20S proteasomes and protein degradation “by default”

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Summary
The degradation of the majority of cellular proteins is mediated by the proteasomes. Ubiquitin-dependent proteasomal protein degradation is executed by a number of enzymes that interact to modify the substrates prior to their engagement with the 26S proteasomes. Alternatively, certain proteins are inherently unstable and undergo “default” degradation by the 20S proteasomes. Puzzlingly, proteins are by large subjected to both degradation pathways. Proteins with unstructured regions have been found to be substrates of the 20S proteasomes in vitro and, therefore, unstructured regions may serve as signals for protein degradation “by default” in the cell. The literature is loaded with examples where engagement of a protein into larger complexes increases protein stability, possibly by escaping degradation “by default”. Our model suggests that formation of protein complexes masks the unstructured regions, making them inaccessible to the 20S proteasomes. This model not only provides molecular explanations for a recent theoretical “cooperative stability” principle, but also provokes new predictions and explanations in the field of protein regulation and functionality. BioEssays 28:844–849, 2006. © 2006 Wiley Periodicals, Inc.

Introduction
Protein degradation plays an important role in almost every basic cellular process. The selective degradation of many short-lived proteins in the cell is mediated via the ubiquitin-26S proteasomal degradation pathway.(1,2) Ubiquitin, a 76 amino acid residue protein, is covalently conjugated in a highly regulated multistep process to the substrate protein, marking it for degradation by the 26S proteasomes.(1,2) Recent exciting discoveries in the field of protein degradation have revealed that several proteins are also susceptible to ubiquitin-independent degradation mediated via the core 20S proteasomes.(3–5) These findings shed new light on our understanding of protein degradation and may unveil new principles with wide biological implications.

Ubiquitin-independent p53 degradation
The tumor suppressor p53 is a short-lived protein that accumulates following exposure to different types of stress and induces cell cycle arrest or apoptosis.(6) Regulation of p53 stability plays a central role in the control of proper function of p53. Degradation of p53 has been intensively studied and indeed p53 has become a hallmark for ubiquitin-dependent 26S proteasomal degradation. Several specific E3 ubiquitin ligases were reported to bind and poly-ubiquitinate p53, marking it for degradation by the 26S proteasomes.(7–10) Interestingly, p53 is also susceptible to degradation in a ubiquitin-independent manner that is mediated via the core 20S proteasomes(3,11–16) (Fig. 1). Experiments both in vitro and in vivo suggest that, in contrast to degradation of p53 by the 26S proteasomes, degradation by the 20S proteasomes does not require interaction with E3 ubiquitin ligases or any tagging such as poly-ubiquitination.(13,16)

It seems that degradation of p53 by the 20S proteasomes is the result of inherent features of the p53 protein and therefore we term it degradation “by default”. The term “by default” refers here to a pathway chosen automatically. In this case, it means that the degradation will occur unless specific intervention prevents it. p73, a p53 family member, was also found to undergo degradation “by default” via the 20S proteasomes in the cells.(16)

Ornithine decarboxylase and degradation “by default”
A mechanistic insight into the phenomenon of degradation “by default” is gained from the study of ornithine decarboxylase (ODC). ODC is the first rate-limiting enzyme in polyamine biosynthesis that catalyses the decarboxylation of ornithine to form putrescine.(4,17) In its active and stable form ODC is a homodimer with two enzymatic active sites. Binding of antizyme, a polyamine-induced protein, to ODC disrupts ODC...
homodimers and exposes the C-terminal region of ODC, inducing ubiquitin-independent 26S proteasomal degradation of ODC.\(^4,17\) Significantly, ODC monomers are also susceptible in the cells to ubiquitin-independent degradation by the core 20S proteasomes\(^18\) (Fig. 1). Degradation of ODC monomers by the 20S proteasomes does not require tagging (such as poly-ubiquitination) or protein–protein interaction, suggesting that these monomers are inherently unstable and are degraded “by default” by the 20S proteasomes in the cells. In contrast, ODC dimers are resistant to degradation by the 20S proteasomes.\(^18\) Furthermore, ODC monomers are protected from ubiquitin-independent 20S proteasomal degradation not only by binding to another ODC monomer but also by binding to antizyme.\(^18\)

