myxococcus bsgA* gene, identified because mutations in it make cells defective early in the developmental pathway to sporulation, encodes a protein with extensive amino acid similarity to *E. coli* Lon (68). The purified BsgA protein, like Lon, catalyzes ATP-dependent degradation of casein. Gill et al. predict that a substrate of BsgA must be depleted or kept at low levels by rapid degradation for the sporulation cascade to proceed. Therefore, future studies on revertants of *bsgA* should identify possible targets for BsgA (Lon). In fact, Gill et al. reports preliminary characterization of an extragenic suppressor of a *bsgA* null mutation which not only restores fruiting-body formation and sporulation but also allows the inappropriate expression during vegetative growth of a gene normally expressed only during sporulation (68). Thus, the suppressor may encode a negative regulator which is normally destroyed by proteolysis during fruiting-body formation. If so, it will be interesting to determine whether the switch from vegetative growth to sporulation/fruiting-body formation involves a change in the levels of synthesis of the putative substrate, an increase in degradation at the onset of development, or both. The recent identification of a human cDNA encoding a Lon-like protein (1) further supports the notion that proteases similar to Lon may play central roles in developmental pathways in many other organisms.

Role of Lon in degradation of abnormal proteins. The original identification of the *lon* gene product as a possible protease stems from the finding by Bukhari and Zipser (22) that *lon* mutants arose in a selection for mutations which allowed intramolecular complementation by unstable fragments of β-galactosidase and that, in the *lon* mutant cells, these fragments were stabilized. Later work demonstrated that a variety of temperature-sensitive mutations could be suppressed by *lon* mutants and that suppression could be correlated with stabilization (84, 87). Many temperature-sensitive and osmotically remedial *Salmonella* mutants can be suppressed by introducing a *lon* mutation into the host, suggesting that these temperature-sensitive proteins are unstable (134). Abnormal proteins made after treatment of cells with amino acid analogs and chain-terminating antibiotics such as puromycin, as well as cloned foreign proteins, are subject to rapid proteolysis in *E. coli*. *lon* mutations significantly slow (but do not abolish) the turnover of these abnormal proteins (152), and increasing the cellular content of Lon increases the rate of degradation of abnormal proteins (72).

Degradation of abnormal proteins by Lon seems to require

the participation of the heat shock chaperone proteins, since mutations in *dnaK*, *dnaJ*, *grpE*, and *groE*, all of which encode chaperone proteins, can have dramatic effects on turnover of abnormal proteins, in some cases reducing degradation significantly more than do *lon* mutations (127, 218). This may suggest that these heat shock proteins are directly involved in presenting abnormal protein substrates to Lon or that these chaperone proteins alter the solubility and therefore the accessibility of abnormal proteins. The *Salmonella* conditional mutants which can be suppressed by *lon* mutations can also be suppressed by providing increased amounts of the molecular chaperones encoded by the *groE* locus (232). This observation suggests that excess GroE allows these unstable proteins to fold properly and therefore escape degradation. At least in vivo, when additional proteins such as the heat shock chaperone proteins may contribute to the ability of Lon to recognize its substrates, the motifs used by Lon may be general enough to appear in most proteins, presumably within usually inaccessible regions of the proteins.

Conditional Degradation

The *lon* targets described above appear to be degraded under essentially all circumstances tested; one can consider them constitutively unstable. In these cases, changes in synthesis levels are likely to be primarily responsible for changes in protein availability. For a number of interesting protease targets, however, degradation varies dramatically under different conditions, so that both degradation and synthesis may be targets for regulation.

Cyclins: eukaryotic timing proteins with limited time of action. The last few years have provided an explosion of new information on the eukaryotic cell cycle, including strong evidence that many of the central elements of the cell cycle are conserved between lower and higher eukaryotes. Among the conserved regulatory proteins in all cells is a family of short-lived proteins called the cyclins, because their abundance in the cell oscillates in concert with each cell cycle. First identified in rapidly dividing *Xenopus* eggs and clam oocytes, the cyclins are a component of maturation-promoting factor, whose activity accumulates during part of the cycle and then begins to disappear rapidly just before mitosis. The loss of activity of maturation-promoting factor correlates with degradation of cyclin (166; reviewed in references 165, 167, and 172). Murray and Kirschner isolated mutants of cyclin, deleted in the amino-terminal region (see below), that are both metabolically stable and active (166). These mutant cyclins disrupt the cell cycle and arrest cells in metaphase. Therefore, wild-type cyclin acts to stimulate division, and its degradation is an essential feedback step for advancement to the next phase of the cell cycle (166). In fact, cells carrying both stable and unstable cyclins have lost control of the timing for cyclin degradation; wild-type cyclin is degraded continuously in such cells, suggesting that cyclin may play a role in signaling its own degradation.

The information required for cell-cycle-specific degradation of cyclin is also sufficient to target other proteins for periodic degradation, since fusion proteins carrying amino acids 13 to 90 from sea urchin cyclin B are degraded in extracts with the same pattern as cyclin itself (69). This provides the most complete evidence that all information necessary for targeting for degradation can reside within a relatively short stretch of amino acids.

A variety of recent evidence suggests that cyclin degradation is dependent on the energy-dependent ubiquitin-targeting system. Ubiquitin-tagged cyclins can be detected in cells at the time of cyclin degradation and are not seen when stable derivatives of cyclin are used (69). Ubiquitin derivatives that are unable to form polyubiquitin trees slow cyclin degradation in a cell-free system (100).

What remains unclear, however, is what changes in the cell to make the cyclins a target for degradation at the appropriate time. Since cyclin synthesis can also be regulated, accumulation may or may not be sufficient to initiate degradation. It seems likely that some additional modification of the proteins by one of the kinases also involved in the cell cycle progression might act as a switch to turn on degradation at the correct time (197). Recent work suggests that of the two classes of cyclins found in clam oocytes, cyclin A and cyclin B, cyclin B is responsible for initiating the degradative cycle. If stable derivatives of either cyclin A or cyclin B are added to lysates which are arrested at a stage at which cyclin degradation is normally low, cyclin B but not cyclin A activates the degradation of the endogenous cyclins, suggesting that some function of cyclin B specifically signals the degradative cascade (141).