### Regulation of the process of degradation “by default”

The process of degradation “by default” must be regulated in order to allow proper protein accumulation and avoid unwarranted degradation. Two different mechanisms were discovered to protect the proteins from degradation “by default”. The ODC example described above clearly demonstrates that assembly of protein into functional protein complexes, namely homodimerization, protects ODC from 20S proteasomal degradation (Fig. 1). Several additional examples as discussed below suggest that large protein complexes support protein stability by protecting from 20S proteasomal degradation; however, this is not the only means of protecting proteins from degradation “by default”. The second mechanism is executed by NAD(P)H quinone oxidoreductase-1 (NQO1), a ubiquitous enzyme that utilizes NAD(P)H to catalyze the reduction of various quinones. Recently NQO1 was proposed to function as the “gate keeper” of the 20S proteasomes, regulating the degradation of certain substrates.\(^16\) Purification of the 20S proteasomes from mice livers\(^16\) and human red blood cells (unpublished data) show that NQO1 is physically associated with the 20S proteasomes but not with the 26S proteasomes. NQO1 also binds a subset of short-lived proteins including p53, p73\(^\alpha\) and ODC and protects them from 20S proteasomal degradation.\(^16,18\) Binding of NQO1 to these proteins is augmented in the presence of NADH and inhibited by dicoumarol, an inhibitor of NQO1, which competes with NADH.\(^16\) Inhibition of NQO1 by dicoumarol or NQO1 knockdown with specific NQO1 siRNA induces ubiquitin-independent degradation of these proteins.\(^16,18\) Several lines of evidence demonstrate that NQO1 regulates p53 stability via this unique pathway that is ubiquitin-independent and is mediated via the core 20S proteasomes. First, NQO1 fails to inhibit p53 degradation specifically mediated by the E3 ubiquitin ligase Mdm2.\(^12\) Furthermore, a mutant p53 (p53\(^\alpha\))\(^22,23\) that is resistant to Mdm2-mediated degradation is susceptible to dicoumarol-induced degradation.\(^13\) Second, unlike Mdm2-mediated degradation, dicoumarol-induced p53 degradation is not associated with accumulation of ubiquitin-conjugated p53.\(^13\) Third, inhibition of NQO1 by dicoumarol or NQO1 knockdown with specific NQO1 siRNA in cells with a temperature-sensitive E1 ubiquitin-activating enzyme induces p53 degradation and inhibits apoptosis at the restrictive temperature under conditions devoid of ubiquitination.\(^13\) Fourth, in vitro degradation...
studies showed that dicoumarol-induced p53 degradation is ubiquitin-independent.\textsuperscript{(13)} In vitro analysis with purified proteasomes further revealed that native p53 is degraded by the 20S, but not the 26S proteasomes. NQO1 together with NADH selectively protects p53 from degradation by the 20S proteasomes and dicoumarol alleviates the protection.\textsuperscript{(16)} Altogether, these findings establish a novel ubiquitin-independent mechanism for proteasomal degradation of p53 that is regulated by NQO1 and is mediated via the 20S proteasomes in cells. In addition to p53, in vitro degradation assays confirmed that NQO1 together with NADH selectively protect p73\textalpha{} and ODC from 20S proteasomal degradation and dicoumarol reduces the protection.\textsuperscript{(16,18)} The binding of NQO1 to the 20S proteasomes and the ability of NQO1 to bind and protect a subset of short-lived proteins from 20S proteasomal degradation suggest that NQO1 is an important regulator of degradation “by default”.

**Degradation “by default” is a wide phenomenon**

Several other examples from the literature demonstrate that degradation “by default” is a pervasive phenomenon. The Cdk inhibitor p21\textsuperscript{CIP}, a naturally unstructured protein, is a critical regulator of cell division and DNA replication.\textsuperscript{(19)} In the cells, p21\textsuperscript{CIP} proteins are rarely found in a “free” form and are almost always detected as a part of a complex, raising the possibility that the “free” p21\textsuperscript{CIP} is rapidly degraded in the cells.