Regulation of cyclin degradation, combined with regulation of cyclin synthesis, allows rapid and dramatic changes in the availability of these proteins. One of the striking characteristics of cyclin turnover is that rapid degradation itself is conditional, presumably dependent on some modification only present during one part of the cell cycle.

Other timing proteins showing conditional degradation are discussed below. For LexA, the activity of the protease changes to allow rapid degradation of the otherwise stable repressor in response to stress. For HtpR, transient stabilization of this highly unstable protein serves to initiate and magnify a stress response; the basis for stabilization is not yet understood. In plant cells, light-induced changes in phytochrome serve to convert it to a metabolically unstable protein. The tumor suppressor p53 becomes subject to rapid degradation when complexed with an oncoprotein. Taken together, these cases of conditional degradation demonstrate models for controlling protein function through degradation. Conditional degradation allows the cell to dispose of a particular regulatory protein rapidly and switch completely to a new regulatory mode; if synthesis of the regulator is continuous, the original regulatory mode will be restored rapidly when degradation slows.

LexA: regulator of DNA damage stress response. A special case of instability of a regulatory protein is the DNA damage-dependent cleavage of LexA in *E. coli*. LexA is a stable repressor of a large number of genes involved in the repair of DNA damage; *sulA*, the gene encoding the unstable cell division inhibitor discussed above, is under LexA repression (Fig. 5). When the cell encounters DNA-damaging agents such as UV light, however, LexA is rapidly inactivated by cleavage at a specific Ala-Gly bond that separates the DNA-binding amino-terminal region from the rest of the molecule. Degradation is dependent on LexA itself and on the RecA protein, which must be activated by a DNA damage-induced signal. Binding of activated RecA to LexA switches LexA from a stable to an unstable form. In this case, as opposed to the others discussed here, the active site for the protease necessary for the single cleavage lies within LexA itself. In vivo, activity of this cryptic protease requires energy consumption and the participation of activated RecA (235).

RecA works with LexA to contribute to its degradation. Lambda and lambdoid repressors are also cleaved during this response, leading to prophage induction, and these

proteolytic cleavages are also dependent on active sites within the repressors themselves. UmuD, a protein synthesized when LexA repression is relieved, depends on RecA-dependent proteolytic processing for its activity; as in the other cases, cleavage of UmuD is dependent on active-site residues within UmuD (171). Therefore, a single ATP-dependent activator protein (RecA) can interact with a number of different "protease" subunits to mediate autodegradation of each specific protein.

HtpR: unstable regulator of prokaryotic heat shock response. The heat shock sigma factor of *E. coli*, sigma 32, encoded by the *htpR* (also called *rpoH*) gene, is responsible for the synthesis of the major heat shock proteins including the chaperone proteins DnaJ, DnaK, GrpE, and GroEL, as well as Lon, ClpP, and ClpB (129, 132, 169, 214). Synthesis of these heat shock proteins is transiently increased after a sudden rise in temperature; this increase in synthesis is correlated with an increase in the amount of sigma 32 in the cell (reviewed in reference 36). Increased accumulation of sigma 32 is dependent on changes in regulation at multiple levels, one of which is the transient stabilization of this normally highly unstable protein (219). The mechanism of this stabilization and its importance in mediating the heat shock response await the identification of mutations, either in sigma 32 itself or in the responsible protease, that stabilize the protein. Although mutations in some of the heat shock genes lead to stabilization, it has been unclear whether this is a direct or an indirect effect; it is not known whether sigma 32 degradation is energy dependent. A current model for stabilization of sigma 32 postulates that heat shock leads to inactivation or inhibition of the responsible protease, possibly because the protease or some essential component of the protease binds to the increased amounts of heat-denatured proteins, and that, after the heat shock response, increased synthesis of the protease restores rapid degradation of sigma 32 (36). In this model, the protease itself or some essential component of the protease should be a heat shock protein-present in limiting amounts. Another possibility, for which there is not yet any evidence, is that sigma 32 is stabilized by increased binding to its target, RNA polymerase core protein. To support this model one would have to demonstrate a mechanism for increased binding after heat shock, for example, a modification in sigma 32 itself or a change in sigma 70 that weakens its interaction with core polymerase.

Light-regulated conditional degradation in plants. Phytochrome, a major plant protein involved in seed germination, chloroplast development, and flowering, exists in two photointerconvertible states with different biological activities and different metabolic lifetimes (reviewed in reference 183). Red-light-activated phytochrome (Pfr) is the physiologically active form but is also subject to rapid degradation. Formation of Pfr is followed rapidly by conjugation of Pfr to ubiquitin, and kinetic studies suggest that the ubiquitin-conjugated Pfr is the target for degradation (115, 207). Since conversion of phytochrome to Pfr both activates it and increases its turnover rate, degradation appears to be designed to modulate the physiological responses to this regulatory protein. Degradation of Pfr limits the length of time that activity can be expressed and ensures that continued expression of activity, which would require new synthesis of phytochrome, can be subject to renewed regulation. What advantages this mechanism of modulating Pfr activity has over conversion to the inactive form, Pr, is not known.

Oncoprotein stimulation of p53 degradation. The p53 tumor suppressor has a relatively short half-life (20 to 40 min) (175). Although some oncogenic proteins seem to both inactivate and stabilize p53, infection with some types of human papillomavirus leads to the formation of complexes between the E6 oncoprotein encoded by the virus and p53, resulting in the increased, ubiquitin-dependent degradation of p53 in reticulocyte extracts and reduced accumulation of p53 in vivo (200). These observations suggest either that an essential element for protease recognition is provided by the complexed E6 or that E6 interaction changes the conformation of p53 sufficiently to allow rapid degradation. Given that the half-life of p53 is relatively short even in normal, uninfected cells, it may be reasonable to assume that all essential degradation recognition elements are present but that E6 may increase their accessibility.

Unstable Regulators and Developmental Switches

Oncogenes. Many cellular and viral oncogenic proteins, including Myc, Myb, and Fos, have been shown to have extremely short half-lives in vivo; degradation of c-Myc has been shown to be energy dependent (4, 184). When Myc and Fos were synthesized in a cell-free system containing ubiquitin-conjugating activity, they were degraded. Degradation was stimulated by ATP and inhibited by antibodies against the ubiquitin-conjugating protein, E1 (32). Thus, these proteins, like the cyclins, are subject to ubiquitin tagging and ubiquitin-dependent degradation, at least when synthesized in vitro, when proper folding and assembly might be slow.

Ftz: developmental timing protein limited in time and site of action. The fushi tarazu (*ftz*) gene of *Drosophila* species (128) encodes a DNA-binding protein that serves as a regulator of essential developmental genes. The sequence of Ftz reveals the presence of a homeo box, suggesting that it acts as a regulator of protein expression. Ftz is expressed in cells that are found at the boundaries of parasegments and is involved in the formation of segments in the developing embryo. Mutations that interfere with Ftz activity result in a failure to form boundaries and a loss of structures from even-numbered parasegments.

The wild-type Ftz protein is highly unstable; after cycloheximide treatment, most of the Ftz protein decays with a half-life of about 6 min (128). In vivo degradation of Ftz is energy dependent and may be due to the ubiquitin-dependent degradative system. Mutations in *ftz* that increase the half-life of Ftz more than sixfold have been identified. These mutations are dominant and lead to the accumulation of increased amounts of Ftz. Mutants with more stable Ftz have a phenotype similar to that previously seen in mutants called anti-*ftz*, which overproduce the protein severalfold; they show homeotic transformations of some segments and defects in odd-numbered segments, which are not affected by *ftz* loss of function mutants. The dominant, stabilizing mutations in Ftz change prolines in a stretch of the protein which has similarities to a region of Myc and other short-lived regulatory proteins, including another pair-rule protein called Eve (128). Eve is responsible for odd-numbered rather than even-numbered parasegments. Whether the corresponding changes would affect the stability of Myc or Eve is not yet known. It is also unclear whether the increased stability of the mutant proteins is due to a direct change in protease recognition of the Ftz protein or to increased interaction of the mutant protein with other, thus far unidentified, proteins, leading indirectly to increased stability and increased activity.

MATα2. α2 is an unstable developmental timing protein of *S. cerevisiae*. Like Ftz, α2 has a homeotic DNA-binding domain (211), but unlike Ftz, which is a regulator for a

unidirectional developmental pathway, α2 regulates genes which must switch on and off under different conditions. α2 is necessary for repression of type **a**-specific genes in α cells and in diploid **a**/α cells. Repression of **a**-specific genes must be relieved whenever the mating type cassette at the *MAT* locus switches from **a** to α or in **a** cells derived from **a**/α diploids. In the case of switches at the *MAT* locus, synthesis of α2 ceases and expression of the genes that α2 normally represses increases within 90 min. Decreased synthesis of α2 and the dilution of preexisting α2 is not sufficient to explain the rate of the decrease in repressive activity of α2. Hochstrasser and Varshavsky (106) demonstrated that the α2 protein has a half-life of only 5 min, indicating that the repressor is depleted rapidly when its synthesis stops. The continuous degradation of α2 and Ftz, both homeotic developmental regulators, ensures that expression of activity will be immediately dependent on the rate of synthesis of the protein. Degradative control may be especially important in developmental pathways in which specific regulatory proteins must be eliminated once a switch toward a new cell type has been made.

Regions of α2 which might target the protein for degradation were defined by using deletions of an α2–β-galactosidase fusion protein (106). Two regions within the protein, one at the front end and one including the DNA-binding region, are each sufficient to cause instability of the fusion protein. Host mutations which stabilize fusion proteins containing only the amino-terminal signal have been identified, and they also stabilize α2, confirming the role of this N-terminal signal in targeting wild-type α2 degradation. In addition, using an epitope-marked ubiquitin derivative, Hochstrasser et al. (105) have been able to demonstrate that ubiquitin becomes conjugated to α2. Although host mutants which disrupt the N-end recognition system of *S. cerevisiae* (see below) still degrade α2 rapidly, mutants which block multiubiquitination lengthen the half-life of α2 (105).

Prokaryotic developmental switch: *c*II of bacteriophage lambda. A prokaryotic analog for α2 is provided by the lambda lysogenization regulator *c*II, which is required to establish the lysogenic state for an infecting lambda phage in *E. coli*. *c*II activates the *pre* promoter, which provides a burst of *c*I repressor synthesis during establishment of repression and also activates the *p*I promoter for integrase protein, which is needed for the stable integration of the repressed prophage into the bacterial chromosome (reviewed by Wulff and Rosenberg [246]). *c*II has a half-life of <1 min in vivo, so that it is available only during the brief period when early genes are transcribed rapidly before repression is established. Additional complexity and control are provided by a third protein required for efficient lysogenization, *c*III. In the absence of *c*III, lambda lysogenization is decreased 100-fold. *c*III may act primarily by stabilizing *c*II, because the half-life of *c*II is increased somewhat when *c*III is present. Protection of *c*II may be the primary mechanism of *c*III action during lambda lysogenization, which would argue that stabilization of *c*II should be critical in tilting the balance between lysogeny and lytic growth toward lysogeny.

E. coli host mutants which lysogenize efficiently even in the absence of *c*III contain mutations in either of two loci, called *hflA* and *hflB*. *c*II has a longer half-life in *hfl* mutants. The *hflA* locus consists of three genes, *hflK*, *hflC*, and *hflX*; purified HflK and HflC can degrade *c*II in vitro (10, 27). Mutations in *hflA* cause multiple changes in the two-dimensional pattern of proteins synthesized in *E. coli*, suggesting a general role for HflA in cell metabolism (26). One puzzling aspect of the in vitro degradation of *c*II by the Hfl system is the absence of an ATP requirement or even activation by ATP. Energy dependence is an essentially universal characteristic of the cytoplasmic degradation of unstable proteins thus far studied. Since the energy dependence of *c*II degradation in vivo has not yet been reported, it is unclear whether the lack of an ATP requirement in vitro reflects the absence of an essential protein component or an exceptional mechanism of degradation of an unstable timing protein.