Degradation studies with p21\textsuperscript{CIP} show that this protein is degraded by the 20S proteasomes in a ubiquitin-independent manner. Ubiquitin-independent 20S proteasomal degradation of p21\textsuperscript{CIP} is inhibited upon binding of p21\textsuperscript{CIP} to the proliferating cell nuclear antigen (PCNA) or in the presence of the Cyclin E and Cdk2 complex.\textsuperscript{(20–22)} It was also reported that p21\textsuperscript{CIP} undergoes ubiquitin-dependent proteasomal degradation\textsuperscript{(22)} in particular following exposure to UV radiation.\textsuperscript{(23)} Another example is IxB\textalpha{} a major regulator of the NF-\textkappa{}B transcription factor. Following exposure to different types of stimuli, IxB\textalpha{} is phosphorylated and rapidly degraded via the ubiquitin–26S proteasome degradation pathway.\textsuperscript{(24)} Independent of its rapid stimulation-induced breakdown, IxB\textalpha{} is inherently unstable and undergoes continuous turnover. Analysis of both the basal degradation and the stimulation-induced breakdown of IxB\textalpha{} revealed that, although in both cases, the degradation is mediated via the proteasome, these two pathways of degradation are distinct. In contrast to the stimulation-induced breakdown, the basal degradation of IxB\textalpha{} does not require poly-ubiquitination of IxB\textalpha{}.\textsuperscript{(25)} Interestingly, expression of p65, which interacts with IxB\textalpha{} significantly reduces the basal turnover of IxB\textalpha{}.

Several studies have shown that IxB\textalpha{} is degraded by the core 20S proteasomes in a ubiquitin-independent manner and that p65 can protect IxB\textalpha{} from 20S proteasomal degradation.\textsuperscript{(25,26)} In these last two examples, the basal degradation of both p21\textsuperscript{CIP} and IxB\textalpha{} is ubiquitin-independent and mediated via the core 20S proteasomes whereas ubiquitin-dependent degradation occurs only upon exposure to certain physiological stimuli. These examples further suggest that the degradation of certain proteins through the ubiquitin-independent 20S degradation pathway is a passive process that depends upon the inherent quality of the substrate protein, whereas ubiquitin-dependent degradation is an active process that enhances the basal level of degradation. Furthermore, assembly of substrate proteins into large protein complexes protects these substrates from 20S proteasomal degradation.

This model can be further expanded not only to protein–protein complexes but also to protein–RNA complexes as is evident in the case of YB-1 (Y-box binding protein-1). YB-1 is a nucleocytoplasmic shuttling protein involved in many DNA- and RNA-dependent events. In the nucleus, YB-1 regulates transcription of many genes involved in cell proliferation and differentiation.\textsuperscript{(27)} Recently, it was demonstrated that YB-1 undergoes a specific proteolytic cleavage by the 20S proteasome. Cleavage of YB-1 by the 20S proteasome is ubiquitin-independent, and is inhibited following association of YB-1 with messenger RNA.\textsuperscript{(27)}

It seems that degradation “by default” by the 20S proteasomes is a general and wide phenomenon and association with any protein complexes protects these protein substrates from 20S proteasomal degradation. However, it is still unclear what constitutes the signal for degradation by the 20S proteasomes and how interaction with different proteins protects proteins from degradation.

**Degradation “by default” and disease**

Many neurodegenerative diseases, including Parkinson’s disease, are caused by the accumulation of aggregated proteins in the neuronal or glial cytoplasm. The inclusions of aggregated and filamentous proteins, called Lewy bodies, are comprised primarily of \textalpha{}-synuclein, a protein of unknown function that is highly expressed in the human brain.\textsuperscript{(28)} Monomeric \textalpha{}-synuclein is a natively unfolded protein without defined structure that undergoes ubiquitin-independent degradation by the 20S proteasomes.\textsuperscript{(28)} \textalpha{}-synuclein spontaneously multimerizes to form highly stable insoluble fibrils that are resistant to degradation. Inhibition of the proteasomes in cells leads to an accumulation of \textalpha{}-synuclein that is phenotypically similar to the inclusions found in diseased tissue.\textsuperscript{(28)} Furthermore, inherited forms of Parkinson’s disease include mutations that increase the copy number of \textalpha{}-synuclein, mutations that impair proteasome function, and mutations in \textalpha{}-synuclein that promote oligomerization of the protein and resistance to proteasomal degradation.\textsuperscript{(29)} These neurodegenerative diseases illustrate the importance of functioning 20S degradation. Proteasomal degradation “by default” of monomeric \textalpha{}-synuclein is likely to be contributing to the maintenance of low cellular protein levels and the prevention of the accumulation and subsequent spontaneous
oligomerization that leads to the protein inclusions associated with these diseases.