*c*III has been postulated to act as a general inhibitor of proteolysis, because overproduction of *c*III leads to stabilization of sigma 32 and induction of the heat shock response (9). To explain this phenotype, one need only postulate that *c*III inhibits the thus far unidentified protease primarily responsible for sigma 32 degradation. Since *hfl* mutations do not stabilize sigma 32, the Hfl protease does not seem to be the target of *c*III. The ATP-dependent proteases, Lon and Clp, are also both unlikely targets for *c*III inhibition since *c*II is degraded in mutants lacking these proteases.

Degradation as part of a lasting commitment: mechanism for plasmid addiction systems. Plasmids frequently have multiple mechanisms for ensuring faithful transmission of the correct number of plasmid copies to dividing cells. These mechanisms include replication control, plasmid partitioning, and, in addition, back-up mechanisms for inhibiting growth of cells that lose the plasmid. The systems responsible for this last mechanism have been called addiction systems by Yarmolinsky (247), because host cells become inviable when the plasmid is withdrawn. The addiction systems studied so far are believed to have at least two components: a stable killer function and an unstable element that prevents expression of the killer function or acts as an antidote to it (64, 161). F, R1, and R100 plasmids and the plasmid prophage P1 possess addiction systems in which the killer and the antidote appear to be proteins (161, 231, 247). Both killer and antidote are encoded by the plasmid and are synthesized while the plasmid is present in the host. When the plasmid is lost from a cell, new synthesis of killer and antidote is no longer possible. Because the killer persists longer than the antidote, the cell that has lost the plasmid (or perhaps its progeny) will be killed. Direct evidence for the turnover of an antidote protein has thus far been found only for R100 (231), leaving open the possibility that antidote activity is lost in some cases by a mechanism other than degradation. For a number of addiction systems, including a second addiction system in plasmids F and R1, the antidote has been shown to be an unstable antisense RNA which acts posttranscriptionally to block the synthesis of killer from a stable mRNA encoding the killer protein (64).

These addiction systems provide intriguing models for the use of an unstable inhibitor to temporarily mask the action of a second, stable protein. When synthesis of the unstable inhibitor ceases (as happens in the addicted cell on loss of the plasmid, or might occur for an inhibitor encoded by a developmentally regulated gene), the rapid disappearance of the inhibitor will result in a burst of expression of a previously stockpiled but inhibited function. The use of such an unstable inhibitor is reminiscent of the decay of SulA, the SOS-induced inhibitor of cell division discussed above (Fig. 5). The recovery of *E. coli* from the effects of the SOS-induced cell division inhibitor SulA illustrates this model. Filaments have DNA nucleoids and incipient septa distributed along their length and are poised for rapid multiple and simultaneous cell divisions when DNA damage is repaired and SulA synthesis ceases. It may be that unstable inhibitors of this sort function to hold development systems poised to

respond to signals for transition to the next stage of development.

Multimeric Complexes: Loss of Free Subunits by Degradation

Many otherwise stable proteins that are found in multicomponent complexes appear unstable when they are not associated in the complex. The presence of free subunits might occur as a result of unequal synthesis of the components of the complex, delays in the assembly of the complex, or the presence of factors or conditions that affect the final stoichiometry of the complex or the affinity of the components. Most adjustment of the stoichiometry of protein complexes is accomplished by coordinate regulation of synthesis of the subunits; for example, ribosomal proteins are under feedback control of synthesis that normally avoids excess synthesis of unneeded subunits (117). As with most examples of degradation of free subunits of multimeric complexes, ribosomal subunits are degraded only when their proper stoichiometry is unbalanced by cloning or mutation of individual subunits (181).

In some cases, however, subunits are not coordinately synthesized or the rate of assembly is limited. The unassembled forms of α- and β-spectrin are both unstable, and the more rapid degradation of the β-spectrin implies that degradation is important in the topogenesis of the erythroid membrane cytoskeleton (135, 245). Subunits of the T-cell receptor are unstable when expressed in the absence of the other components of the complex, and in some normal cells the low level of ζ-subunit expression results in turnover of nearly 90% of newly synthesized receptor (130). In *E. coli*, the regulatory subunit of Clp protease, ClpA, is degraded whereas the proteolytic component, ClpP, is not, suggesting that degradation of ClpA may be used to adjust the stoichiometry of the two components (81). Dissociation of a complex in response to metabolic effectors would also give rise to free subunits. The catalytic subunit of the cAMP-dependent protein kinase is degraded when the regulatory subunit dissociates in response to cAMP binding (5). Whether this mechanism is generally important has not been ascertained, but it is possible that degradation is used to modulate the activity of a complex whenever the components must dissociate and come back together during an activation cycle. An additional advantage of the degradation of excess subunits by the processive energy-dependent proteases is that the processivity of the degradation will help ensure that protein domains or subunits with partial activity do not accumulate in the cell.

DEGRADATION SIGNALS AND SELECTIVITY OF PROTEOLYSIS

How do proteolytic systems distinguish between proteins so as to degrade appropriate substrates and avoid damage to other cellular proteins? Appropriate targets for proteolysis include both naturally unstable proteins, which can be defined as wild-type proteins that are genetically programmed for degradation under physiological conditions, and abnormal proteins, which are proteins with structures or sequences unlike those found in the wild type. Abnormal proteins include missense and nonsense mutant proteins, incorrectly synthesized proteins, proteins that have incorporated amino acids analogs, misfolded proteins, proteins damaged by heat or chemical reactions, many foreign gene products, and excess subunits of multimeric proteins arising from cloning or genetic manipulations. Degradation of both classes of proteins is very rapid, indicating that abnormal proteins are recognized as efficiently as naturally unstable proteins, and selective, since it occurs in the presence of a large excess of cellular proteins that are not degraded.

There are three major considerations that should help define the basis for selectivity of protein degradation. First, not all naturally unstable proteins are degraded by the same protease or targeted by the same degradative system. As mentioned above, Lon protease degrades SulA and RcsA but not sigma 32 or the λ O or *c*II proteins (82, 152, 162, 219, 229). Thus, different proteases (e.g., Lon and Clp) or protease mediators (e.g., E2 and E3 proteins) recognize different features of proteins. Alternatively, specific proteases and their substrates could be compartmentalized. as with the endoplasmic reticulum degradative system. Second, some naturally unstable proteins and abnormal proteins are degraded by the same protease. For example, Lon is required for about 50% of the energy-dependent degradation of canavanyl proteins, as well as for degradation of the specific proteins mentioned above (152), and the ubiquitin system carries out degradation of abnormal proteins as well as cyclin and other regulatory proteins. Thus, some naturally unstable proteins and abnormal proteins could have chemical or structural features in common, or there are components of the degradative machinery that can recognize different features of unstable or abnormal proteins and present the proteins in a degradable form to a common protease (a protease adaptor function). Third, many if not all proteins can be altered or damaged sufficiently to become susceptible to degradation, and in most cases merely unfolding or changing the conformation of a protein will lead to its degradation. Thus, all proteins must contain degradation signals which either are buried in the native protein or must be formed or assembled from elements in the damaged or denatured protein. From this last consideration it is also clear that the rate of degradation of a protein depends not only on the presence of a degradation signal in a protein but also on properties of the protein that might determine how readily the signal becomes exposed or accessible to the degradative system.

Proteins can have one or more degradation signals, and in some cases more than one degradation signal may be necessary for recognition and targeting by the degradation machinery. For multimeric proteins or proteins that can associate with other macromolecules, degradation signals may be present on different subunits within the complex and multimerization might in some cases provide the necessary information to allow degradation of an otherwise stable protein. An interesting example of *trans* recognition is seen with mixed multimers of β-galactosidase fusion proteins in which the ubiquitination signal (a destabilizing amino terminus) and the ubiquitination target (an internal lysine of a subunit with a stabilizing amino terminus) reside on different subunits (118). Examples of *trans* targeting have yet to be demonstrated in natural systems, but the destabilization of p53 by association with the papillomavirus E6 protein (200) could reflect such a mechanism.

Below, we consider several features that have been shown to be important in determining the susceptibility of proteins to intracellular degradation. We will first discuss recognition motifs and then discuss general structural or compositional features of proteins that correlate with susceptibility to degradation. Degradation signals have been defined for the amino terminus and the carboxy terminus of proteins, and there are a few specific degradation signals that involve

sequences that might be found anywhere in a protein. Recognition by proteases and recognition by protease mediators, such as E2 and E3 proteins, will be discussed together, since the molecular principles involved should be similar for both.

Sequence-Specific Degradation Signals

The idea that naturally unstable proteins could contain unique sequences that can be recognized by specific proteases or components of a proteolytic system is attractive. Cleavage of the proteins could occur at the unique sequence or at a site elsewhere in the protein while it is tethered to the protease by tight binding at the unique recognition sequence. There are, in fact, numerous examples of proteins that cleave a limited number of proteins at unique or rare sites; these proteases are generally ATP independent. Most sequence-specific cleavage involves limited proteolysis or processing of proteins, as in zymogen activation, prohormone processing, secretion, and retroviral protein maturation; frequently the cleaved region is within a linker between domains, suggesting that it is particularly accessible to the protease. In some cases it is clear that the protease recognizes a specific sequence of amino acids that occurs infrequently in proteins, ensuring a severely limited substrate range for the protease. The recognition site for one processing protease, factor Xa, contains a 5-amino-acid sequence or a closely related sequence. This motif has been used to introduce a specific factor Xa cleavage site into exposed regions of a number of cloned proteins, indicating that the protease specifically recognizes that amino acid sequence in a variety of longer sequence contexts. Reports of such specific cleavage in the degradation of naturally unstable proteins are, however, very few.

The spore protease from *Bacillus megaterium* provides an example of one of the most stringent specificities in a degradative reaction. During germination of spores, the spore protease (Gpr), which is not energy dependent, initiates degradation of a number of storage proteins. The storage proteins are a homologous family of small, acid-soluble proteins that are cleaved by the Gpr at a specific site in an extended sequence motif consisting of seven amino acids T E (F, I) A S E F (204). Following the specific cleavage in the spore proteins, other peptidases further degrade the fragments to amino acids. Another specialized, and probably rare, example of specific degradation is the autocatalytic cleavage of the *E. coli* LexA repressor (138). When complexed with RecA, the LexA protein carries out an intramolecular site-specific cleavage of a particular Ala-Gly bond. In this case, it is not clear whether the cleavage is specific for the Ala-Gly bond or whether, because of structural constraints within LexA, that bond is the only one available for the intramolecular cleavage reaction.

The unique sequence recognized by the protease need not be the site at which the protein is cleaved. There is accumulating evidence that proteases, particularly ATP-dependent proteases, and certain protease mediators may have more than one site for binding substrates and that proteases might be held in place by a specific binding interaction and positioned for cleavage or modification at other, possibly less specific, sites. Analysis of the stability of deletion derivatives of cyclin led to the identification of a sequence found at the N terminus, between amino acids 13 and 91 in *Xenopus* cyclin, that appears to be necessary and sufficient for degradation (69). Within this region, a segment, R A A L G N I S N, called the destruction box by Glotzer et al. (69), is highly conserved in cyclins and is found in a modified version, R D I L V F L S R, in the unstable yeast mating factor, MATα2 (106), although the relevance of this similar sequence in α2 is unclear (104). Sequences containing the cyclin destruction box have been inserted into the amino-terminal region of a completely heterologous protein, protein A, rendering it unstable in vivo. The basis for the effect of the destruction box is not understood, but, since cyclin can be ubiquitinated and degraded in an energy-dependent fashion, it is possible that the destruction box is recognized by an E2 or E3 protein which mediates the conjugation of cyclin to ubiquitin. In addition to the destruction box itself, a nearby lysine-rich region, possibly the site for attachment of ubiquitin, is required for instability (69). In MATα2, the destruction box is located at a similar distance from the amino terminus and is required presumably along with a nearby lysine residue.