**Unstructured region—the common signal**

A common feature of ubiquitin-independent 20S proteasomal degradation substrates is the presence of an unstructured protein region,\(^{(30)}\) a region that lacks a specific three-dimensional structure.\(^{(31)}\) These unstructured regions are inherent features of certain functional proteins and are not due to improper or aberrant folding. It should be noted that proteins unfolded due to stress, mild oxidation, and heat have been shown to be substrates of the 20S proteasomes (reviewed in Refs 32,33). Various oxidized proteins were reported as in vitro substrates of the 20S proteasomes, including oxidized glutamine synthetase, calmodulin, casein, superoxide dismutase, hemoglobin, myoglobin, albumin and oxidized histones.\(^{(5)}\) Experiments in cells have shown that mild oxidation inactivates the ubiquitin-dependent system and the activity of the 26S proteasome but not of the 20S proteasome.\(^{(34)}\) Study with cells harboring a thermolabile E1 ubiquitin-activating enzyme demonstrated that these cells effectively degrade oxidized proteins in an ubiquitin-independent manner, further supporting the possibility that oxidized proteins are degraded by the 20S proteasomes in vivo.\(^{(35)}\) Degradation of these proteins represents a very important aspect of 20S activity, and this subject has been discussed in a number of excellent reviews.\(^{(32–34)}\) However, our current focus is disordered protein regions that exist as natural parts of certain proteins, and are not caused by damage or misfolding. Many disordered protein regions fold upon binding to their biological targets (coupled folding and binding), whereas others constitute flexible linkers that have a role in the assembly of multi-protein complexes.\(^{(31)}\) Both the p53 N-terminal trans-activation domain and the C-terminal regulatory domain were identified as unstructured regions.\(^{(31)}\) These unstructured regions may facilitate and serve as a signal for degradation “by default” by the core 20S proteasomes. Additionally, analyses of ODC using different algorithms that predict unstructured regions revealed that ODC monomers contain an unstructured region (unpublished data). Like p53, the unstructured region of ODC may facilitate and serve as a signal for degradation. Our observation that ODC dimers resist 20S digestion may suggest that, upon dimerization, the unstructured regions are either folded or masked and therefore are not accessible for degradation by the core 20S proteasomes.\(^{(31)}\) Similarly, p21\(^{(30)}\) and \(\alpha\)-synuclein are unstructured proteins, and are susceptible to 20S proteasomal degradation.\(^{(19,28)}\) Here again, it is possible that these proteins are protected from 20S proteasomal degradation upon interaction with other proteins, due to masking or folding of their unstructured regions. Because many unstructured regions fold upon binding to their biological targets in the cells, it seems nature has developed an elegant way to link protein stability with biological functionality.\(^{(31)}\) Such a mechanism is extremely important in cases of functional multi-protein complexes, in which any excess of one of the subunits might interfere with the proper biological function of the complex.

The ability of the core 20S proteasomes to degrade primarily unstructured proteins and protein regions can be utilized as an assay for the identification of such protein regions. The protein of interest can be incubated *in vitro* with purified 20S proteasomes and the degradation products can be analyzed by SDS-PAGE. Furthermore, in cases when a protein folds upon binding to its biological counterpart such an assay can be a valuable tool for identifying the interacting target protein or molecule. More importantly, such a biological assay enables us to examine whether a suspected candidate protein is degraded “by default” by the 20S proteasomes and protected upon binding to its biological counterpart. For example, the protein \(\alpha\)-catenin was also reported to undergo ubiquitin-independent proteasomal degradation, and binding of \(\beta\)-catenin to \(\alpha\)-catenin blocked \(\alpha\)-catenin proteasomal degradation.\(^{(36)}\) Although it is not yet clear whether ubiquitin-independent degradation of \(\alpha\)-catenin is mediated via the 20S proteasomes, our analyses of \(\alpha\)-catenin using different algorithms that predict unstructured regions suggest that \(\alpha\)-catenin contains several unstructured regions and therefore might be a substrate for the 20S proteasomes in the cells. This possibility can be verified by using the in vitro assay described above.