Studies with Lon protease in vitro identified six sites of cleavage within the physiological substrate, λ N protein (147). Cleavage sites are found primarily at bonds in which the P1 position is occupied by leucine or alanine and there is a basic amino acid in positions P1 to P4. Alignments of N protein to other physiological substrates of Lon, including SulA, RcsA, and Tn*903* transposase, revealed that sequences similar to that surrounding one of the cleavage sites were found in all of them. Whether cleavage in substrates other than N protein occurs at this site remains to be shown. The consensus derived from the comparison, $\Phi\ X_{3-4}$ L S (L,X) X_5 S X Φ (where Φ represents a hydrophobic side chain), suggests that the binding pocket for substrates on Lon might accommodate an extended region of the polypeptide chain around the cleavage sites. This consensus should occur about once in every 2,000 leucines, or in about 1% of proteins, and might reflect the stringent recognition of naturally unstable proteins rather than the broadly specific interaction with which Lon can degrade unfolded forms of many proteins.

N-End Rule

The clearest example of a simplifying principle underlying the selectivity of protein degradation is illustrated by the N-end rule, originally enunciated by Bachmair et al. (7) and since supported by a variety of biochemical and genetic observations from several laboratories (77, 99, 186, 234). The N-end rule was originally formulated to explain why certain β-galactosidase fusion proteins, either in yeast cells or in extracts of *S. cerevisiae* or reticulocytes, were degraded at drastically different rates (7). Degradation of the unstable fusion proteins in *S. cerevisiae* is both ATP and ubiquitin dependent, and the rate depends on the amino-terminal amino acid of the fusion protein (7). In general, fusion proteins with methionine, glycine, valine, or proline at the amino terminus are stable, whereas proteins with bulky hydrophobic or basic amino-terminal amino acids are highly unstable (7). Rapid degradation of the fusion proteins also depends on the presence of a lysine residue at position 15 or 17 of the sequence, which has been shown to be the site at which ubiquitin is conjugated (8). These observations formed the basis for the N-end rule, which states that a recognition signal for degradation by the ubiquitin-dependent pathway is the presence of a destabilizing amino acid at the amino terminus of the protein, coupled with a nearby lysine to allow ubiquitin conjugation. Major support for the N-end principle came from two independent sets of studies. It was shown that the E3 proteins from the ubiquitin system

of *S. cerevisiae* and mammals are N-end-recognizing proteins, which specifically bind to proteins with "destabilizing" amino-terminal amino acids (77, 97, 188). The E3α protein has separate binding sites for type I and type II amino termini, and the E3β protein has a binding site for type III amino termini (see reviews by Varshavsky [234] and Hershko and Ciechanover [99] for definitions of types of amino termini). In addition, it was shown that another set of enzymes can modify proteins with acid or amide side chains at the amino terminus by deamidation where necessary, followed by addition of the destabilizing amino acid arginine to the amino terminus (58, 77). Proteins altered in this way are then recognized by the appropriate E3 protein, ubiquitinated, and degraded (99, 234). These observations firmly establish the biochemical basis for selection by the N-end rule.

Recently, Varshavsky and colleagues showed that the principle of the N-end rule is not limited to eukaryotes or to ubiquitin-dependent systems. In *E. coli*, which lacks a ubiquitination system, β-galactosidase fusion proteins are degraded at different rates depending on the amino-terminal amino acid (228). The N-end rule is more restrictive in *E. coli*, with far fewer destabilizing amino acids (tryptophan, tyrosine, phenylalanine, and leucine, as well as arginine and lysine, which are modified by addition of an N-terminal leucine). Cells with mutations in *clpA* accumulate the unstable fusion proteins, indicating that Clp protease is involved in degradation of the fusion proteins. Whether ClpA itself can bind proteins with specific amino termini or whether there is another N-end-recognizing protein that interacts with Clp protease has not been established.

The physiological role of the N-end-recognizing system is still a matter of some speculation. At the very least, it is designed to recognize proteins with abnormal amino termini. The amino acids recognized by the N-end rule system are not found at the amino termini of most intracellular or cytoplasmic proteins, inasmuch as the methionine aminopeptidases of both eukaryotes and prokaryotes remove methionine from proteins primarily when the second amino acid is small or hydrophilic, i.e., stabilizing (102). The N-end-recognizing system might be used to screen proteins that have been improperly processed at the amino terminus. Also, since removal of the signal peptide from secreted proteins often leaves a destabilizing amino acid at the amino terminus, the N-end system could eliminate proteins that are incompletely secreted or are taken back into the cytoplasm (7). Proteases that carry out the initial cleavage of proteins might specifically cut at bonds resulting in products with destabilizing amino acids at the amino termini. For example, Lon and Clp both tend to cleave hydrophobic regions of proteins, often (but not always) yielding products with hydrophobic amino-terminal amino acids. Other endoproteolytic or oxidative cleavages of proteins might also generate shortened proteins with destabilizing amino-terminal amino acids. Thus, the N-end pathway could function as a secondary system to completely degrade proteins that have been partially degraded by other proteases or by chemical damage.

The amino-terminal amino acid of proteins is a genetically programmed feature of the protein. By restricting the nature of the amino acids found at the amino termini of normal proteins, the cell has a simple and elegant means of distinguishing certain classes of abnormal proteins. In addition, the N-end recognition system could be involved in the programmed regulation of stability of certain naturally unstable proteins. It is possible that the methionine aminopeptidase or another exopeptidase removes the methionine from specific proteins even when the next amino acid is destabilizing. Thus, the metabolically active form of some proteins might have abnormal amino termini. For example, the mature form of the highly unstable *c*II protein of phage λ has an amino-terminal arginine (103), although Clp, the N-end rule protease in *E. coli* (229), does not seem to be responsible for *c*II degradation (125). Sindbis virus polymerase is formed by cleavage of a polyprotein to give an unstable N end and has been shown to be a substrate for ubiquitin-dependent degradation (41).

Mutational studies indicate that the N-end recognition system is not involved in all ubiquitin-dependent functions or even in all ubiquitin-dependent protein degradation, e.g., the degradation of cyclins and MATα2 mentioned above. The importance of the N-end rule in *E. coli* is also unclear, since much protein degradation is independent of ClpA. Since most known unstable proteins and abnormal proteins do not have destabilizing amino acids at the amino terminus, degradative systems must be able to recognize degradation signals in addition to the N end. The N-end rule may serve primarily as an example of the kind of information sufficient to specifically target a protein for degradation.

C-Terminal Determinants of Stability

No system comparable to the N-end system has been found for the carboxy terminus of proteins. There have been several reports, however, that sequences or structures at the C-terminal regions can affect the stability of proteins in vivo. The degradation rate of a cloned amino-terminal fragment of λ repressor is affected by the composition of the five carboxy-terminal amino acids (179). The presence of hydrophobic amino acids in the last five positions resulted in a highly unstable protein. Replacement of any of these five amino acids with hydrophilic amino acids led to slower degradation in vivo, with the greatest effect observed for the carboxy-terminal amino acid itself (179). An ATP-independent protease that preferentially degraded proteins with a hydrophobic tail was isolated (tail-specific protease) (210), and it was found to be identical to a periplasmic protease that processes penicillin-binding protein (93). Endogenous intracellular protein substrates for a protease that prefers hydrophobic carboxy termini have not been demonstrated. In a related study, it was shown that addition of amino acids to the carboxy terminus of an unstable mutant of the Arc repressor protected it from degradation in vivo (21). The unstable Arc protein has a hydrophobic carboxy-terminal region, and the protecting extensions tended to decrease the hydrophobic character of the carboxy terminus (21). Since the number of proteins with hydrophobic carboxy-terminal regions is probably limited, proteases that recognize such a degradation signal might have a role in degrading proteins that have been cleaved by other proteases to generate such regions or proteins whose translation has been interrupted prematurely. We know, however, that many prematurely terminated proteins are degraded rapidly in a Lon-dependent, energy-dependent fashion (22, 218), which is presumably distinct from and independent of the tail-specific protease identified by Silber et al. (209).

In mammalian cells, degradation of mouse ornithine decarboxylase, which is one of the most unstable metabolic enzymes, is dependent on sequences found near the carboxy terminus (67). Deletion of the last five amino acids (ARIVN) results in an ornithine decarboxylase that is resistant to constitutive degradation (67). In addition, a PEST sequence

(see below) present in the carboxy-terminal region may play some part in the constitutive degradation, because ornithine decarboxylase derivatives with deletions of the carboxy-terminal PEST sequence but retaining the last five amino acids are stable (66). The identity of the destabilizing sequence in ornithine decarboxylase has not been defined.

Studies with the catalytic subunit of cAMP-dependent protein kinase indicated that rapid degradation of this kinase requires a stretch of acidic amino acids near the carboxy terminus as well as an adjacent region containing hydrophobic amino acids (5). A similar requirement was noted for degradation of the epidermal growth factor receptor protein. A protease that recognizes both proteins as well as a chimeric protein made by attaching the hydrophobic and acidic regions to the carboxy terminus of dihydrofolate reductase has been isolated. This protease, as with the prokaryotic tail-specific protease, does not appear to require ATP for activity in vitro (5).

Unsatisfied Protein-Protein Bonding Domains

The free subunits of multimeric proteins are usually unstable in vivo. Although some of the reported examples arise from overproduction of individual subunits expressed from cloned genes, degradation of separated subunits, "proteins without partners," also has important functions under normal physiological conditions (see the section on multimeric complexes above). Intersubunit bonding domains have characteristics of both solvent-exposed surfaces and buried regions of proteins (98). Although subunit bonding domains must be very heterogeneous, the amphipathic nature of the exposed surface in dissociated subunits might be recognized by specific proteases or protease mediators. Alternatively, subunit interactions within the complex might stabilize the tertiary structure of individual subunits, and dissociation of the subunits would then lead to unfolding and exposure of degradation signals within the subunit.

It is interesting that most of the naturally unstable proteins normally function within multimeric complexes or by interacting with other macromolecules within the cell, raising the possibility that the regions of the protein targeted for ubiquitination or degradation are those involved in protein-protein interactions, which would be unavailable in the multimers but would be exposed on dissociation. The recognition of protein-protein interaction domains as degradation signals would also be consistent with the observation that essentially all proteins can become subject to proteolysis when unfolded by mutation or stress. It is possible that the proteins are in an equilibrium that favors a significant concentration of the free form, which would be rapidly degraded. Completion of single rounds of activity or changes in metabolic conditions could result in release of the protein and subsequent degradation. Myc and Myb, for instance, form different multimers depending on physiological conditions, suggesting that these proteins may dissociate from their partners in vivo (16, 120). One might predict, then, for unstable proteins that are part of multimeric complexes, that (i) the degradation motifs will be in or close to the multimerization domains (for instance, one possible candidate for a degradation motif would be leucine zippers) and (ii) mutants which increase protein-protein interaction may lead to increased stability. Observations with the unstable *E. coli* positive regulator, RcsA, are consistent with these predictions; mutations that increase the interaction of RcsA with RcsB apparently shield protease-sensitive portions of RcsA (see the section on RcsA, above).