**Degradation “by default” and “Cooperative stability”**

Over the years, it has become more and more evident that almost every major process in the cell is carried out by assemblies of protein molecules: dimers, oligomers or even large protein complexes. As these protein complexes carry out their biological functions, each of these protein assemblies can disassemble or interact with several other complexes of proteins. It is clear that such protein assemblies can carry out more complicated tasks with higher efficacy and accuracy. One advantage to this arrangement is that substrates can be channeled from one enzyme to the next, which greatly increases the efficiency of the reaction. The assembly of proteins into large complexes also has important implications in terms of protein stability. Over 30 years ago, Goldberg et al showed that free subunits of multimeric complexes undergo rapid degradation (reviewed in Refs 32,37). Numerous examples demonstrate that proteins assembled into complexes are more stable than the free monomer subunits.\(^{(38)}\) This tendency of multi-protein complexes to support protein stability might be explained by the proposed degradation “by default” mechanism. For example, both the tumor suppressor p14\(^{ARF}\) and the viral oncogene SV40 LT inhibit dicoumarol-induced p53 degradation.\(^{(13)}\) The underlying mechanisms remain to be uncovered, but a likely possibility is that these
proteins generate, together with p53, larger protein complexes and therefore protect p53 form 20S proteasomal degradation.

A recent theoretical analysis of gene circuits in bacteria presents a model for how selective degradation of monomers could be advantageous to the cells. This study analyzes the nonlinear dependence of degradation rate on the protein concentration. The degradation rate of proteins at lower concentrations is elevated compared to the degradation rate at higher protein concentrations. Since monomers predominate at low protein concentrations and dimerization is favored at higher concentrations, the model proposes that multimeric complexes support protein stability and terms it “cooperative stability”. The authors of the “cooperative stability” model suggest that it improves the functioning of genetic networks and provides the means for evolutionary changes. This is based on the premise that genetic networks can be better regulated if the proteins involved are at higher concentrations. For example, if a protein dimer is the active species, then a low protein concentration would require high affinity for the dimer to be formed, and also would demand very stringent control of the protein expression. Higher protein concentrations offer the cell more flexibility in the regulation of expression, allow for lower affinity interactions and allow for the variability that is essential for evolution. However, high protein concentrations and lower affinity mean that excess protein monomers may interfere with reactions catalyzed by the active dimers. Therefore, there must be a means to selectively degrade the excess monomers. Our degradation “by default” model explains how excess monomers are selectively degraded without requiring any additional molecular components.

Future prospects
Throughout this review, we have described several examples of protein substrates that are completely degraded by the 20S proteasomes. However, it is well known that unstructured regions are preferentially degraded by the 20S proteasomes. An intriguing possibility is that, under certain conditions, such selectivity might result in protein processing and not complete degradation. The ability of the 20S proteasomes to preferentially degrade unstructured regions, sparing fragments of folded domains, might result in the generation of a large repertoire of processed products. These processed products might play a role in regulation of various biological functions of the target protein substrate. Since binding to other proteins protects from 20S degradation, such processing possesses tremendous potential for diversity. Binding of different molecules to different domains might protect certain domains while others are susceptible to degradation, generating a large variety of processed products. Such a scenario is certainly feasible as was nicely demonstrated by Liu et al who showed that p21 fused to GFP at both termini is processed by the 20S proteasomes generating free GFP products. Such a possibility will certainly enlarge the cellular protein repertoire and will result in many important biological implications. It is still not clear what determines whether a certain protein is completely degraded by the 20S proteasomes or only partially digested generating different new products.

Conclusions
We propose a model whereby a subset of proteins are inherently unstable and degraded “by default” by the 20S proteasomes in the cells (Fig. 2). These substrate proteins can be protected by interacting with each other as in the case of ODC homodimers or by interacting with other proteins or even upon their assembly into large functional protein complexes. In addition, this model proposes that certain proteins, such as NQO1, function as “professional stabilizers” that bind and protect proteins from 20S proteasomal degradation. It is possible that molecular chaperones also function in the capacity of a “professional stabilizer.” One implication of this
model is the importance of studying and identifying new protein stabilizers and not focusing only on inducers of degradation such as different E3 ligases in order to fully understand the determinants of protein half-life.

The degradation "by default mechanism" is distinct from the current "modification to destabilization" mechanism that is mediated by poly-ubiquitination. The nascent protein that manages to escape 20S proteasomal breakdown matures and assembles into larger functional protein complexes. At this stage, the degradation of the protein can occur only via ubiquitin-dependent 26S proteasomal degradation. Our model suggests that degradation "by default" is important for maintaining the proper steady-state levels of proteins, and for reducing the interference of excess protein monomers with the functioning of multiprotein complexes.

References