Structural and Compositional Correlations with Susceptibility to Degradation

A number of correlations of in vivo half-lives with general properties of proteins have been made. When the bulk of cellular proteins are examined, larger, more hydrophobic, more acidic proteins tend to be degraded at higher overall rates (73). These correlations have not provided much insight into the control of degradation, particularly for extremely short-lived regulatory proteins. A more intriguing correlation with protein stability was identified by Rogers et al. (193), who noted that metabolically unstable proteins contained regions of amino acid sequence, called PEST sequences, that are bounded by basic amino acids and are rich in four amino acids, proline (P), glutamate (E), serine (S), and threonine (T). PEST regions were found in unstable proteins for eukaryotic cells, often with several PEST regions in a single protein, but not in unstable proteins from bacterial cells (193). Attempts to delete PEST sequences from proteins to stabilize them (67, 194) or to add PEST sequences to proteins to increase their susceptibility to degradation (140) have given mixed results. It appears that PEST sequences do not act as a simple tag that is recognized by the degradative machinery, and so far no proteases or protease mediators have been found to bind to or cleave within PEST regions. The presence of a PEST sequence may have a more general structural significance in a protein, perhaps reflecting a tendency to fold in certain ways or to display flexibility in a way that contributes to metabolic instability. Although a PEST sequence may not constitute a degradation signal per se, it might dictate the frequency of exposure of such a signal within a given protein. The recent report that insertion of a PEST sequence in dihydrofolate reductase does not destabilize the protein unless a second unrelated modification is made in the protein (140) is consistent with this model. In addition, the last five amino acids of mouse ornithine decarboxylase, not part of the PEST region, are necessary but not sufficient for degradation, indicating that a specific C-terminal determinant in addition to an internal PEST region may be required for degradation.

Another statistical analysis of metabolically unstable proteins presented a surprising correlation between the dipeptide content and the in vivo half-life of a protein (89). There is some overlap in the predictions made by the dipeptide content and the PEST hypothesis in that 39 of the 81 destabilizing dipeptides contain PEST amino acids. The dipeptide model, however, predicts not only that other amino acids can contribute to instability depending on the sequence context, but also that PEST amino acids are not destabilizing in all sequence contexts. The physical basis for the correspondence between dipeptide content and degradation rates is obscure but may relate to fundamental properties such as folding patterns and pathways.

CONCLUSIONS

Most rapid degradation of intracellular abnormal and normally unstable proteins in both prokaryotic and eukaryotic cells is energy dependent. Energy is used at multiple steps in the degradation process: for the assembly of the multicomponent proteases such as Clp and the 26S protease complex, for ubiquitin tagging of proteins in eukaryotic cells, and for the processive degradation of protein substrates, as well as for chaperone-dependent interaction with abnormal proteins. We suggest that the energy dependence of the degradation process reflects an energy-dependent proofread-

ing/scanning which increases the selectivity of substrate targeting and increases the processivity of degradation of large substrates.

Protein-degradative machinery is only one of several surveillance systems whose functions are to recognize abnormality in or misplacement of macromolecules and to initiate or perform corrective reactions. Enzymatic systems for the repair of damaged DNA, folding enzymes, and transport chaperones share with the ATP-dependent proteases described here the ability to bind a wide range of targets and to use the energy of ATP hydrolysis to alter the interactions between the enzyme or chaperone and its target. This use of nucleotide hydrolysis to increase the rate of binding interactions is also seen with the ubiquitous GTPases, which act similarly to chaperones in promoting many highly specific macromolecular interactions.

The surveillance functions shared by the ATP-dependent proteases and other scanning ATPases and GTPases are critical to the cell for timed responses, reactions to stress, secretion and transport, and macromolecular organization. In addition, scanning ATPases are involved in recognition of abnormal protein structures, that is, in discriminating between correct and incorrect or self and nonself. The different remedies applied—refolding, presentation for degradation, secretion—presumably depend on the other enzymatic factors recruited by the ATPases. One can imagine that the nature and mechanism of reversible binding of unfolded regions of proteins to different ATPases might be similar. Recent findings of structural similarities between peptide-binding regions of major histocompatibility complex molecules and HSP70-like chaperones suggests that the biochemical basis for recognizing abnormal or nonself protein structures may be widely conserved (65, 190). An intriguing possibility is that the allosteric site on the bacterial ATP-dependent proteases, where proteins bind but are not cleaved, may be analogous to the peptide-binding regions of molecular chaperones and may have a functional equivalent in the eukaryotic proteases that process antigens to yield protected peptides that interact with major histocompatibility complex molecules.

We have discussed a variety of specific substrates that are rapidly degraded in both prokaryotic and eukaryotic cells. These highly unstable proteins are frequently at control points for cell cycle control and other developmental switches or are part of emergency responses which require rapid destruction of the emergency repair apparatus during recovery and return to normal growth. They include cell cycle regulators and both positive and negative regulators of transcription. They generally, share, in addition to their short half-lives, a low abundance in the cell, multiple levels of regulation of both synthesis and activity, and essential protein-protein interactions as part of their mode of action.

These protein-protein interactions can play critical roles in modulating degradation, either by providing elements of the degradation recognition signals (such as in the *trans* targeting described by Johnson et al. [118]) or in shielding degradative signals (as for SulA and RcsA). The existence of these interactions provides another variable in the regulation of these proteins, since mutations which increase or decrease interactions can have profound effects on both the stability and activity of such proteins. The use of ubiquitin for targeting proteins to the 26S protease can be considered an exquisite example of a protein-protein interaction that modulates degradation. The covalently attached polyubiquitin chain could mediate degradation of a protein either by binding to the protease (a type of *trans* targeting) or by disrupting the structure of the substrate, exposing regions that are recognized by the protease.

In addition to the rapid degradation of regulatory proteins, a number of metabolic proteins undergo a somewhat slower but still significant degradation under particular metabolic conditions. Particularly in bacterial cells where reversible covalent modifications of metabolic enzymes is rare, slower degradation of enzymes could be used to modulate their activities and could represent a system poised to respond to changes in nutritional conditions or other environmental pressures. Slower degradation might also reflect the tendency of certain proteins to be damaged or denatured in some way.

We are just beginning to appreciate the importance of rapid degradation as an essential element of the regulatory circuitry in both prokaryotic and eukaryotic cells and to understand the complex, energy-dependent proteases which carry out this degradation. Major questions remain unanswered: How is the recognition of substrates and the in vivo regulation of proteolytic activity carried out? What is the precise role of ATP in proteolysis? The answers to these questions promise to be entertaining.

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